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THE ARX ANAEROBIC ARSENITE-OXIDIZATION PATHWAY IS CONSERVED IN HALOMONAS AND ECTOTHIORHODOSPIRA STRAINS ISOLATED FROM BIG SODA LAKE, NEVADA

A thesis submitted in partial satisfaction
of the requirements for the degree of

MASTER OF SCIENCE

in

MICROBIOLOGY AND ENVIRONMENTAL TOXICOLOGY

by

ALISON CONRAD

June 2014

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TABLE OF CONTENTS

ABSTRACT

THE ARX ANAEROBIC ARSENITE-OXIDIZATION PATHWAY IS CONSERVED IN

HALOMONAS AND ECTOTHIORHODOSPIRA STRAINS ISOLATED FROM BIG SODA

LAKE, NEVADA

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ALISON CONRAD

Microorganisms play a significant role in environmental arsenic cycling. The most recent discovery to the ever growing collection of known arsenic metabolisms is photosynthesis-linked arsenite oxidation (photoarsenotrophy). However, it is poorly understood and has only been identified in thermal springs on Paoha Island of Mono Lake, CA. The arsenite oxidase ArxA is thought to be responsible for the oxidation of arsenite in photoarsenotrophy. However, the first and only isolated photoarsenotroph, *Ectothiorhodospira sp* PHS-1, has not proven amenable to genetic manipulations, making genetic confirmation impossible. Water, sediment, and tufa collected from Big Soda Lake and Mono County were used for enrichment culturing and functional gene analyses. *arxA*-specific primers successfully amplified products from 15 out of 18 environmental samples, and cloned products show high homology to existing *arxA*-like sequences. Strains containing *arxA*-like sequences and capable of arsenite oxidation belonging to the genera *Ectothiorhodospira* and *Halomonas* were isolated. This work demonstrates that *arxA* sequences and photoarsenotrophs can also be found in Big Soda Lake, NV, and presents two novel *arxA*-containing, arsenite-oxidizing strains: the chemoautotroph *Halomonas* sp. BSL-1 and the photoarsenotroph *Ectothiorhodospira* sp. BSL-9.

ACKNOWLEDGEMENTS

INTRODUCTION

Arsenic is a common natural contaminant of both geothermal waters and freshwater aquifers. It is of particular concern in southeastern Asia where millions of people consume quantities of arsenic up to 100 times greater than the World Health Organization's standard of 10 ppb (Ahmed et al., 2006). Previous studies have shown that in many environments microbes are the principle driving force behind arsenic cycling (Harvey et al., 2002; Islam et al., 2004; Oremland et al., 2004; Salmassi et al., 2006). Bacteria have been demonstrated to use arsenic for energy conservation; using arsenite as a source of electrons, or arsenate as an electron acceptor. They are able to do so using proteins belonging to the DMSO reductase family of enzymes (McEwan et al., 2002). The best understood clades of arsenic redox enzymes are the arsenate reductase, ArrA, and the arsenite oxidase, AioA, which have both been found in numerous prokaryotes (Oremland and Stolz, 2003). Less well understood is ArxA, a recently discovered clade of arsenite oxidases distinct from AioA found in autotrophic bacteria from Mono Lake (Zargar et al., 2010, 2012).

A particularly interesting aspect of ArxA is that it thought to be the arsenite oxidase involved in the recently discovered microbial metabolism photoarsenotrophy (Kulp et al., 2008). By this process photosynthetic bacteria are able to use solar energy to convert the more toxic form of arsenic, arsenite, to the less toxic arsenate. Photoarsenotrophy was discovered in the anaerobic, purple sulfur bacterium, *Ectothiorhodospira sp.* str. PHS-1, isolated from an arsenic-rich hot spring microbial mat on Paoha Island, Mono Lake, a hypersaline, alkaline lake in California (Kulp et al., 2008). This phototroph contains an *arxA* homolog which is upregulated in the presence of arsenite (Zargar et al., 2012) . However ArxA itself is newly discovered and has only been genetically confirmed in a non-phototrophic arsenite oxidizer called *Alkalilimnicola ehrlichii* str. MLHE-1, also isolated from Mono Lake (Oremland et al., 2002; Zargar et al., 2010). PHS-1 has not proven amenable to genetic manipulations, thus the anaerobic arsenite oxidase involved in photoarsenotrophy has yet to

be genetically confirmed. The isolation of a photoarsenotroph that can be developed as a genetic model is essential to eliminating this bottle neck and allowing further genetic examination of *arxA* to continue. Additionally, as *arxA*-containing bacteria known to oxidize arsenite have only be isolated from Mono Lake, it is unknown whether these bacteria are limited to this region, or are instead common to arsenic-rich environments. This knowledge would help us begin to understand how large of an influence ArxA-mediated arsenite oxidation has on arsenic cycling in the environment.

The purpose of this work is to provide evidence that proteins belonging to the ArxA clade are found outside of the Mono Lake area and demonstrate that they are found in bacteria outside the family Ectothiorhodospiracea. Samples collected from Big Soda Lake near Fallon, NV were used for environmental PCR and enrichment cultures. Two novel *arxA*-containing strains, the photoautotroph *Ectothiorhodospira sp.* strain BSL-9, and the chemoautotroph *Halomonas sp.* strain BSL-1 are presented along with their draft genomes.

MATERIALS AND METHODS

Sampling sites

Samples were collected from two areas: Big Soda Lake, NV (BSL) in June of 2011, and Mono County, CA in September of 2011. GPS coordinates are noted for each sampling location. At BSL (39.525, -118.879), samples were collected from the water column, tufas, lake sediment, and a connected swamp. In Mono County, tufa and lake sediments were collected from Mono Lake (38.008, -119.018), while sediment and microbial mat samples were collected from Hot Creek (37.6613, -118.8286), Little Hot Creek (37.690915, -118.844307, and 37.690197, -118.842469), and various hot springs such as Navy Beach (37.941062, -119.022976), Warm Springs (38.033535, -118.904325), Wild Willy's (37.661039, -118.767818), Wild Willy's Evaporative Lake (37.658834, -118.769809), Bath Tub (37.646972, -118.809444), and Shepherd's (37.666928, -118.803295). Both Big Soda and Mono Lake are alkaline (pH 9.7), hypersaline (ML 90 g/L and BSL 26-88 g/L) and contain

high concentrations of inorganic arsenic, 20 and 200 μM respectively. Hot Creek, Little Hot Creek and the various hot springs sampled from Mono County were freshwater with neutral pH. Depth profile of BSL was collected using a Seacat SBE 19-03 multiprobe and analyzed using SeaSoft V2 software (Sea-Bird Electronics).

Environmental sample processing

Samples collected for DNA extraction were stored on dry ice after collection and analyzed within a week of collection. At least 60 mL of each depth of BSL water column sample was filtered through 0.7 μm GF/F filter before being stored. All samples were frozen at -80°C for long-term storage. DNA was extracted from sediment, tufa, and microbial mat samples using the PowerSoil DNA Isolation Kit (MoBio). Tufa was comminuted using a sterile hammer prior to DNA extraction. DNA was extracted from aqueous samples using the WizardGenomic DNA Purification Kit (Promega). Aluminum ammonium sulfate at 100 mM was added to all cell lysis steps of DNA extractions to reduce the co-precipitation of PCR inhibitors with DNA (Braid et al., 2003). The DNA samples were stored at -20°C until use. Samples for arsenic analysis were stored in vacutainer tubes and analyzed by high-performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC-ICP-MS).

Detection of arxA*,* aioA*,* arrA *and 16S rRNA genes*
Functional genes were amplified using the Malasarn *et al.* *arrA* primers [ArrAfwd (5'-AAGGTGTA-TGGAATAAAGCGTTT-gtbgghgaytt-3') and ArrArev (5'-CCTGTGATTTCAGGTGCC-caytyvggngt-3')], Inskeep *et al. aioA* primers [first set (5′-GTSGGBTGYGGMTAYC ABGYCTA-3′ and 5′- TTGTASGCBGGNCGRTTRTGRAT-3′)], and *arxA* primers developed within the Saltikov lab [PHS-1 specific (5′-CGGTCTTGGGATTGATCTCG-3′ and 5′-TGATCTTTACATG-GTCACCCC- 3′), and

3

degenerate (5′-CCATCWSCTGGRACGAGGCCYTSG- 3′ and 5′-

GTWGTTGTAGGGGCGGAAS- 3′)] (Malasarn et al., 2004; Inskeep et al., 2007; Zargar et al.,

2012). The 16S rRNA gene was amplified using the 8F/1492R primer set (Reysenbach et al.,

1994). The PCR reactions consisted of: 12.5 ml of PCR Master Mix (Promega), 200 nM of

each forward and reverse primers, 30 ng of DNA (environmental or pure culture) and sterile

water (as needed). For *arxA* and *arrA* primers the samples were incubated with the following

touchdown thermocycler profile: 95°C for 30 s, 1 cycle of 65°C for 30 s, 72°C for 1 min,

repeating 4 times with the annealing temperature decreasing 2°C each cycle. This is followed

by 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, repeating 29 times. For amplification of 16S

ribosomal gene an initial annealing temperature of 60°C was used with a final annealing

temperature of 50°C, and elongation was extended to 90 s. For every PCR reaction the

following organisms were used as controls: *Ectothiorhodospira* sp. str. PHS-1 (*arxA*),

Alkalilimnicoli ehrlichii str. MLHE-1 (*arxA*), *Shewanella* sp. str. ANA-2 (*arrA*), *Citrobacter* sp.

str. TSA-1 (*arrA*), *Agrobacterium albertimagni* str. AOL15 (*aioA*), and *Alcaligenes faecalis*

(*aioA*). PCR products were cloned using the TOPO TA Cloning Kit (Life Technologies) and

sequenced by Sanger sequencing (Sequetech).

Phylogenetic analyses

The predicted protein sequences of ArxA-like sequences as well as representative DMSO

reductase family enzymes (McEwan et al., 2002) were aligned using CLUSTALW (Chenna et

al., 2003). Sequences and methods used for phylogenetic inferences have been described

elsewhere (Zargar et al., 2010). The phylogenetic tree in Figure 9 was generated using

CLUSTALW within the sequence data analysis software package, ARB, and distance

methods.

Isolation of arsenotrophs and growth conditions

Anaerobic Balch tubes of Big Soda minimal media (BSM) were prepared prior to sampling (g/L^{-1}): NaAsO$_2$ (0.26) yeast extract (0.02), NaCl (25.0), KH$_2$PO$_4$ (0.24), K$_2$HPO$_4$ (0.3) (NH$_4$)$_2$SO$_4$ (0.23), MgSO$_4$ (0.12), Na$_2$CO$_3$ (5.3), NaHCO$_3$ (2.1), vitamin B12 (0.0002), SL10 trace element solution (1 ml) and Wolfe's vitamin mix (10 ml) (Widdel et al., 1981; Kostka and Nealson, 1998). Tubes containing media with and without nitrate (0.85) as an electron acceptor were inoculated to select for arsenotrophic chemotrophs and phototrophs, respectively. At BSL, 1 mL of each water column sample was used to directly inoculate tubes at the time of sampling, while sediment and microbial mat samples were anaerobically introduced to media within 48 hours. Samples collected in Mono County were used to inoculate cultures the same day as collected. Cultures were incubated at either room temperature or at 37°C, depending on temperature of sampling site. Cultures selecting for phototrophs were grown in the light, typically at 2000 lux, and those selecting for chemotrophs were incubated in the dark. Cultures were streaked on plates and incubated anaerobically at room temperature. Colonies were repeatedly picked, transferred to liquid culture, and restreaked to ensure strains consisted of isolates. Once established, both BSL-1 and BSL-9 were typically grown at 30°C. When BSL-1 was grown at different pH conditions, pH was altered by addition of HCl and NaOH. Unless otherwise stated, organic acids were used at concentrations of 10 mM.

Transmission electron microscopy

BSL-1 was grown in LB media at pH 10, and BSL-9 was grown in BSM with the addition of 5 g/L yeast extract and using malate as an electron donor (20 mM). Near the end of exponential growth, cells were collected and fixed in 0.1 M cacodylate buffer containing 2.5% gluteraldehyde (Fisher), and post fixed in 2% osmium tetroxide containing 1.25% potassium ferricyanide in 0.2 M cacodylate buffer. Samples were washed in 0.1 M phosphate buffer, followed by Type I reagent grade water. The pellet was enbloc stained with 2% uranyl acetate in 30% ethanol and a dehydration series was performed with ethanol 30%, 50%, 70%, 80%,

90%, 95% and 100%. Samples were then infiltrated by adding Spurr's resin, embedded in Beem® capsules and incubated overnight at 65° C for polymerization. Survey sections at 1 μm thickness were collected with a Leica EM UC7 ultramicrotome, stained with 1% toluidine blue and mounted for light microscopy to determine an area of interest. Subsequent thin sections at 0.18μm thickness were observed under a Hitachi H9500 TEM.

Growth experiments

Both BSL-1 and BSL-9 were grown using BSM, with an initial arsenite concentration of 0.5 M. For BSL-1, 10 mM of nitrate was provided as an electron acceptor. BSL-9 was grown in the light at approximately 2000 lx. Growth was measured by absorbance at 600 nm. As growth increased, arsenite additions were made to cultures to provide a constant source of electron donor, while avoiding high concentrations of initial arsenite from preventing growth. Arsenic analysis was performed using high performance liquid chromatography (HPLC).

Draft genomes sequencing and assembly

Total genomic DNA of BSL-1 and BSL-9 was prepared from cultures originating from a single colony obtained from a streak plate. Liquid cultures were prepared from a single colony. Cells were harvest from early stationary phase cultures. DNA from cell pellets was extracted using the DNEasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. DNA samples were processed for sequencing by Illumina HiSeq. Trimmomatic program was used to remover and/or trim the 5' and 3' ends poor quality reads (Bolger et al., 2014). Reads with adapter sequences contamination were either trimmed or removed. The filtered DNA sequence data was assembled into contigs using ABySS 1.3.5 (Simpson et al., 2009). For BSL-1 and BSL-9, this process yielded 231 and 248 contigs with N50 of 83,990 and 114,790 kb and an average sequencing coverage of 204X and 296X, , respectively. The contigs were annotated using RAST: Rapid Annotation using Subsystem Technology (Overbeek et al., 2014). The Whole Genome Shotgun projects were deposited at DDBJ/

EMBL/GenBank under the Accession # PRJNA2397 (BSL-1) and PRJNA232800 (BSL-9). The final genome annotations were generated using the Public Genome Annotation Pipeline (PGAP) service provided by NCBI.

RESULTS AND DISCUSSION

Characterization of sampling sites

We collected samples from several sites in order to study the distribution of *arxA* in arsenic-rich environments and search for novel *arxA*-containing organisms. In Mono County, samples were collected from Mono Lake (ML), ML tufa, Hot Creek, Little Hot Creek, and several hot springs in the area (Table 1). Total arsenic concentrations of hot springs in the Mono County area ranged from 0.06-13.1 µM. In comparison, arsenic levels in the hot springs of Yellowstone typically range from ~1-50 µM (Nordstrom et al., 2001). Arsenic concentrations in Mono Lake have previously been determined to be ~200 µM (Oren, 2002; Oremland et al., 2004). Arsenite concentrations in hot springs varied, with it being the dominate species in some springs (Navy Beach) but very low in others (Shepherd's hot spring). Overall, As[III/(III+V)] ratios ranged from 0.01–0.84. Arsenic concentrations in Little Hot Creek were previously reported as 9.8 µM, similar to our results of 8.9 and 9.6 µM (Vick et al., 2010). Our work additionally determined arsenite and arsenate concentrations, and found that downstream arsenite concentrations were very low relative to the sample collected nearer the source of the spring, suggesting oxidation is occurring. This observation is similar to reports showing rapid arsenite oxidation in nearby Hot Creek, and also in some hot springs of Yellowstone National Park (Langner et al., 2001; Salmassi et al., 2002).

When selecting sampling sites, we collected samples in sites where photosynthesis-linked arsenite oxidation was most likely occurring. Temperatures of the hot springs varied from 29-59 ºC, and all had a neutral pH (Table 1). Sampling was restricted to lower temperature springs in order to increase the likelihood of culturing phototrophs, which

become increasingly rare at higher temperatures and typically do not grow at temperatures above 73ºC (Castenholz and Pierson, 1995).

To examine the distribution of *arxA* and photoarsenotrophs outside of vicinity of ML, we also collected samples near Fallon, NV, from Big Soda Lake (BSL), BSL tufa, and an attached sulfidic swamp. This provided samples from a site even further from ML in an environment particularly suited to photoarsenotrophs due to the presence of a previously reported purple bacteria layer in the anoxic mixolimnion (Zehr et al., 1987). In BSL, total arsenic concentrations were found to be approximately 22 μM (Table 1), similar to previously reported values, and approximately 10-fold less than ML (Oremland et al., 2004). As expected, arsenite was absent from the upper regions of the lake, where arsenate appears to dominate (Figure 1). The oxycline occurred between 7-18 m, and the thermocline between ~7-25 m. Even at 35 m arsenate was abundant, despite this being in anoxic waters where arsenic is expected to be found in a reduced form such as arsenite (Oremland and Stolz, 2003). This could be explained by prevalent microbial-mediated, anaerobic arsenite oxidation in the anoxic mixolimnion of BSL. Additional sampling is planned to confirm this unexpected result. Below the chemocline, where sulfide concentrations are high (12.8 mM) (Cloern et al., 1983b), we failed to measure arsenate or arsenite by HPLC-ICP-MS. This is can most likely be explained by arsenic being found as thioarsenites in these samples. In alkaline waters with this high sulfide concentration, arsenic is expected to exists primarily as trithioarsenite (Wilkin et al., 2003; Planer-Friedrich et al., 2010), which we were not able to separate using HPLC.

The potential for photoarsenotrophy differs greatly between BSL and ML. In BSL, fluorescence, indicative of photosynthetic organisms, spiked below the oxycline, correlating with previous reports of a layer of purple sulfur bacteria dominated by purple sulfur bacteria, *Ectothiorhodospira vacuolata* and *Chromatium sp,.* below the oxycline of BSL at levels below 1% surface irradiance during summer and fall (Cloern et al., 1983a; Zehr et al., 1987). In the winter and spring the oxycline reportedly increases in depth to 25-29 m, below the photic

region (Zehr et al., 1987). This removes the niche occupied by anoxygenic phototrophs and instead phytoplankton, such as *Nitzschia* and *Oocystis*, dominate the photic region during the colder months (Cloern et al., 1987). Previous work has shown that BSL and ML are similar in profile (Oren, 2002). ML has an oxycline of 13-18 m and thermocline of approximately 8-24 m (Humayoun et al., 2003). In Mono Lake, the green alga *Picocystis sp.* str. ML dominates the photic region of the lake, ranging down into portions of the anoxic monimolimnion . Presumably, this abundant growth of algae prevents the formation of a plate of purple bacteria by reducing the amount of light that penetrates the anoxic monimolimnion (Roesler et al., 2002).

Amplification of environmental arxA-*like sequences*

　　　To determine whether the gene for the arsenite oxidase *arxA* can be found outside of ML, and to examine its distribution in arsenic-rich environments, samples were screened using *arxA*-specific primers. At Big Soda Lake, all samples collected yielded PCR products when screened with *arxA*-specific primers (Figure 2). PCR products were confirmed to be *arxA*-like sequences by cloning, sequencing and comparison of sequences against known ArxA sequences. Of particular note, the intensity of amplified PCR product relative to 16S rRNA PCR product is highest at 30 m in the water column, suggesting that of the depths sampled 30 m contains the highest proportion of *arxA*-containing organisms. Unfortunately, samples were not collected just below the oxycline, determined to be 18-21 m (Figure 1), where a plate of purple bacteria would have been expected to develop and photoarsenotrophs most likely to occur. However of the samples collected, the 30 m sample is nearest sample beneath this region. Several reasons could account to spike in *arxA* signal at 30 m. First, this could be due to amplification from *arxA*-containing microbes originating from the above bacterial layer at ~18 m which were sinking. Alternatively, the source could be *arxA*-containing chemotrophs living at 30 m in the anoxic mixolimnion, although it is unclear what electron acceptors they might use. Just 5 m below at 35 m, and in the proximity of the

chemocline (Oren, 2002), *arxA* amplification drops of sharply (Figure 2). Previous work has suggested that the bacterial population in the monimolimnion of BSL are primarily inactive or slow growing cells, likely inhibited by high levels of reduced sulfur compounds, such as sulfide (~13 mM) (Cloern et al., 1983a; Zehr et al., 1987). The intensity of *arxA* PCR product was also high in tufa, lake sediment, swamp crust and swamp sediment samples. These are also environments expected to host anaerobic phototrophs due to their proximity to sunlight and lack of oxygen (Imhoff, 2006).

       Although MLHE-1, which oxidizes arsenite using ArxA, and the photoarsenotroph PHS-1, which is suspected of also using ArxA, have been isolated from ML and Paoha Island, respectively (Oremland et al., 2002; Kulp et al., 2008), no one has investigated the distribution of *arxA*-sequences in the area. In the ML area, all samples except for the Bath Tub hot spring and Navy Beach hot spring yielded PCR products when screened with *arxA*-specific primers (Figure 2). There was no clear correlation between the amplification of PCR product and environmental factors such as temperature or pH. Cloned PCR products amplified from BSL environmental DNA with primers designed for *arxA*, *arrA*, and *aioA* fell within their respective protein clades with one exception (Figure 3). Despite reported difficulties using PCR to amplify *aioA* sequences from the alkaline, saline Lake Tukh (Hamamura et al., 2014), we were able to amplify *aioA* sequences from lake tufa, sediment and the near swamp. This suggests that the *aioA* primers from Inskeep 2007 may be superior to those from Quéméneur 2008 in saline and alkaline environments (Inskeep et al., 2007; Quéméneur et al., 2008). The one sequence amplified with Malasarn *arrA* primers grouped with the ArxA clade, indicating that these primers detect both *arrA* and *arxA* sequences (Malasarn et al., 2004). *arxA*-like sequences were amplified from environmental DNA extracted from the BSL water column, lake sediment, tufa, and swamp crust. This confirms that *arxA*-like sequences can be found outside of the Mono County area and suggests that they may be widespread in arsenic-rich environments.

Isolation and identification of novel arsenite oxidizing photo- and chemotrophs

We isolated several arsenite oxidizing strains belonging to the genera *Ectothiorhodospira* and *Halomonas* from BSL samples (Figure 4). All 14 arsenite oxidizing isolates were able to grow anaerobically using arsenite a sole electron donor and oxidizing it completely to arsenate when grown autotrophically. The *Ectothiorhodospira* isolates oxidized arsenite when grown photoautotrophically. The *Halomonas* isolates oxidized arsenite when grown chemoautotrophically with nitrate as the electron acceptor. *Ectothiorhodospira* strains were isolated from swamp samples and lake tufa. Based on 16S rDNA analysis, *Ectothiorhodospira* isolates BSL-6 and -11 grouped most closely with *E. shaposhnikovii*, 99.4% and 99.1%, respectively. *E. shaposhnikovii* has a NaCl optimum of 3%, pH optimum of 8-8.5 and is capable of microaerophilic growth in the dark (Imhoff, 2006). The rest of the *Ectothiorhodospira* isolates grouped closely with *E. variabilis*, (99.4-99.5%), a species isolated from several different soda lakes such as Mongolia, Egypt, and Siberia (Gorlenko et al., 2009). *E. variabilis* has a salt optima of 5-8%, pH optimum of 9.0-9.5 and is strictly anaerobic. The *Halomonas* isolates were isolated from swamp samples, lake sediment, and lake tufa, and were more diverse based on 16S rRNA gene homologies. For example, strain BSL-1 is most similar to *H. campaniensis* (97.1%), while other BSL *Halomonas* showed 16S rRNA gene similarities to *H. johnsoniae* (BSL-2, 97.0%), *H. kenyensis* (BSL-3, 95.7%), and *H. mongoliensis* (BSL-4, 98.6%). From our limited strain culture collection, Big Soda Lake *Ectothiorhodospira* species appear to have low diversity compared to *Halomonas* isolates. This matches previous studies which have shown exceptionally high diversity of Halomonas in soda lakes (Duckworth et al., 1996; Oueriaghli et al., 2014). A search of the Ribosome Database Project found 33 total entries for the genus *Ectothiorhodospira*, 5 of which were type strains (Cole et al., 2009). In contrast, *Halomonas* has 1627 isolates, of which 64 are type strains.

Characterization of isolates

Due to our ability to amplify an *arxA* sequence from *Ectothiorhodospira* sp. BSL-9 by PCR, it was selected for further analysis. Although *arxA* was also amplified from strains BSL-6 and -10 as well, they grew heterogeneously as films along the glass of their Balch tubes, making analysis by growth curve difficult. *Halomonas* sp. BSL-1 was selected for further analysis based on its rapid growth. Based on transmission electron microscopy (Figure 5), BSL-9 cells appeared as ~2 µm bent rods. While the morphology of BSL-9 is typical of the genus *Ectothiorhodospira*, it is considered large for the genus, which typically range from 0.7 – 1.5 µm (Gorlenko et al., 2009). BSL-1 is also ~2 µm and rod-shaped, similar to *H. campaniensis* (Romano et al., 2005). The colonies of BSL-9 are dark red in color, circular, shiny, and ~5 mm in diameter when grown photoheterotrophically with malate as an electron donor. When grown photoautotrophically with arsenite as an electron donor, colonies are also red-pigmented but smaller, approximately 2 µm. When grown photoheterotrophically, colonies appeared within 2 days, but when grown photoautotrophically took 7 days to appear. The differences in colony size and growth rate are likely because arsenite is less energetically favorable as an electron donor than malate as well as being toxic. The colonies of BSL-1 appeared overnight and are cream to grey in color, circular, shiny and ~ 6 mm when grown heterotrophically on LB media, at pH 9.8. Similar to BLS-9, colonies took longer to grow (3-4 days) and were smaller (~3 µm) when grown on a minimal medium with arsenite as an electron donor and nitrate as an electron acceptor.

When grown with arsenite as the sole electron donor both isolates were able to oxidize it fully to arsenate (Figure 6 and Figure 7). In both cases, additional arsenite (0.5-3 mM) was spiked into cultures as initial arsenite became depleted. This is necessary because arsenite concentrations above 2 and 3 mM inhibited growth of BSL-9 and -1, respectively. BSL-1 is able to grow on Luria broth under aerobic conditions, but grows best if the pH of the media is adjusted to 9.8. BSL-9 grew optimally at 33ºC at a salinity of 2% and within pH ranges of 7.0-9.5. The growth of BSL-9 with other electron donors was also analyzed and generation times determined. BSL-9 grew anaerobically on sulfide, thiosulfate (9.5 h), malate

(13.6 h), acetate (6.9 h), proprionate (5.2 h), lactate (55.0 h), fumarate (23.3 h), pyruvate

(28.9 h), and succinate (10.8 h). These characteristics are similar to other members of the

genus *Ectothiorhodospira* (Gorlenko et al., 2009). The fastest growth was seen on acetate,

when BSL-9 reached stationary phase at an OD_{600} of 1.7 at 38 h. The abilities of BSL-9 to

both grow on agar plates and grow quickly on organic acids make it so far the most facile

photoarsenotroph to work with in laboratory conditions, and a potential model organism for

future genetic studies.

Genomic analysis

Draft genome sequences were determined for BSL-1 and BSL-9. BSL-9 has a

genome length of 3.4 Mbp and GC content of 63.0%, which is similar to that of *E.*

haloalkaliphila ATCC 51935, but larger than that of PHS-1 (Zargar et al., 2012). BSL-1 has a

genome length of 4.4 Mbp and GC content of 66.8%. This genome size is similar to other

members of the genus *Halomonas*, including *Halmonas* sp. str. HAL1 and GFAJ (Lin et al.,

2012; Phung et al., 2012; Sogutcu et al., 2012). The high GC contents of the BSL-1 and BSL-

9 are typical of the *Ectothiorhodospira* and *Halomonas* genera (Romano et al., 2005; Imhoff,

2006). Both genomes contain *arx* operons homologous to those found in members of the

family Ectothiorhodospiraceae (Figure 8). BSL-1, however, lacks the putative arsenite

oxidase regulatory gene cluster *arxRSX,* which is found in PHS-1, MLHE-1, and BSL-9,

indicating it uses an alternative method to regulate expression of its arsenite oxidase (Liu et

al., 2011; Zargar et al., 2012). When the predicted ArxA protein sequences are mapped to

the DMSO reductase family phylogenetic tree they fall within the ArxA clade (Figure 9). The

ArxA sequences of BSL-1 and BSL-9 matched most closely with the ArxA of MLHE-1 (72%

and 78%, respectively). Both genomes also contain arsenic resistance operons (*ars*) and the

cytoplasmic arsenate reductase *arsC.* BSL-9 appears to have a single arsenic resistance

operon containing the genes *arsD*, *arsA*, *arsB*, and *arsC*, as well as a histidine kinase. ArsB

is an integral membrane pump, and ArsA is an ATPase which, in combination with ArsB,

makes arsenix export more efficient (Slyemi and Bonnefoy, 2011). ArsD is a chaperone that

brings arsenite to ArsA. BSL-1, in comparison appears to have several arsenic resistance

operons. One operon contains *arsR*, a repressor of arsenic-resistance genes (Murphy and

Saltikov, 2009), with several genes predicted to form a phosphate transporter. Approximately

10 kb downstream of this is another arsenic-related gene cluster containing 2 *arsH* genes, an

arsC, *arsB*, and a gene predicted to encode for a universal stress protein. ArsH is a

NAD(P)H-dependent flavoprotein predicted to protect against arsenite-induced oxidative

stress (Hervás et al., 2012). On a separate contig is an operon containing *arsC*, *arsH,* and a

hypothetical protein, and distributed through BSL-1's genome are 2 genes expected to

encodes ArsB proteins as well as an ArsA. Based on currently available *Halomonas*

genomes, the arrangement of arsenic resistance genes in this genus appears to vary greatly

(Kawata et al., 2012; Lin et al., 2012; Phung et al., 2012). BSL-1 has the first sequenced

genome of an *arxA*-containing organism outside of the family Ectothiorhodospiraceae. The

genus *Halomonas* is of particular interest in the field microbe-arsenic interactions. It contains

species with the AioA arsenite oxidase and species with the ArxA arsenite oxidase (Lin et al.,

2012; Hamamura et al., 2014), as well as the highly arsenic-resistant and controversial strain

GFAJ which had been reported to incorporate arsenic into its nucleic acids (Wolfe-Simon et

al., 2011; Erb et al., 2012).

Conclusions

This work provides the first detailed study of the distribution of *arxA*-like sequences in an

arsenic-rich alkaline saline lake (Big Soda Lake) and demonstrates that *arxA* can be found

throughout this environment, as well as various thermal environments throughout Mono

County. This adds to the growing evidence that ArxA plays an important role in arsenic

cycling in these environments. Additionally, the isolation of *Ectothiorhodospira* sp. BSL-9 and

Halomonas sp. BSL-1 add to the growing number of known isolates that contain *arx* operons

and are capable of arsenite oxidation. BSL-1's ability to grow quickly aerobically on rich

media like LB make it an appealing organism to work with for further study of ArxA during chemotrophic growth. BSL-9, unlike PHS-1, grows easily on agar plates, is aerotolerant, and is a mesophile. This comparative ease of working with BSL-9 makes it a promising organism for genetic work to elucidate the molecular mechanism behind photoarsenotrophy.

However, many questions remain. What are the phylogenies of *arxA*-containing organisms in the water column of BSL, and is photoarsenotrophy occurring in the water column? What are the contributions of *arxA*-mediated arsenite oxidation on arsenic cycling and productivity in BSL? Future work will focus on these questions as well as on developing a model organism for the study of photoarsenotrophy.
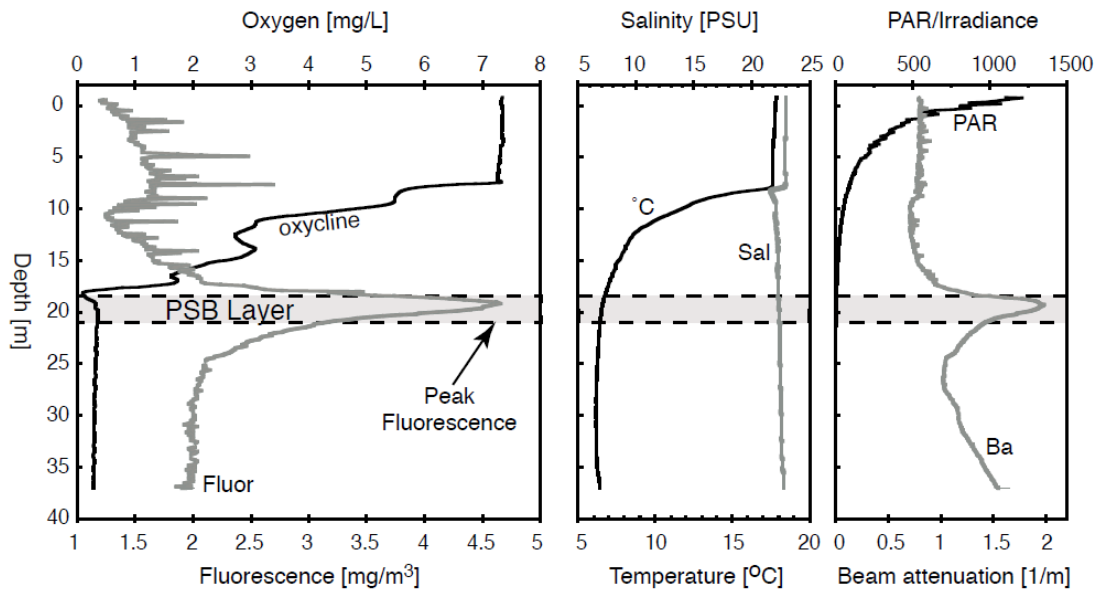
Figure 1. Depth profiles of Big Soda Lake, NV depicting dissolved oxygen, fluorescence, salinity, temperature, PAR/Irradiance, and beam attenuation (transmittance). The horizontal bar depicts the predicted location of purple sulfur bacteria. Values were collected using a Seacat SBE 19-03 multiprobe.
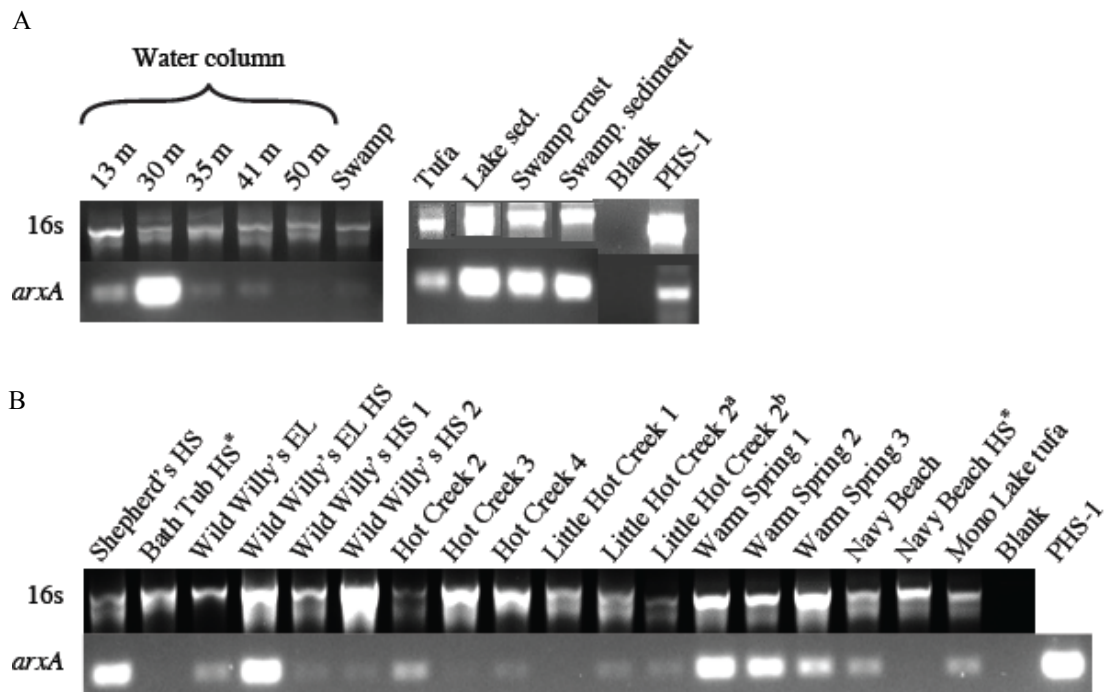
Figure 2. PCR analysis for the 16S rRNA gene and *arxA* of environmental samples. Blank consists of nuclease-free water, and PHS-1 DNA is used as a positive control. The ribosomal16s gene products are ~1500 bp, and the *arxA* products are ~175 bp. A. Big Soda Lake samples. B. Mono County samples. Asterisks denote samples from which no detectible *arxA* products were amplified. Superscript letters are used to denote upstream ([a]) and downstream ([b]) samples.
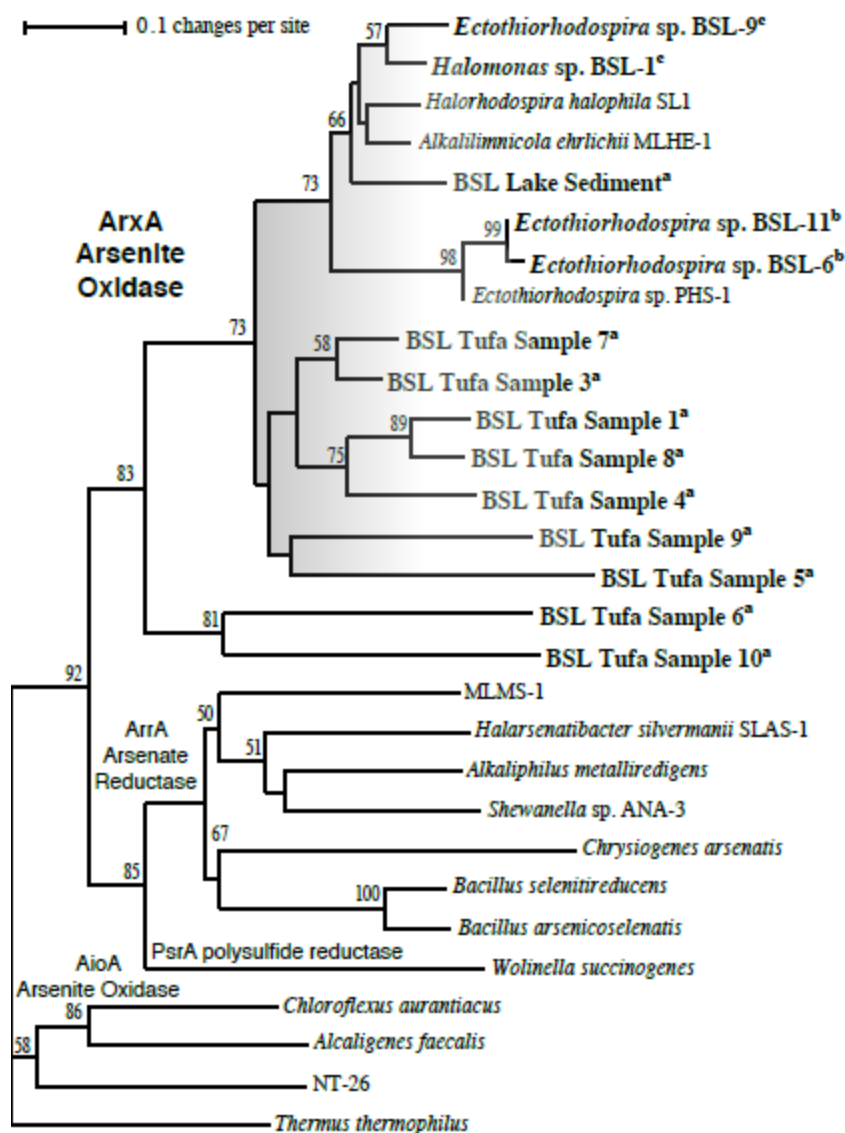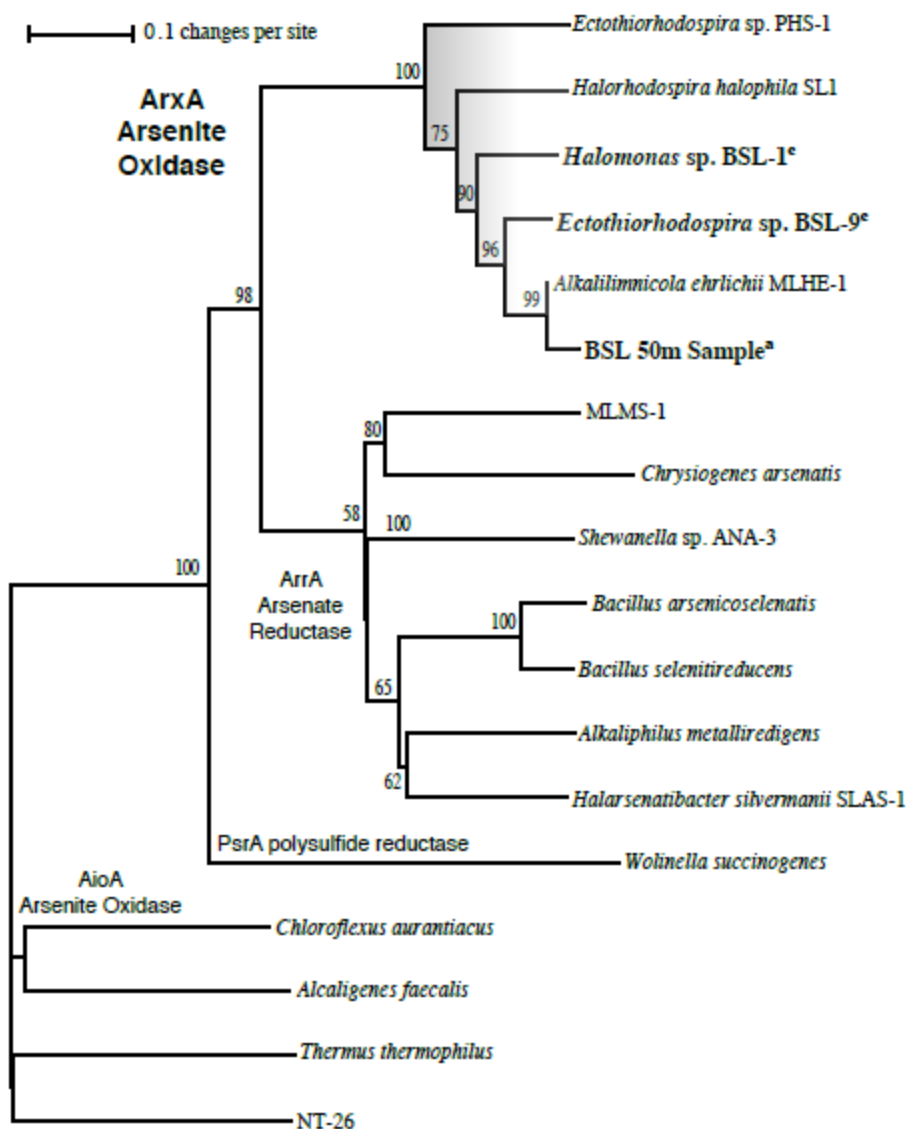
Figure 3. A. Phylogenetic analysis of the amino acid sequence translation of the partial *arxA* PCR amplicons from BSL environmental samples ([a]) and isolates ([b]). Sequences were amplified using primers derived from the PHS-1 *arxA* sequence (Zargar et al., 2012). Also compared are complete *arxA* sequences from sequences isolates ([c]). The phylogeny was constructed using the distance criterion and neighbor joining with the AioA clade as an outgroup. Bootstrap analysis with 1000 replications was performed to test the significance of the tree topology. The bootstrap values are included on the nodes that have values greater than 50%. Reference sequences were also included in the phylogeny. These include haloalkiliphilic *arxA*-containing bacteria, arsenate respiring bacteria, polysulfide reductase and several *aioA*-containing arsenite oxidizers.
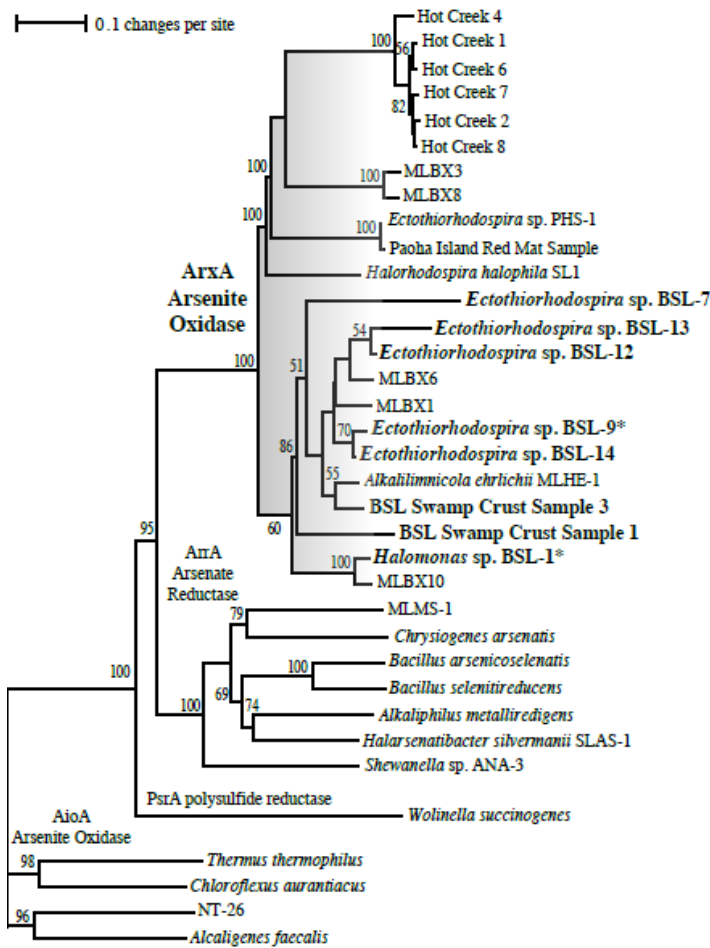
Figure 3. B. Phylogenetic analysis of the amino acid sequence translation of the partial *arxA* PCR amplicons from BSL environmental samples. Sequences were amplified using degenerate *arxA* primers (Zargar et al., 2012). Also compared are complete *arxA* sequences from sequences isolates ( ). The phylogeny was constructed using the distance criterion and neighbor joining with the AioA clade as an outgroup. Bootstrap analysis with 1000 replications was performed to test the significance of the tree topology. The bootstrap values are included on the nodes that have values greater than 50%. Reference sequences were also included in the phylogeny. These include haloalkiliphilic *arxA*-containing bacteria, arsenate respiring bacteria, polysulfide reductase and several *aioA*-containing arsenite oxidizers.

Figure 3. C. Phylogenetic analysis of the amino acid sequence translation of the partial *arrA* PCR amplicon from BSL 50 m water column sample ([a]). Sequence was amplified using *arrA* primers, despite it showing greatest homology to the ArxA clade (Malasarn et al., 2004). Also compared are complete *arxA* sequences from sequences isolates ([c]). The phylogeny was constructed using the distance criterion and neighbor joining with the AioA clade as an outgroup. Bootstrap analysis with 1000 replications was performed to test the significance of the tree topology. The bootstrap values are included on the nodes that have values greater than 50%. Reference sequences were also included in the phylogeny. These include haloalkiliphilic *arxA*-containing bacteria, arsenate respiring bacteria, polysulfide reductase and several *aioA*-containing arsenite oxidizers.
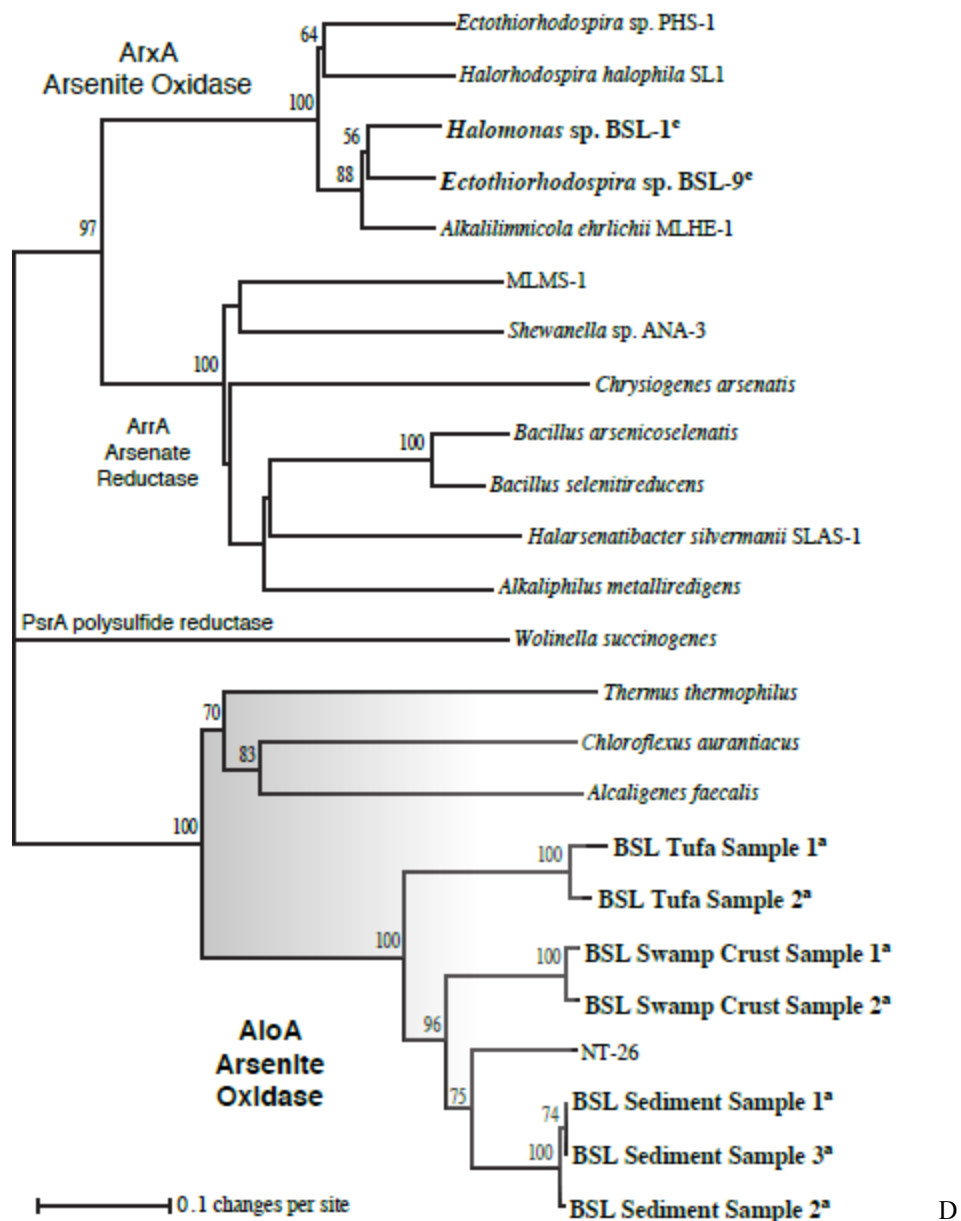
Figure 3. D. Phylogenetic analysis of the amino acid sequence translation of the partial *aioA* PCR amplicons from BSL environmental samples ([a]). Sequences were amplified using *aioA* primers (Inskeep et al., 2007). Also compared are complete *arxA* sequences from sequences isolates ([c]). The phylogeny was constructed using the distance criterion and neighbor joining with the ArrA clade as an outgroup. Bootstrap analysis with 1000 replications was performed to test the significance of the tree topology. The bootstrap values are included on the nodes that have values greater than 50%. Reference sequences were also included in the phylogeny. These include haloalkiliphilic *arxA*-containing bacteria, arsenate respiring bacteria, polysulfide reductase and several *aioA*-containing arsenite oxidizers.
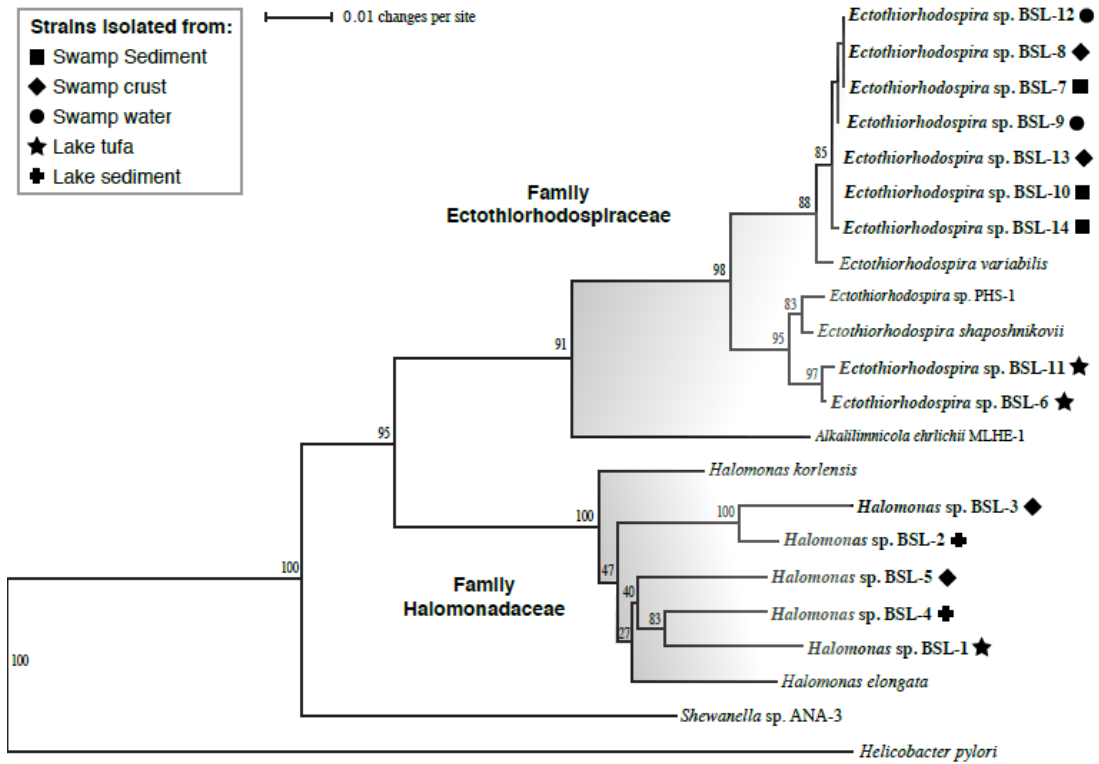
Figure 4. 16S rRNA-based tree showing phylogenetic relationships between BSL isolates and related organisms. Bootstrap values are given at branch points. The sample each organism is isolated from is indicated by symbol. Tree was made using the Ribosome Database Tree Builder (Cole et al., 2009).
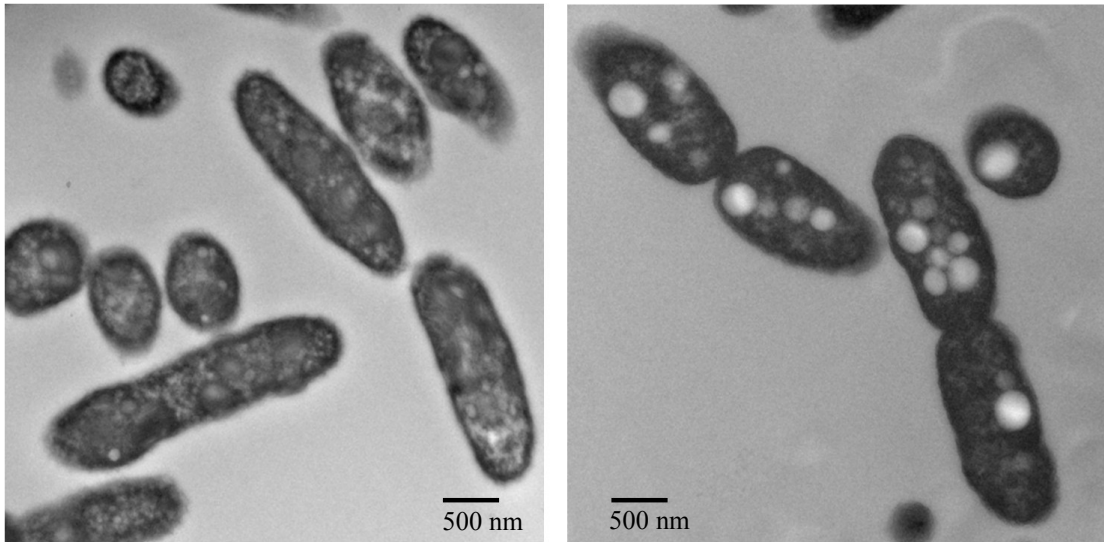
Figure 5. Transmission electron micrographs of (A) *Ectothiorhodospira* sp. strain BSL-9, and (B) *Halomonas* sp. strain BSL-1. BSL-1 was grown in LB media at pH 10, and BSL-9 was grown in BSM with the addition of 5 g/L yeast extract and using malate as an electron donor (20 mM). Bar denotes 500 nm.
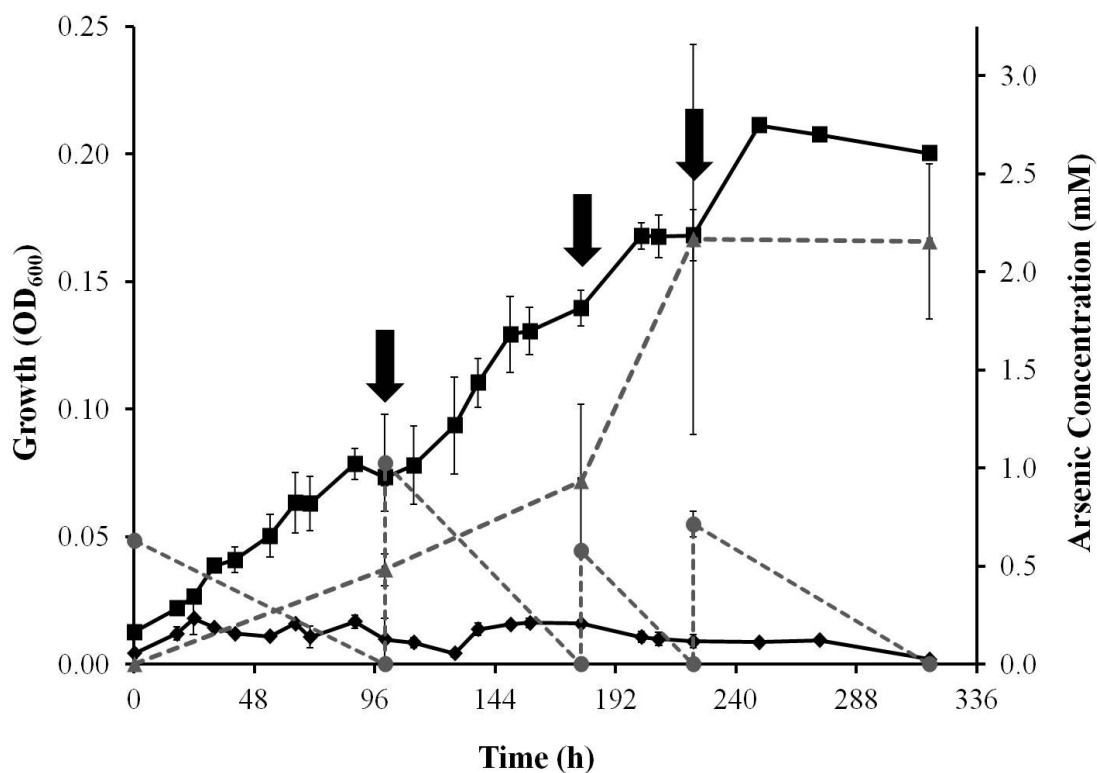
Figure 6. Anaerobic growth of BSL-9 grown phototrophically with As(III) as the electron donor (■), or with no added electron donor (◆) correspond with the left axis (Growth). Arsenic speciation and concentration were determined by HPLC: As(V), (▲); As(III), (●), and correspond with the right axis (Arsenic Concentration). Symbols represent the means for three separate cultures, and error bars indicate ±1 standard deviation. Arrows indicate when arsenite was added to the cultures.
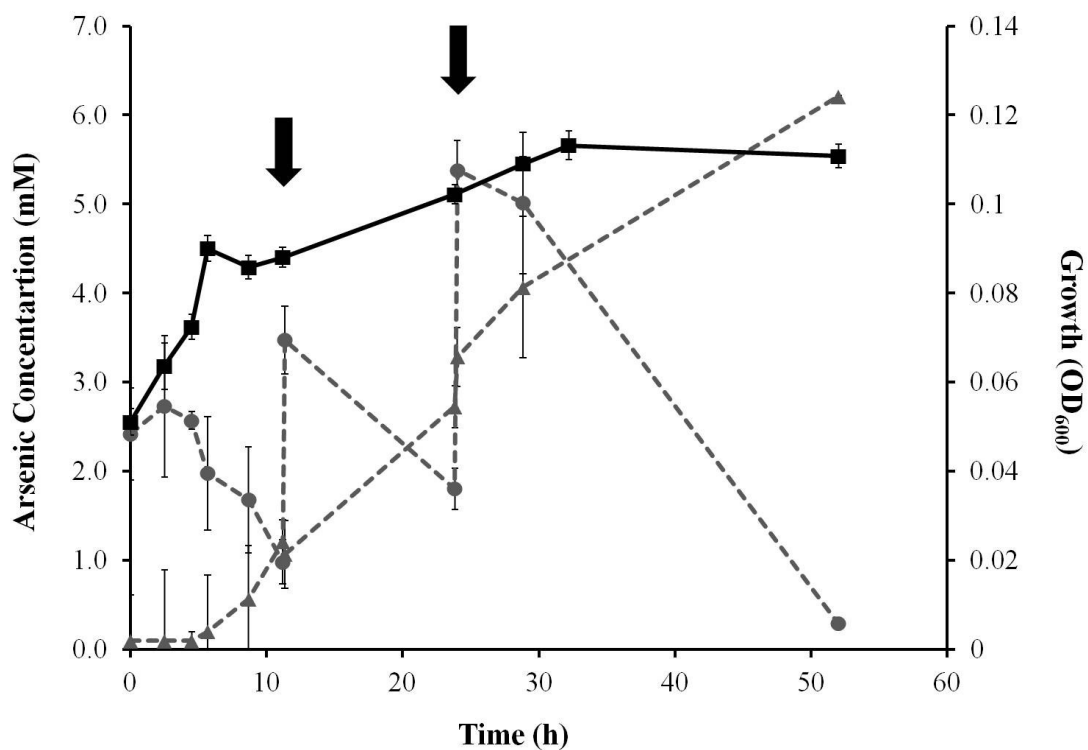
Figure 7. Anaerobic growth of BSL-1 grown with As(III) as the electron donor and nitrate as the electron acceptor (■) corresponds with the right axis (Growth). Arsenic speciation and concentration were determined by HPLC: As(V), (▲); As(III), (●), and correspond with the left axis (Arsenic Concentration). Symbols represent the means for three separate cultures, and error bars indicate ±1 standard deviation.
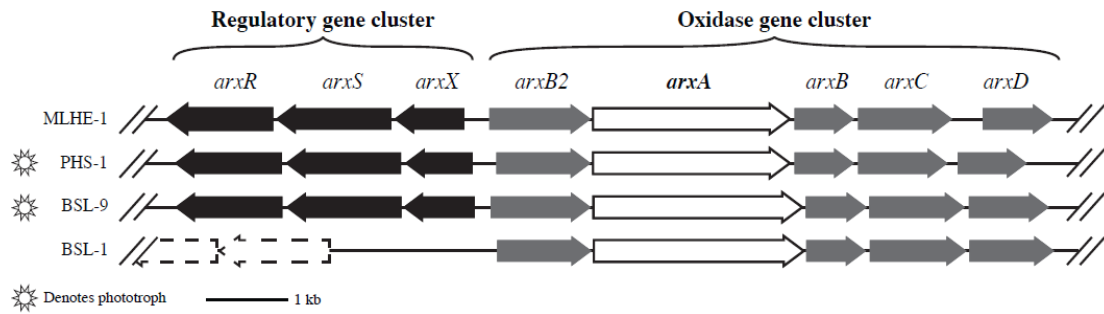
Figure 8. A. BSL-1 and BSL-9 arx operons in comparison to those of PHS-1 and MLHE-1. Genes are shown as arrows to scale, with arrows indicating the orientation of the coding sequence. The BSL-9 and BSL-1 arx operons are predicted to contain: *arxB2*, a [4Fe-4S] containing protein; *arxA*, the molybdopterin containing arsenite oxidase alpha subunit; *arxB*, another [4Fe-4S] containing protein; *arxC*, a membrane anchoring and quinol oxidoreductase subunit; and *arxD*, a TorD-like molybdoenzyme chaperone. BSL-1 appears to lack the *arxXSR* gene cluster predicted to encode regulatory proteins, which has been found in other *arxA*-containing bacteria.

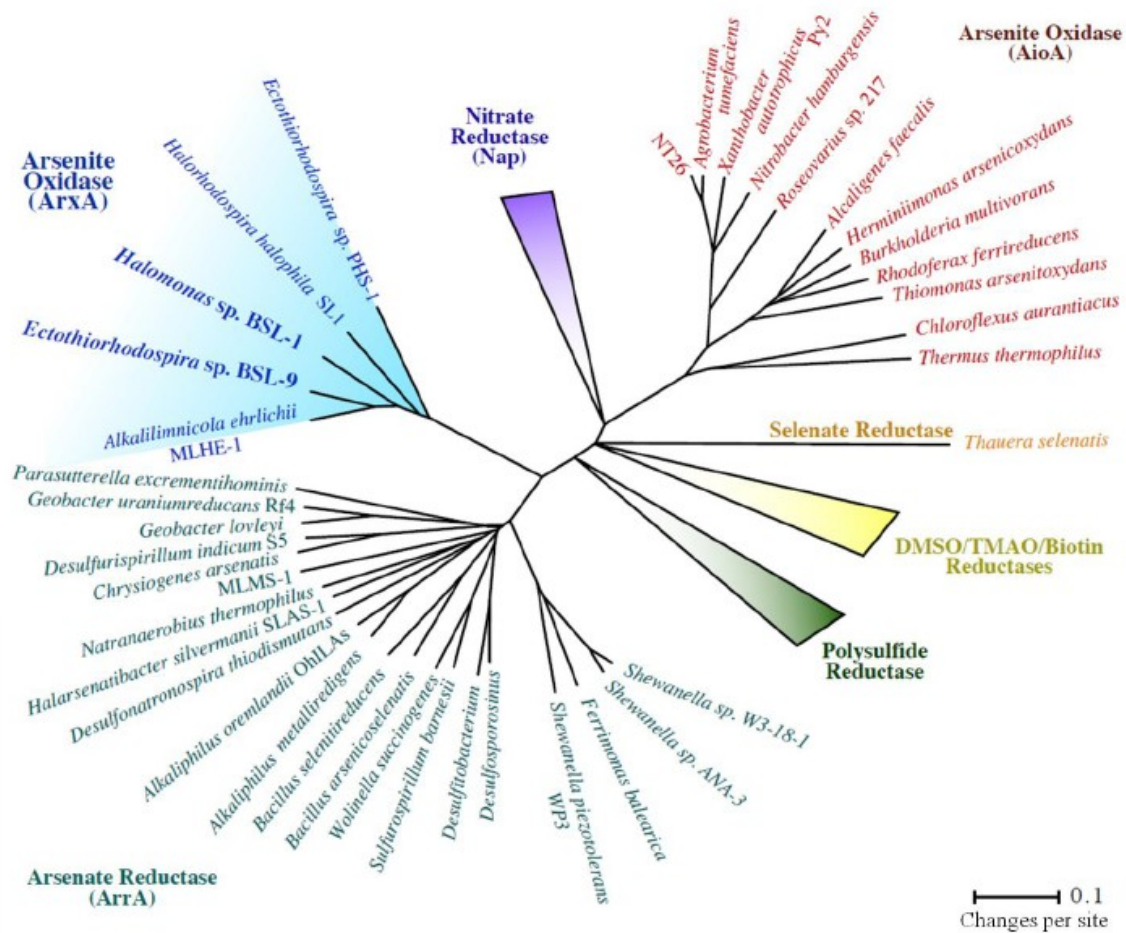**Figure 9.** Phylogenetic analysis of the ArxA of PSH-1 and BSL-1 in relation to other oxidoreductases within the dimethyl sulfoxide (DMSO) reductase family of molybdenum containing enzymes. Phylogenic analysis was conducted using ARB. A CLUSTALW multisequence alignment was generated followed by phylogenetic analysis using distance criteria. Scale bar corresponds to 0.1 amino acid changes per site.

Mono County Site:	Dissolved As μM	As(III) μM	As[III/(III+V)]	Temp. (^{0}C)
Evaporative lake hot spring	5.8	1.03	0.18	40
Wild Willy's hot spring	5.31	3.64	0.69	29
Bath tub hot spring	13.1	2.13	0.16	50
Shepherds' hot spring	6.63	0.43	0.06	50
Navy Beach hot spring	8.85	5.35	0.60	32
Warm Springs	1.19	1	0.84	30
Little Hot Creek #1	8.93	4.84	0.54	50
Little Hot Creek #2	9.62	0.06	0.01	49

Big Soda Lake Depth (BSL)	As(V) μM	As(III) μM
13 m	22.3	0
30 m	23.5	0
35 m	22	0
41 m	0	*
50 m	0	*

Table 1. ICP-MS analysis of total dissolved arsenic and arsenite using the Ficklin method of anion exchange to separate As(V) from As(III). Big Soda Lake (BSL) samples were analyzed by HPLC-ICP-MS. Asterisks indicate values likely to be ~22 μM arsenite because sulfide interfered with the analysis (Hollibaugh et al. 2005)

Appendix A: Work with *Ectothiorhodospira* sp. str. PHS-1

Arsenite-induced arxA *expression in PHS-1*

      To determine if *arxA* expression is induced by exposure to arsenite, *arxA* expression was determined by qRT-PCR. Two groups of PHS-1 cultures were grown using acetate as an electron donor and samples were collected for RNA analysis. After 47 hours, one group of cultures was exposed to 1 mM arsenite. qRT-PCR analysis determined that *arxA* was strongly upregulated in comparison to *gyrB* after arsenite exposure, but not in controls (Figure A1). This suggests that *arxA* has a role in arsenic metabolism in PHS-1, and supports the hypothesis that ArxA is the arsenite oxidase involved in photoarsenotrophy.

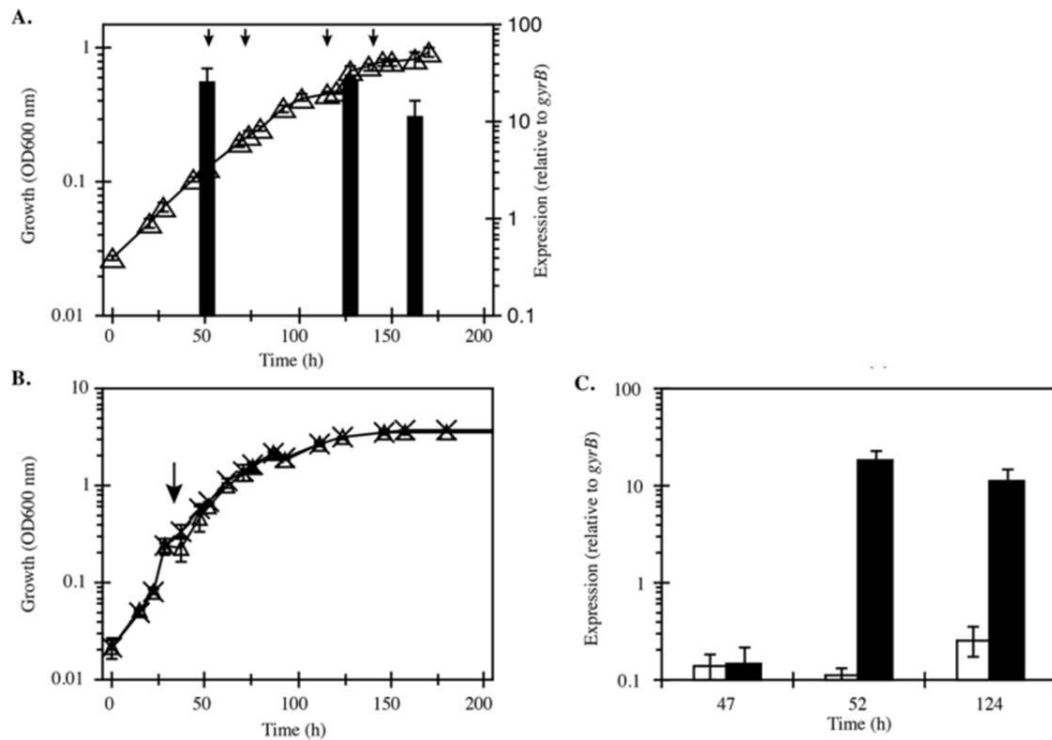   Methods outlined in Zargar et al. 2012.

Figure A1. Gene expression analysis of *arxA* in PHS-1. A. Arsenite-dependent photoautotrophic growth (triangles). The dark bars represent *arxA* gene expression. The

arrows indicate when arsenite was spiked into the cultures. B. The X symbols indicate the growth curve of PHS-1 grown with acetate and spiked with 1 mM arsenite. The arrow indicates when arsenite was spiked into the cultures. Triangles represent growth curve of control cultures that were not spiked with arsenite. C. Expression of *arxA* (relative to *gyrB* housekeeping gene) pre-arsenite induction (47 h) and several time points post arsenite addition (dark bars). The clear bars represent control cultures that did not receive arsenite. Data and error bars represent means and standard deviations respectively of triplicate cultures.

Conjugational DNA transfer experiments with PHS-1

Attempts were made to transfer the plasmid pBBR1MCS-1 (Cm^r) into PHS-1 using

the donor *Escherichia coli* strain WM3064 (Zargar et al., 2010). No chloramphenicol-resistant

colonies of PHS-1 were ever recovered, indicating that either conjugation between the two

organisms did not occur or that pBBR1MCS-1 cannot replicate in PHS-1.

A conjugation protocol derived from Pattaragulwanit et al. 1995 was used

(Pattaragulwanit and Dahl, 1995). PHS-1 and WM3064-pBBRMCS-1 were grown overnight,

spun down and resuspended in 100 µL combined media. Combined cells were pipetted onto

a 0.45 µm HA nitrocellulose membrane (Millipore) and placed on a 0.3% Paoha Island Media

(PIM) agar plate (Zargar et al., 2010), and incubated overnight aerobically in the light at 45ºC.

The filter was used to inoculate an anaerobic liquid PIM culture, which was incubated

overnight in the light at 45ºC. The resulting culture was used to perform an agar dilution

series in PIM media, both with and without chloramphenicol.

Appendix B: Developing *Ectothiorhodospira* sp. str. BSL-9 as a model organism

BSL-9 grows easily on agar plates, is aerotolerant, and has a sequenced genome. Because of this, efforts were made to develop it as a genetic model with which to determine the molecular mechanisms behind photoarsenotrophy.

Antibiotic resistance profile with BSL-9

In order to identify an antibiotic effective against BSL-9, the organism was grown with serial dilutions of the antibiotics chloramphenicol, kanamycin and ampicillin in liquid media. Cultures were grown photoheterotrophically using malate as an electron donor and incubated in the light, anaerobically, at 30°C. BSL-9 was sensitive to chloramphenicol and ampicillin, and resistant to kanamycin (Figure B1). When grown on varying concentrations of chloramphenicol and ampicillin, no colonies were seen at the lowest concentration of chloramphenicol used, 5 μg/mL, but lawns formed on the highest concentration of ampicillin used, 50 μg/mL. Chloramphenicol resistance was picked as the selectable marker of choice for future experiments.
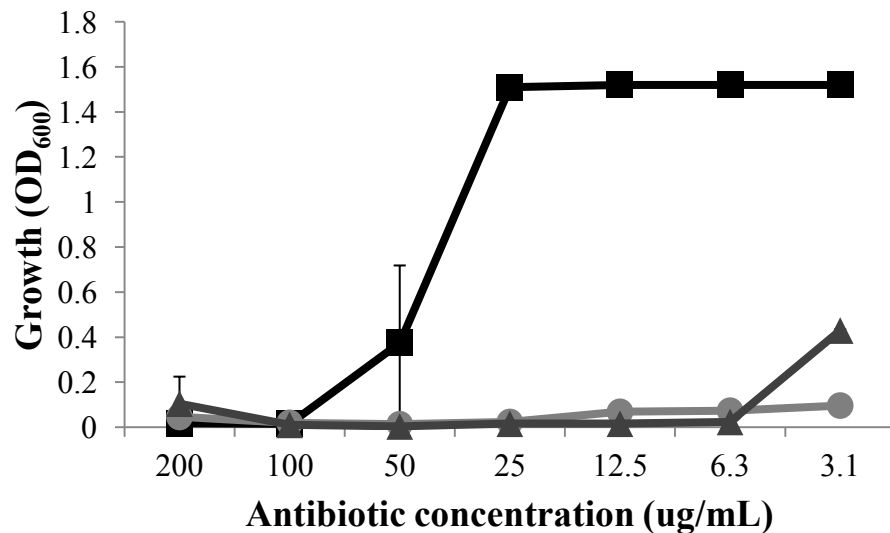
Figure B1. Overnight growth of BSL-9 photoheterotrophically on different antibiotics: kanamycin (■), ampicillin (●) and chloramphenicol (▲). Symbols represent the means for two separate cultures, and error bars indicate ±1 standard deviation.

DNA transfer experiments with BSL-9

Attempts were made to transfer the plasmid pBBR1MCS-1 (Cm^r) into BSL-9 using 1) conjugation with the donor *E. coli* strain WM3064, and 2) by electroporation. DNA transfer by electroporation was never successful. On one occasion, conjugation did result in recovery of chloramphenicol-resistant colonies of BSL-9 (Figure B2). This demonstrated that BSL-9 is capable of uptaking plasmids via conjugation with *E. coli*.

Conjugation was performed when BSL-9 and *E.coli* had reached OD$_{600}$ values of 2.0 and 4.4, respectively. Equal volumes (1.5 mL) of cultures were spun down together and resuspended in 1 mL LB. Bacteria were filtered through 0.45 µm HA nitrocellulose membrane (Millipore) until no more culture could be passed through filter. Filters were placed on top of LB agar plates using sterile forceps and incubated anaerobically at 32ºC in the light for 12 hours. Filters were then rinsed into 1 mL LB, and plated on BSM agar plates with 20 mM acetate as electron donor and containing 20 µg/L chloramphenicol. Plates were incubated anaerobically at 32ºC in the light until colonies developed.

For electroporation, cells were prepared as described elsewhere (Rogers, 1999), taking care to limit their exposure to oxygen. 1 uL of pBBR1MCS-1 DNA was added to 50 µL prepared cells, mixed, and incubated on ice for 15 minutes in pre-chilled electroporation cuvettes with 1 mm gap (Fisherbrand). Electroporation apparatus was set to 2.5 kV, 25 µF and the pulse controller to 200 ohms. Pulse was applied and cells immediately injected into 1 mL anaerobic BSM with 5 g/L yeast extract and incubated overnight in the light at 30ºC. They were then spread plated onto BSM agar plates containing 20 µg/L chloramphenicol and incubated anaerobically at 32ºC in the light
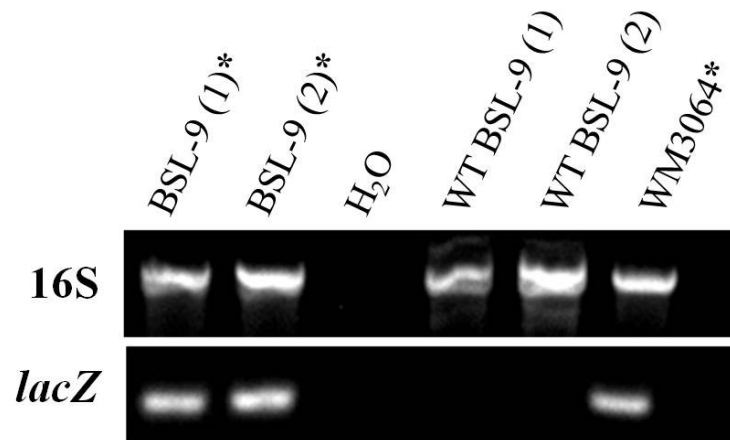
32

Figure B2. PCR amplicons resulting from the amplification of ribosomal 16S and *lacZ* genes from genomic DNA extracts. Presence of *lacZ* indicates the presence of the plasmid pBBR1MCS-1. PCR was carried out using the 8F/1492R and M13 primer sets, respectively. Asterisks indicate DNA originating from a chloramphenicol-resistant organism.

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