UNIVERSITY OF CALIFORNIA
SANTA CRUZ

Ecology and molecular genetics of anoxygenic photosynthetic arsenite oxidation by *arxA*

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

DOCTOR OF PHILOSOPHY

in

MICROBIOLOGY AND ENVIRONMENTAL TOXICOLOGY

by

Jaime Hernandez-Maldonado

June 2017

Dissertation of Jaime Hernandez-Maldonado is approved:

_____

Professor Chad W. Saltikov

_____

Professor. Ronald S. Oremland

_____

Professor Jonathan P. Zehr

_____

Professor Karen M. Ottemann

_____

Tyrus Miller

Vie Provost and Dean of Graduate Studies

Table of contents

List of Figures

Abstract

Ecology and molecular genetics of anoxygenic photosynthetic arsenite oxidation by *arxA*

Jaime Hernandez-Maldonado

Thesis statement:

Anoxygenic photosynthetic arsenite oxidation encoded by *arxA* is a bacterial arsenic metabolism that contributes to the biogeochemical cycle of arsenic in extreme environments.

This dissertation provides molecular genetics and environmental insight into the poorly-understood phenomenon of a photosynthetic microbial metabolism fueled by arsenic. The hypothesis is that *arxA* is critical for photosynthetic arsenite oxidation and actively found in the environment, which has an impact on the arsenic cycle in euphotic arsenite rich extreme environments.

The discovery of light dependent arsenic metabolisms that allows bacteria to conserve energy by oxidizing arsenite to arsenate in the absence of oxygen was described in 2008. This light dependent (photo) arsenic fueled (arsenotrophy) metabolism is referred to as "photoarsenotrophy" or more specifically anoxygenic photosynthetic arsenite oxidation. These earlier findings have yielded interesting molecular genetics and environmental ecology questions that are address in this

dissertation. The first question is that of a molecular genetic nature, namely: Is the arsenite oxidase, ArxA, essential to the photoarsenotrophy mechanism? This question was validated after a genetic system was developed in *Ectothiorhodospira* sp. strain BSL-9, since there were no genetic models to study photoarsenotrophy.

The second question that was investigated was: Do anoxygenic photosynthetic arsenite oxidizing bacteria transform arsenite to arsenate in arsenic rich environments? We surveyed Paoha Island Mono Lake in California and Big Soda Lake, NV, two extreme environments rich in arsenic known to have ideal physicochemical conditions, photosynthetic purple sulfur bacterial and the arsenite oxidase *arxA* gene. The results provide evidence of anoxygenic photosynthetic arsenite oxidation activity by demonstrating in situ *arxA* gene expression within the hot spring pool biofilms of Paoha Island, and also provide evidence for a microbial arsenic cycle that was light dependent within anoxic water column samples that were collected from Big Soda Lake, Nevada. The environmental-functional gene ecology study demonstrated that Big Soda Lake contains microbial populations that can perform anoxygenic photosynthesis coupled to arsenite oxidation and arsenate reduction. These ecological studies further elucidate the biogeochemical cycle of photoarsenotrophy in extreme environments, lay the foundation for future fundamental molecular genetics and raising many questions that have yet to be addressed.

Dedication

This is dedicated to my dear family. Thank you for the tremendous support, patience and guidance throughout this journey. You have all allowed me become a young scientist and pursue my passion for microbiology. Con mucho amor y cariño esto es dedicado a mi querida familia. Gracias por todo el apoyo, paciencia y guia con esta trayectoria en perseguir mi passion por la microbiologia.

Acknowledgments

I would like to thank my advisor Chad W. Saltikov for giving me the opportunity to join his laboratory. He has been fundamental and a great role model in my training to become a young scientist. I am forever grateful for his guidance, training, patience, and allowing me to work independent. I will miss his laboratory and time that we spend mountain biking. My time as a graduate student in his laboratory has been enjoyable. Thank you Chad!!!

I would like to acknowledge all the programs and fellowships that have supported me financially. This dissertation has been possible thanks to funding that I have received over the years from the National Science Foundation (NSF), Initiative for Maximizing Student Development (IMSD) Fellowship, Research Mentoring Initiative (RMI) Fellowship, and the UCSC President's Dissertation Year Fellowship. ACCESS and Maximizing Access to Research Careers (MARC) at UCSC were fundamental to sparking my interest to research. I also would like to thank all the staff that run these program. Many of them who have provided support and mentorship.

I would like to thank all of the USGS group members from California and Nevada. Thank you Ron Oremland, Laurence Miller, Michael Rosen, Shelley McCann for making the environmental sampling trips memorable. It was a pleasure to spend time environmental sampling Mono Lake, CA and Big Soda Lake, NV. Thanks all the past and present Saltikov lab members, specially Alison Boren for your

friendship, support and guidance with the photoarsenotrophy project. Thank you Brendon for your commitment and help with the sequencing project.

I would like to acknowledge Rob Franks who provided guidance and tremendous help in developing an automated system to analyze arsenic. I would also like to thank Stephen Hauskins for all the ITS support and help in installing and running programs that were key to analyzing illumina Next Generation Sequencing (NGS) in order to determine the microbial ecology in Big Soda Lake, NV.

Finally, I would like to thank my entire Hernandez-Ortega family for all their support and unconditional love that they have provided to my daughters, Anareli and Isabel. Special thanks to my wife and daughters for all their support through these years. They have been my inspiration and motivation to pursue science. Muchas gracias Yuli, por todo tu amor y poyo todos estos años.

Chapter 1. Thesis overview

Section 1.01 Introduction

The general field of interest of this dissertation is environmental microbiology. This dissertation, covers the specific aspects of the biology and ecology of a photosynthetic microbial arsenic metabolism. The main focus is to understand the genetics and ecological properties of a light dependent arsenite oxidation metabolism (Budinoff and Hollibaugh, 2008; Kulp *et al.*, 2008), refereed to as "anoxygenic photosynthetic arsenite oxidation" or "photoarsenotrophy" (Hernandez-Maldonado *et al.*, 2017). The objectives were to identify essential genes involved in photosynthetic arsenite oxidation, and investigate the impacts of these metabolisms on the environment. I aim to provide basic biological principles specific to photosynthetic arsenic metabolisms and offer evidence for the activity of this arsenic that may influence arsenic cycling in the environment.

Section 1.02 Current Knowledge

The activity of microorganisms to convert arsenite to arsenate with sunlight was first described in 2008 by two independent groups (Budinoff and Hollibaugh, 2008; Kulp *et al.*, 2008). In one case environmental enrichment cultures collected from sulfidic sediments in Mono Lake, CA, a hypersaline alkaline lake (Budinoff and Hollibaugh, 2008) and were monitored for light dependent arsenite oxidation coupled to photoautotrophy. Alternatively Red-pigmented biofilms in hot springs of Paoha Island Mono Lake, CA, also a hypersaline alkaline environment, were collected (Kulp *et al.*, 2008) examined for photoarsenotrophy. In both independent cases light

dependent arsenite oxidation coupled to photoautotrophy was shown under laboratory conditions. The first photoarsenotrophic bacterium *Ectothiorhodospira* sp. strain PHS-1 was isolated from Paoha Island containing the arsenite oxidase *arxA* gene. To-date no photoarsenotrophic bacteria have been isolated from pH-neutral environments also rich in arsenic and are limited to Mono Lake, California and Big Soda Lake, Nevada. Thus, little is known about the mechanism and impacts of photoarsenotrophy upon arsenic cycling biogeochemical cycling. Anaerobic arsenite oxidation was first described in the chemoautotrophic bacterium *Alkalilimnicola ehrlichii* sp. strain MLHE-1 (Oremland *et al.*, 2002; Hoeft *et al.*, 2007), where the *arxA* gene was shown to encode a novel molybdopterin enzyme, ArxA, essential for arsenite oxidation that coupled with nitrate (Zargar *et al.*, 2010; 2012). Computational biology approaches were key for identifying *arx* gene clusters (*arxB2ABCDE*) homologs in photosynthetic and chemoautotrophic bacterium. Apart from Paoha Island in Mono Lake, CA, Big Soda Lake was also known to have purple bacterial water column blooms (Priscu *et al.*, 1982) and preliminary data (unpublished) indicated the presence of the arsenite oxidase gene, *arxA* genes. Below the oxycline of Big Soda Lake, ideal physicochemical conditions also favored anoxygenic photosynthetic bacteria (Kimmel *et al.*, 1978; Priscu *et al.*, 1982; Cloern *et al.*, 1983; 1987; Zehr *et al.*, 1987). However, no studies had previously investigated the role of these photosynthetic purple sulfur bacteria to anaerobic arsenite oxidation.

Section 1.03 Knowledge gaps

Prior to this work, little was known about the molecular mechanism and biogeochemical cycling of photosynthetic arsenite oxidation. A molybdopterin enzyme, ArxA was shown to be conserved and was identified within the *arxB2ABCDE* gene cluster (Zargar *et al.*, 2012). These genes were identified as potential functional genes essential to photoarsenotrophy but were difficult to study due to the lack of a genetic system. The ecology and activity of photoarsenotrophy is currently limited to investigations of photoarsenotrophy conducted under controlled laboratory conditions, however less is known about the of this metabolism in the environment.

Section 1.04 Hypothesis

The hypothesize are (i) *arxA* is essential for both arsenite oxidation and growth on arsenite and (ii) photosynthetic microbes containing, *arxA* may actively transform arsenite to arsenate in the photic zones of arsenic-rich soda lakes.

Section 1.05 Primary research questions

The primary questions are (i) is *arxA* essential for the photoarsenotrophy to function? and (ii) Can anoxygenic photosynthetic arsenite-oxidizing bacteria oxidize arsenite to arsenate *in situ* in arsenic rich soda lakes?

Section 1.06 Research Design

In order to test our hypothesis and validate *arx* gene clusters essential for photoarsenotrophy a genetic system was developed. A photosynthetic purple sulfur bacterium *Ectothiorhodospira* sp. strain BSL-9 was previously isolated from Big Soda Lake sediments and selected as the candidate bacterium to study

photoarsenotrophy. The *arx* gene cluster is conserved in strain BSL-9. Preliminary data also indicated strain BSL-9 sensitive to chloramphenicol which facilitated screening for strain BSL-9 candidates that uptake foreign DNA (plasmids). Lastly strain BSL-9 has the ability to grow in both liquid and agar media, which facilitated DNA transfer by conjugation. These characteristics facilitated developing a genetic system where a strain BSL-9 *arxA* disruption mutant was generated and shown to inhibit both growth and arsenite oxidation when arsenite was provided as an electron donor (Hernandez-Maldonado *et al.*, 2017).

To study photoarsenotrophy in the environment two alkaline soda environments rich in arsenic were identified, Paoha Island in Mono Lake, CA and Big Soda Lake, NV, as potential model system where photoarsenotrophy likely occurs. Investigations for *arxA* gene expression was determined within Paoha Island hot springs known to be rich in arsenite and dominated by photosynthetic purple sulfur bacteria. The photoarsenotrophy metabolism was also studied in Big Soda Lake, Nevada where there had been reported evidence for *arxA* and purple sulfur bacteria below the oxycline. This dissertation provides evidence for photoarsenotrophy, which was determined in microcosms composed of lake water taken from below the oxycline (~20 m) of Big Soda Lake.

Section 1.07 Purpose of the study

In order to advance our understanding of the biological processes regarding light-dependent arsenite oxidizing bacteria and validate the role of photoarsenotrophy in the environmental arsenic cycle this work was critical. The purpose of this study

was to provide genetic evidence for the arsenite oxidase *arxA* involved in photoarsenotrophy and validate the activity of this metabolism in soda lakes rich in arsenic. This work has provided evidence of light dependent microbial arsenic metabolisms that can impact the arsenic cycle. This work also paves the path for on going questions on the molecular genetics and ecology of photoarsenotrophy. Bioinformatics and computational analysis may further facilitate understanding the occurrence of photoarsenotrophy in the environment by further developing next generation sequencing *arxA* primers as new photoarsenotrophs are isolated from the environment. Photoarsenotrophy adds another layer of complexity to the arsenic biogeochemical cycle that may occur in other euphotic non-extreme environments, rich in arsenic. Understanding these basic biological principles aids in studying microbial arsenic metabolisms at the molecular level in the lab and out in the environment as well, both of which are critical for solving and making future decisions to study the role of microbes on aquatic systems that may impact people that depend on the consumption of arsenic contaminated water.

Section 1.08 Chapter overviews

Chapter 2 includes a review of the biogeochemistry of arsenic, and an overview of arsenic toxicity and distribution. Chapter 3 aims to address the ecology and activity of photoarsenotrophy that may occur in Big Soda Lake, Nevada, an arsenic-rich soda lake. Lake water collected below the oxycline of Big Soda Lake was monitored for light dependent microbial arsenic transformations in microcosm incubations that received multiple arsenite additions over time. At the end of the

experiment the microbial community was analyzed by 16S rRNA and the presence of arsenic transforming genes such as *arxA* and *arrA* was investigated. These studies provide evidence for a light dependent arsenic cycle by native microbes found in the anoxic water column of Big Soda Lake. Chapter 4 expands on the study of photoarsenotrophic activity by proving evidence of *arxA* gene expression within Paoha Island (Mono Lake, CA) hot spring biofilms dominated by purple sulfur bacteria containing *arxA*. It is probable that the presence of *arxA* within these hot spring pools leads to the production of the active functional arsenite oxidase enzyme, ArxA. Most importantly Chapter 4 is a published manuscript where the photoarsenotrophic mechanism is further discussed and molecular genetic studies validate ArxA as the sole arsenite oxidase present in *Ectothiorhodospira* sp. strain BSL-9. Chapter 5 provides a brief genomic evaluation of reported photoarsenotrophs in culture and includes a comparison to other chemoautotrophic microbial genomes containing *arxA*. An analysis of the strain BSL-9 genome discussed which facilitated molecular genetic studies reported in chapter 4 and chapter 5. Chapters 6 is a conclusion of what we learned from these studies and provides remarks on future research directions in arsenic metabolisms with a focus on photoarsenotrophy.

Section 1.09 References

Budinoff, C.R. and Hollibaugh, J.T. (2008) Arsenite-dependent photoautotrophy by an *Ectothiorhodospira*-dominated consortium. *The ISME Journal* **2**: 340–343.

Cloern, J.E., Cole, B.E., and Oremland, R.S. (1983) Seasonal changes in the chemistry and biology of a meromictic lake (Big Soda Lake, Nevada, U.S.A.). *Hydrobiologia* **105**: 195–206.

Cloern, J.E., Cole, B.E., and Wienke, S.M. (1987) Big Soda Lake (Nevada) .4. Vertical fluxes of particulate matter - seasonality and variations across the chemocline. *Limnology and Oceanography* **32**: 815–824.

Hernandez-Maldonado, J., Sanchez-Sedillo, B., Stoneburner, B., Boren, A., Miller, L., McCann, S., et al. (2017) The genetic basis of anoxygenic photosynthetic arsenite oxidation. *Environ Microbiol* **19**: 130–141.

Hoeft, S.E., Blum, J.S., Stolz, J.F., Tabita, F.R., Witte, B., King, G.M., et al. (2007) *Alkalilimnicola ehrlichii* sp. nov., a novel, arsenite-oxidizing haloalkaliphilic gammaproteobacterium capable of chemoautotrophic or heterotrophic growth with nitrate or oxygen as the electron acceptor. *Int. J. Syst. Evol. Microbiol* **57**: 504–512.

Kimmel, B.L., Gersberg, R.M., Paulson, L.J., Axler, R.P., and Goldman, C.R. (1978) Recent changes in the meromictic status of Big Soda Lake, Nevada. *Limnology and Oceanography* **23**: 1021–1025.

Kulp, T.R., Hoeft, S.E., Asao, M., Madigan, M.T., Hollibaugh, J.T., Fisher, J.C., et al. (2008) Arsenic(III) fuels anoxygenic photosynthesis in hot spring biofilms from

Mono Lake, California. *Science* **321**: 967–970.

Oremland, R.S., Hoeft, S.E., Santini, J.M., Bano, N., Hollibaugh, R.A., and Hollibaugh, J.T. (2002) Anaerobic oxidation of arsenite in Mono Lake Water and by a facultative, Arsenite-Oxidizing Chemoautotroph, strain MLHE-1. *Applied and Environmental Microbiology* **68**: 4795–4802.

Priscu, J.C., Axler, R.P., Carlton, R.G., Reuter, J.E., Arneson, P.A., and Goldman, C.R. (1982) Vertical profiles of primary productivity, biomass and physico-chemical properties in meromictic Big Soda Lake, Nevada, U.S.A. *Hydrobiologia* **96**: 113–120.

Zargar, K., Conrad, A., Bernick, D.L., Lowe, T.M., Stolc, V., Hoeft, S., et al. (2012) ArxA, a new clade of arsenite oxidase within the DMSO reductase family of molybdenum oxidoreductases. *Environ Microbiol* **14**: 1635–1645.

Zargar, K., Hoeft, S., Oremland, R., and Saltikov, C.W. (2010) Identification of a novel arsenite oxidase gene, *arxA*, in the haloalkaliphilic, arsenite-oxidizing bacterium *Alkalilimnicola ehrlichii* strain MLHE-1. *Journal of Bacteriology* **192**: 3755–3762.

Zehr, J.P., Harvey, R.W., and Oremland, R.S. (1987) Big Soda Lake (Nevada). 1. Pelagic bacterial heterotrophy and biomass. *Limnol. Oceanogr*.

Chapter 2. Microbial arsenic review

Section 2.01 Introduction

Arsenic (As) is the 33rd element and occurs naturally in the environment (Smedley and Kinniburgh, 2002). The biogeochemical cycle of arsenic is complex. The speciation, toxicity and mobility of arsenic can be affected by hydrology, mineralogy, chemical speciation and biological reactions (Dowling *et al.*, 2002; Fendorf *et al.*, 2010); (Pallud *et al.*, 2010); (Rakhunde *et al.*, 2012; Dhuldhaj *et al.*, 2013) (Oremland, 2003; Polizzotto *et al.*, 2006; Sharma *et al.*, 2011). The two major inorganic arsenic species in the environment are arsenite (As^{III}) and arsenate (As^{V}) (Ferguson and Gavis, 1972; Smedley and Kinniburgh, 2002; Polizzotto *et al.*, 2005; Hohmann-Marriott and Blankenship, 2011). The health of millions of people in regions such as the Bengal area are at greater risk due to consumption of contaminated arsenic well water (Harvey *et al.*, 2005; Benner *et al.*, 2008; Fendorf *et al.*, 2010; Burgess *et al.*, 2010; Mailloux and Trembath-Reichert, 2013). However abundant prokaryotes have evolved favorable diverse biochemical mechanisms that allow them to cope with arsenic and in some cases microbes can gain energy by metabolizing arsenic (Mukhopadhyay *et al.*, 2002; Oremland, 2003; Stolz *et al.*, 2010; Cavalca *et al.*, 2013; Amend *et al.*, 2014). In order to understand the impact of microbial arsenic metabolisms to the biogeochemical cycle of arsenic, this review will focus on prokaryotic arsenic metabolisms. This chapter provides a review of arsenic distribution, abundance, toxicity, and microbial metabolisms. The underlying work of this dissertation is to study the ecology and molecular genetics of a light

dependent arsenic fueled mechanisms that allow photosynthetic bacteria to thrive in arsenic rich extreme environments. These light-dependent microbial arsenic metabolisms convert a toxic arsenic form, arsenite, to a less toxic form, arsenate.

Section 2.02 Arsenic distribution and speciation

Arsenic is naturally found on the earths crust and both anthropogenic and natural sources play a role in the release, transport, mobilization and transformation of arsenic in the environment. In most environment arsenic occurs mostly in trace amounts and in certain areas arsenic is found in high concentrations (Mukhopadhyay *et al.*, 2002; LiEvremont *et al.*, 2009). On average soils have about 7.2 mg kg^{-}, and aquatic environments about 0.4-2.6 µg l^{-} on the contrary high soluble levels of arsenic are a great concern in groundwater (Radloff *et al.*, 2011). The highest levels of arsenic occur naturally in groundwater in parts of India, Bangladesh and in other regions of South East Asia (Harvey *et al.*, 2002). These regions rely on groundwater for irrigation purposes and drinking water since these aquatic systems serves as pathogen free water sources (Mandal and Suzuki, 2002; LiEvremont *et al.*, 2009). Other countries have reported arsenic levels higher than the Environmental Protection Agency (EPA) standard drinking water level such as Chile, Argentina, México and in the U.S (Ferguson and Gavis, 1972; Smedley and Kinniburgh, 2002). Determining the distribution of arsenic overtime is challenging to study since each environment is unique and its governed by complex biogeochemical factors.

Biotic factors that can alter arsenic solubility and speciation are redox potential (electron potential) and pH (hydrogen potential) (Smedley and Kinniburgh,

2002). Usually oxic and anoxic environments have different redox potential, which can impacts arsenic cycling in the environment (Masscheleyn *et al.*, 1991). Typically under neutral pH oxic environments, arsenate is the dominant arsenic species (Smedley and Kinniburgh, 2002). As environments become anoxic, arsenite becomes the dominant species and mobile. Besides the redox potential and pH, which impact arsenic speciation, a few minerals have a high affinity for arsenate, the most common being iron, aluminum, and magnesium oxides/hydroxides (Pallud *et al.*, 2010) and to a less extent arsenite. The greatest natural occurrence of arsenic poisoning of groundwater is in South East Asia regions (Quiñones, 2000). Unlike geothermal environments where arsenic comes from sub-surface volcanic activity, in South East Asia arsenic is believed to come from the weathering of minerals from the Himalaya Mountains (Acharyya *et al.*, 1999). Redox potential changes(Polizzotto *et al.*, 2006; Jung *et al.*, 2012) and iron dissolution (Polizzotto *et al.*, 2005) are thought to be important factors in arsenic cycling and mobility in groundwater environments. Hydrological activity adds another layer of complexity to factors that regulate arsenic mobility in groundwater environments consequently. Hydrological activities can mobilize high levels of arsenic by advective transport properties, which are dominant in solute mobility over diffusive properties (Harvey *et al.*, 2006; Tufano *et al.*, 2009). Arsenic distribution, availability, stability and mobility (Goldberg, 2002) can increase or decrease as the arsenic speciation changes which in the end affect toxicity.

Section 2.03 Arsenic toxicity

Some of the characteristics and properties of arsenic in aqueous solutions are odorless, colorless and tasteless, therefore challenging to detect adequate concentrations without sophisticated equipment. In 2011 the Agency for Toxic Substances and Disease Registry (ATSDR) placed arsenic as the number one toxic substance on their priority list. Toxic substances are ranked based on frequency, toxicity and potential for human exposure. According to the EPA the standard drinking level of arsenic is currently 10 μg/l. Various countries on the other hand have a higher limit of 50 μg/l. Arsenic exists in many stable oxidation states: +5, +3, 0, and -3, equivalent to pentavalent arsenate [$HAsO_4^{2-}$; $H_2AsO_4^-$; As^V], trivalent arsenite [H_3AsO_3 ; As^{III}], elemental arsenic [As], and arsine gas [AsH_3] (Smedley and Kinniburgh, 2002), respectively. Arsenic is also found in many organic compounds but are less toxic and abundant than inorganic arsenic; therefore, this review will greatly focus on inorganic arsenic. In the ecosystem, As^V and As^{III} are the dominant inorganic arsenic forms and consequently of most concern in public health (Mandal and Suzuki, 2002), as arsenic contaminates water supplies (Mandal and Suzuki, 2002; LiEvremont *et al.*, 2009), such as the Bengal region. Arsenic can cause acute symptoms can lead to lesions through out the body, skin pigmentation, diarrhea, and even affect other systems such as neurological, developmental and cardiovascular (Mandal and Suzuki, 2002). Moreover chronic arsenic consumption can lead to tumors and eventually skin, lung, liver and bladder cancers (Cuzick *et al.*, 1992; Chen *et al.*, 1992; Lai *et al.*, 1994; Nickson *et al.*, 1998; Acharyya *et al.*, 1999; Smith *et al.*, 2000; Mandal and Suzuki, 2002).

Arsenic toxicity and bioavailability depends on the oxidation state of arsenic (Rakhunde *et al.*, 2012). Soluble pentavalent arsenate, As^{V}, and trivalent arsenite, As^{III}, are toxic and the most abundant forms of arsenic to living organisms, since being in a soluble form facilitates arsenic uptake. Arsenate molecules have similar atomic radius, electronegative properties and can mimic phosphate (Wolfe-Simon *et al.*, 2011). This is problematic because cells cant distinguish arsenate from phosphate. Furthermore in the presence of As^{V}, it competes with phosphate molecules and can inhibit ATP synthesis (Crane and Lipmann, 1953), chemical energy production. This in return decouples oxidative phosphorylation where less energy is yield. Differences in the reactivity and the short lifetime of arsenate molecules can lead to hydrolysis in arsenate vs. phosphate molecules (Wolfe-Simon *et al.*, 2011). Arsenite on the other hand has a greater affinity for sulfhydryl compounds, such as enzymes involved in energy production, pyruvate dehydrogenase (Oremland, 2003). Arsenite can induce reactive oxygen species which can disturb protein structure and function within living cells, causing DNA damage, and eventually cell death (Mandal and Suzuki, 2002; Cleiss-Arnold *et al.*, 2010; Dhuldhaj *et al.*, 2013). Prokaryotes have evolved beneficial arsenic metabolizing and detoxification mechanism which allow microorganism to cope with this metalloid by decreasing toxicity and in some cases providing energy for growth.

Section 2.04 Prokaryotic arsenic metabolisms

Even though arsenic is toxic to most of life forms (eukaryotes), prokaryotes have evolved unique pathways that allow them to detoxify (Rosen, 2002) and in some

cases yield energy by utilizing arsenic during metabolism (Oremland *et al.*, 2009). Arsenic islands are regions where genes specific to arsenic metabolisms are found, and can located on plasmids and in others cases they are found in the chromosomes of prokaryotes (Lloyd and Oremland, 2006; Cavalca *et al.*, 2013; Andres and Bertin, 2016a). These gene pathways fit into four groups: (1) anaerobic arsenate respiration by *arr* genes (Saltikov and Newman, 2003), (2) aerobic arsenite oxidation by *aio* genes (Warelow, 2013), anaerobic arsenite oxidation by *arx* genes (Oremland *et al.*, 2002; Zargar *et al.*, 2010), (3) arsenate detoxification by *ars* genes (Rosen, 2002), and (4) arsenic detoxification by methylation (Mukhopadhyay *et al.*, 2002). The most recent bacterial arsenic metabolisms are anaerobic arsenite oxidation by *arx* genes. These mechanism have been observed in chemoautotrophic (Oremland *et al.*, 2002; Hoeft *et al.*, 2007; Zargar *et al.*, 2010; 2012) and anoxygenic photosynthetic bacteria (Kulp *et al.*, 2008; Budinoff and Hollibaugh, 2008; Hernandez-Maldonado *et al.*, 2017). There are a numerous prokaryotes known to have diverse mechanisms that assist in metabolizing arsenic, and have been detected in extensive environments.

Prokaryotic that are able to utilize arsenic as an energy source are referred to as "arsenotrophs" (Oremland *et al.*, 2009). Later have distinct enzyme that can catalyze arsenic in order to conserve energy. These enzymes, arsenite oxidase (AioA), anaerobic arsenite oxidase (ArxA), and arsenate reductases (ArrA) form three distinct clades within the DMSO family of molybdopterin enzymes (McEwan *et al.*, 2010). Respiratory arsenate reductase ArrA (Krafft and Macy, 1998; Saltikov and Newman, 2003; Afkar *et al.*, 2003; Malasarn *et al.*, 2004), and arsenite oxidase AioA

(Anderson *et al.*, 1992; Ellis *et al.*, 2001; Santini and vanden Hoven, 2004; Warelow *et al.*, 2013) which have been greatly characterized. The biochemical pathway of anaerobic arsenite oxidation by ArxA, has yet to be fully elucidated. The three oxidoreductases are heterodimers (ArrAB, AioAB, ArxAB2/B1) (McEwan *et al.*, 2010). Generally the small subunits (ArrB, AioB, ArxB2/B1) contain Rieske-type [2Fe-2S] clusters, which facilitate electron transfer, this complex is typically found in the periplasm (Saltikov, 2011). Arsenate reductase, ArrA, is composed of a large subunit (~95kD) which contains molybdenum cofactor and [4Fe-4S] in the catalytic site and a twin arginine motif (TAT) that guides ArrA to the periplasm after being synthesized in cytoplasm. Arsenite oxidase also has two subunits that are now referred as to AioA and AioB (Lett *et al.*, 2011). Similarly AioA, has [4Fe-3S] instead of [4Fe-4S] in the catalytic site. AioB is the other subunit containing a Rieske-Type [2Fe-2S] cluster and unlike the arsenate reductase, AioB is the subunit that contains the TAT sequence (Muller *et al.*, 2003). Lastly, the new arsenite oxidase ArxA has the TAT sequence in the subunit ArxA and the [4Fe-4S] motif is more similar to ArrA than AioA. Genomic evidence also show two proteins, ArxB' and ArxB, which one is upstream and the other one is downstream of ArxA. These two proteins, ArxB' and ArxB contain [4Fe-4S] and are different than the small subunit AioB which only contain [2Fe-2S] (Zargar *et al.*, 2010; 2012). Differences in their small subunit may explain why *arxAB* function under anoxic conditions, but further experiments are needed in order to characterize the role of *arxB2* and *arxB1*. Interestingly enough all three, AioAB, ArrAB, ArxAB, enzyme complexes have

similarities such as molybdenum active sites, Fe-S clusters that facilitate electron shuttling and all three enzymes are associated with the periplasm and the inner membrane. ArxA clusters more closely with ArrA than Aio on a phylogenetic analysis (Zargar *et al.*, 2012) however the three clades are separate from one another and have distinct activities.

Section 2.05 Anaerobic arsenate respiratory reductase (ArrAB)

Anaerobic arsenate breathing bacteria that utilize arsenic as a terminal electron donor are commonly referred to as dissimilatory arsenate-reducing prokaryotes (DARP's), or arsenate respiring bacteria. These organisms couple arsenate reduction to organic (lactate, acetate e.g.) or inorganic (H_2, and HS) oxidation (Newman *et al.*, 2009). The first bacterium known to respire As^V anaerobically was MIT-13, which was isolated in 1994 (Ahmann *et al.*, 1994). However the purification and characterization of the arsenate reductase (ArrA) was first shown in *Chrysiogenes arsenatis* (Krafft and Macy, 1998). The crystal structure of ArrA is still yet to be crystallized. Some of the questions that arose from the *C. arsenatis* work was the specific characteristics of both ArrA and ArrB and the potential role of ArrB to transfer electrons to ArrA in the electron transport chain (Macy *et al.*, 1996; Krafft and Macy, 1998). Another arsenate reducing bacterium that has characterized ArrAB, is *Bacillus selenitireducens* MLS-10. This gram-positive bacterium was isolated from Mono Lake, CA. The respiratory arsenate reductase of MLS-10 (Afkar *et al.*, 2003) has a higher specificity or arsenate, moreover the Km for arsenate is 34 μM vs. the ArrA in *C. arsenatis* Km for arsenate is 300 μM (use this infor to talk

about arrA in BSL). Future crystal structures of ArrAB enzymes could provide insights to their activity. Later *Shewanella* sp. strain ANA-3 was isolated, which served as a good tractable genetic system, to study *arrA* (Saltikov and Newman, 2003). Genetic manipulation with ArrAB established that ArrA is the sole arsenate reductase during anaerobic respiration on arsenate. Speculation about ArrAB being acquired through horizontal gene transfer was raised since other bacteria from distinct phyla also contain homologues of ArrAB. The ArrAB where shown to be on the same operon and containing a terminal loop which terminates this polycistronic mRNA product (Saltikov and Newman, 2003; Croal *et al.*, 2004; Saltikov, 2011), and a FNR binding site upstream of the operon. Since the ArrAB is a soluble periplasmic enzyme it require *cymA*, a membrane bound cytochrome that links electron transfer from the quinone pool to the terminal electron acceptor, arsenate, which is essential to arsenate respiration and also short circuits other anaerobic respiration pathways (Murphy and Saltikov, 2007). An interesting regulation that is noticed in *Shewanella* sp. strain ANA-3 but not in *C. arsenatis* is that nitrate inhibits *arr* synthesis in *Shewanella* but this may not be the case for *C. arsenatis.* It is thought that nitrate is the better terminal electron acceptor thermodynamically therefore inhibits *arr* expression. Moreover specific arsenate sensing proteins have yet to be identified and characterized, which could give insights into arsenate respiratory regulation. Countless DARP phyla have been identified (Oremland and Stolz, 2005; Cavalca *et al.*, 2013) in microbial genomes, but a globally understanding of arsenate respiration

is need to better understand how bacteria cope with arsenic and regulate pathways that allow bacteria to be resistant high levels of arsenic

Section 2.06 Arsenite oxidase (AioAB)

Arsenic oxidation is carried out by arsenite oxidase named (Aio) which is the current nomenclature (Lett *et al.*, 2011) and has been referred to as Aro, Aox and Aso as well (Silver and Phung, 2005). Many bacteria have been identified that contain this enzyme which allows bacteria to either yield energy (chemoautotrophic arsenite oxidizers- CAO) or detoxify (Heterotrophic arsenite oxidizers- HAO) arsenic away from the cell (Cavalca *et al.*, 2013), in both cases by altering As^{III} to As^{V}, a less toxic form of arsenic. Arsenite oxidation was first noticed in 1918, but it was purified and characterized by *Alcaligenes faecalis,* a heterotrophic prokaryote in 1992 (Anderson *et al.*, 1992). Later AioAB was purified and identified in the aerobic strain NT-26 (Santini *et al.*, 2000; Santini and vanden Hoven, 2004). Both AioAB from the *A. faecalis* and NT-26 are homologous and carry out the same reaction with the exception that *A. faecalis* has a $\alpha_1\beta_1$ and NT-26 has a $\alpha_2\beta_2$ conformation (Santini and vanden Hoven, 2004). Recent work shed light on possible reasons why the arsenite oxidase, Aio, of heterotrophs that cannot gain energy for growth and moreover function as detoxifying mechanism. The crystal structure of Aio in the chemoautotrophic bacterium, *Alphaproteobacterium Rhizobium* sp. NT-2,6 was compared with the Aio crystal structure from heterotroph *Alcaligenes faecalis.* Typically the Aio in *A. faecalis* serves as a mean for detoxification purposes whereas the Aio from NT-26 gain energy when As^{III} is oxidized to As^{V}. Differences in

specific residues that surround the small subunit (AioB) contains the Rieske small subunit which facilitate electron transfer in the electron transport chain. These differences allow the enzymes to acquire different redox potentials which may explain the difference in their activity (Warelow *et al.*, 2013). Current work indicate the detection of AioA in photosynthetic bacteria such as *Chloroflexus* and *Proteobacteria* within microbial mats (Engel *et al.*, 2012), which add complexity to the biogeochemical cycle of arsenic, but the genetics and ecological impact remain unknown.

Microarray studies in response to arsenite stress in *Herminiimonas arsenicoxidans* suggests that the bacterium goes through two different types of response in order to cope with arsenite. Gene expression was compared after 15 minutes of adding As^{III} and at a later phase, 8 hrs. This bacterium is known to have both *aio* and *ars*. In early phase general stress response mechanisms are unregulated such as genes involved in protecting the cell for oxidative stress are expressed along with glutathione (GSH) biosynthesis pathways. In both cases the cell is protected when arsenite is dominant in the cell environment. In the later phase after 8hrs, *aio,* chemotactic pathway related genes and other specific phosphate transporters pathways are expressed. This work suggests that the two distinct global regulation patterns in early vs. late exposure are different, where in early phase general stress response pathways are expressed and later phase expresses pathways more specific to arsenic metabolisms (Cleiss-Arnold *et al.*, 2010).

Section 2.07 Anaerobic arsenite oxidase (ArxAB)

Anaerobic arsenite oxidation mechanisms are now divided into two groups, both of which can conserve energy by oxidizing arsenite (1) arsenite oxidation coupled to nitrate respiration has been reported in the chemoautotrophic bacterium, *Alkalilimnicola ehrlichii* sp. strain MLHE-1 (Zargar *et al.*, 2010) and (2) light dependent arsenite oxidation in anoxygenic photosynthetic bacteria has been reported in *Ectothiorhodospira* sp. strain PHS-1 and *Ectothiorhodospira* sp. strain BSL-9 (Hernandez-Maldonado *et al.*, 2017). Due to the arsenite oxidase from strain MLHE-1 having both arsenate reductase and arsenite oxidase enzymatic properties *in vitro*, *arx* was selected as the nomenclature for the anaerobic arsenite oxidase gene. In fact, ArxA is distinct enzyme, diverging away from ArrA and AioA (Zargar *et al.*, 2012). The arsenite oxidase gene *arxA* seems to be conserved in photosynthetic bacteria and in chemoautotrophic bacteria (Zargar *et al.*, 2012; Andres and Bertin, 2016b). Arsenic detoxification mechanisms by *arxA* may exist but have yet to be identified. The discovery of anaerobic arsenite oxidation encoded by *arxA* is an arsenic metabolism that adds to the biogeochemical cycle of arsenic.

Anaerobic arsenite oxidation was first observed in 2002 by the U.S. Geological Survey Menlo Park, California, USA. Typically, arsenite is stable under anoxic conditions however in the anoxic regions of the Mono Lake water column arsenate was detected as the dominant arsenic species. To investigate microbial anaerobic arsenite oxidation occurring in Mono Lake, microcosm experiments provided evidence for this hypothesis (Hoeft *et al.*, 2010). *Alkalilimnicola ehrlichii* sp. strain MLHE-1, was the first haloalkaliphilic anaerobic arsenite-oxidizing

chemoautotrophic bacterium isolated (Oremland *et al.*, 2002). It is now well established that *arxA* forms a a distinct clade in the DMSO enzymes family (Zargar *et al.*, 2010; 2012). Zargar et al elucidate the role of ArxA as the arsenite oxidase responsible for oxidizing arsenic in strain MLHE-1 by disturbing *arx* (*mlg_0216*). The genetic studies showed that ArxA, is an essential enzyme that allow bacteria to couple arsenite oxidation to nitrate reduction. The disruption of *arxA* in strain MLHE-1 was unable to grow solely on arsenite, nor was it able to oxidize arenite to arsenate or reduce nitrate. Two other chemoautotrophic bacteria known to conserve energy through the coupling of arsenite oxidizing and denitrification are DAO1 and DAO10 (Rhine *et al.*, 2006), but the mechanisms remains unknown.

Light dependent arsenite oxidation was first observed in Mono Lake (Budinoff and Hollibaugh, 2008; Kulp *et al.*, 2008). *Ectothiorhodospira* sp. strain PHS-1 was shown to produce chemical energy by utilizing light energy and arsenite alone, conserving energy by a different electron transport chain than chemoautotrophic bacteria containing *arxA* such as MLHE-1 (Kulp *et al.*, 2008). The molecular mechanism by *arxA* was elucidated in this dissertation (Hernandez-Maldonado *et al.*, 2017), also referred to as "photoarsenotrophy". The proposed model for ARX-dependent arsenite oxidation coupled to anoxygenic photosynthesis to generate ATP an NADP(H) includes a cyclic and non-cyclic electron flow mechanism. Arsenite oxidation is believed to generate a proton motive force that in return produce by reverse electron transport chain of NADH dehydrogenase (NDH) in order to generate NADP(H) and ATP. Electron flow from arsenite oxidation

(dashed lines) to the photosynthetic reaction center (RC) may be mediated by a quinone (Q) or a *c*-type cytochrome such as cyt-c_2 (Fig. 1). Furthermore, the ecological impact of photoarsenotrophy is highlighted by in this dissertation. Evidence of photoarsenotrophy activity in the environment was provided by the detection of *arxA* mRNA *in situ* of Paoha Island. Bioinformatics and computational analysis indicate that *arxA* may be wide spread due to detection of *arxA* like sequences in other hot springs such as Yellowstone, and other non extreme environments (Zargar *et al.*, 2012; Hernandez-Maldonado *et al.*, 2017). The characterization of *arxA* will facilitate ongoing metagenomic and transcriptomic analysis to further validate the ecology of *arxA* containing photosynthetic bacteria in non-extreme environments, which is unknown. Apart from these prokaryotes arsenic metabolisms that aid in energy conservation other detoxification mechanisms are involved in metabolizing arsenic.

Section 2.08 Arsenic Resistance Microorganisms (*arsC*)

Prokaryotes that are unable to generate energy when metabolizing arsenic, typically have detoxifying (*ars*) mechanisms which are the most abundant gene pathways found in prokaryotes base on genomic evidence (Rosen, 2002; Dhuldhaj *et al.*, 2013). There are two common arsenate reductase homologs that prokaryotes utilize to detoxify arsenic in the cytoplasm. The first *ars* pathway has been characterized in *E. coli* where *arsC* is located in a R773 plasmid. To reduce As^{III} to As^{V} glutaredoxin and glutathione are needed to accept electrons from As^{III}. The second arsenate reductase studied is in *S. aureus* which also has *arsC* on a plasmid

p1258. This enzyme uses thioredoxin in order to reduce As^{III}. In *Shewanella* sp. strain ANA-3 *arsC* has been shown to be induced by As^{III} in both oxic and anoxic conditions. Under arsenate conditions *arsC* is expressed after As^{III} has accumulated typically at exponential phase if cells are grown anaerobically under As^{V}. The *ars* mechanisms are effective in converting arsenate to arsenite in order to remove arsenite away from the cell by energy requiring pumps. These mechanisms are advantageous and critical for prokaryotes to cope with high arsenic concentration in the environment.

Section 2.09 Arsenate uptake and exclusion (*pit*, *pst*, *glf and arsAB*)

In the presence of arsenate and arsenite there are two common ways cell can uptake arsenic adventitiously. Arsenate can be up taken through phosphate transporters since it mimics phosphate quite well. Two well-known phosphate transporters are the *pst* and *pit* systems in *E. coli* (Rosen, 2002). In neutral pH arsenite is uncharged and it is suggested to enter the cell through aqua-glyceroporins (Meng *et al.*, 2004). Uniporter *arsB*, can pump As^{III} away from the cell through chemiosmosis, where as in other cases ArsA, an ATPase can facilitate arsenic removal by utilizing chemical energy to pump arsenic away from the cell. ArsB is an antiporter that catalyzes the exchange of trivalent metalloid for protons, coupling As^{III} efflux to the electrochemical proton gradient (Meng *et al.*, 2004). There are a few regulatory mechanisms that have been observed to control these diverse arsenic metabolisms.

Section 2.10 Arsenic regulatory pathways (*arsR*, *aioXSR, arxXSR*)

Arsenate respiratory regulation has been well characterized in *Shewanella* sp. strain ANA-3. The two major regulatory genes that play a role in activating *arr* and *ars* are *arsR* and *crp*. The repressor, *arsR,* binds to the operon region of *arr*, and only in the presence of As^{III}, can the repression be relieved, where *arr* can then be expressed anaerobically (Murphy and Saltikov, 2009). The carbon catabolite repression protein *crp* can also regulate arsenate *arr,* arsenate respiration, by bind to its operon region and activating *arr* expression (Murphy *et al.*, 2009). Under oxic conditions the absence of *arsR* allows *arsC* to be induced but not *arr*. Genomic data show evidence of *arrSR* as potential arsenate regulatory proteins in other organisms, where *arrS* a histidine kinase and *arrR*, phosphorylated response regulator encode for a two-component regulatory system (Saltikov, 2011).

Arsenite oxidation regulatory mechanisms are complex, and in *Agrobacterium tumefaciens* a two component regulatory system has been well characterized. A more complex regulation of *aio* is seen in *Agrobacterium tumefaciens* where a two component regulatory system along with quorum sensing both play a role in activating the *aio* pathway (Kashyap *et al.*, 2006). Moreover, *aio* was induced in the presence of arsenite. Opposite to *arsR*, *aioR* positively regulates *aio*. In the chemoautotrophic strain NT-26, arsenite and expression of the two-component regulatory system is needed in order to induce *aio* genes. The regulation of *arxA* has yet to be characterized however, *arxA* expression has been shown to be induced by arsenite in chemoautotrophic and photosynthetic bacteria (Zargar *et al.*, 2012; Hernandez-Maldonado *et al.*, 2017). Genomic data comparing the two component

regulatory system, *arxXSR* in photosynthetic to chemoautotrophic bacteria indicate similar characteristics. The proposed anaerobic arsenite oxidation regulation by ArxXRS is starts by having arsenite present, where the periplasmic binding protein, ArxX can bind to arsenite and deliver a signal to the two component regulatory system ArxS/ArxR. The interaction of ArxX with ArxS leads to autophosphorylation in ArxS. A phosphotransfer from ArxS to ArxR can lead to a phosphorylated ArxR. Lastly phosphorylated ArxR can lead to DNA binding downstream of the *arx* gene cluster (*arxB2AB1CD*), leading to transcription activation of *arxB2AB1CD* (Fig. 2). Further work is needed to determine pathway.

Section 2.11 Conclusion and Remarks

The arsenic biogeochemical cycle is complex and ongoing bacterial metabolisms continue to be discovered. Light has been an important factor that may affect the arsenic biogeochemical cycle in diverse environments outside non-extreme environments. Currently the ecology of photoarsenotrophy has been shown to widespread (Zargar *et al.*, 2012; Hernandez-Maldonado *et al.*, 2017) and previously shown to be active in the environment (Fig. 3).

As we continue to learn about the impact of these photoarsenotrophic metabolisms to the arsenic biogeochemical cycle next generation sequencing technologies, computational and bioinformatics analysis will help gain a thorough understanding of these metabolisms in diverse environments. Finding other possible arsenic related mechanism that remain unknown such as anaerobic microorganisms that can couple As^{III} to Fe^{III} for either detoxification purposes or to conserve energy

has yet to be identified and would have valuable implication in the geochemistry or arsenic since this mechanism is not something typical that is considered when thinking about arsenic mobility by microorganisms. For this reason, further microbial isolations are needed in order to expand our knowledge of microbes in environmental arsenic cycling. Similar microbial arsenic metabolisms that occur in the environment in environments such as the human gut. These environmental arsenic metabolisms insights may one day help understand the microbiology within the microbiota of humans that consume arsenic contaminated waters. Understanding these diverse arsenic metabolisms that affect the health of millions of people that are at risk in developing diseases related to arsenic poisoning are critical in understanding. Finding a trustworthy model organism is needed to study these complex problems and further understand arsenic toxicity in eukaryotic systems, which impact public health.

Section 2.12 Figures

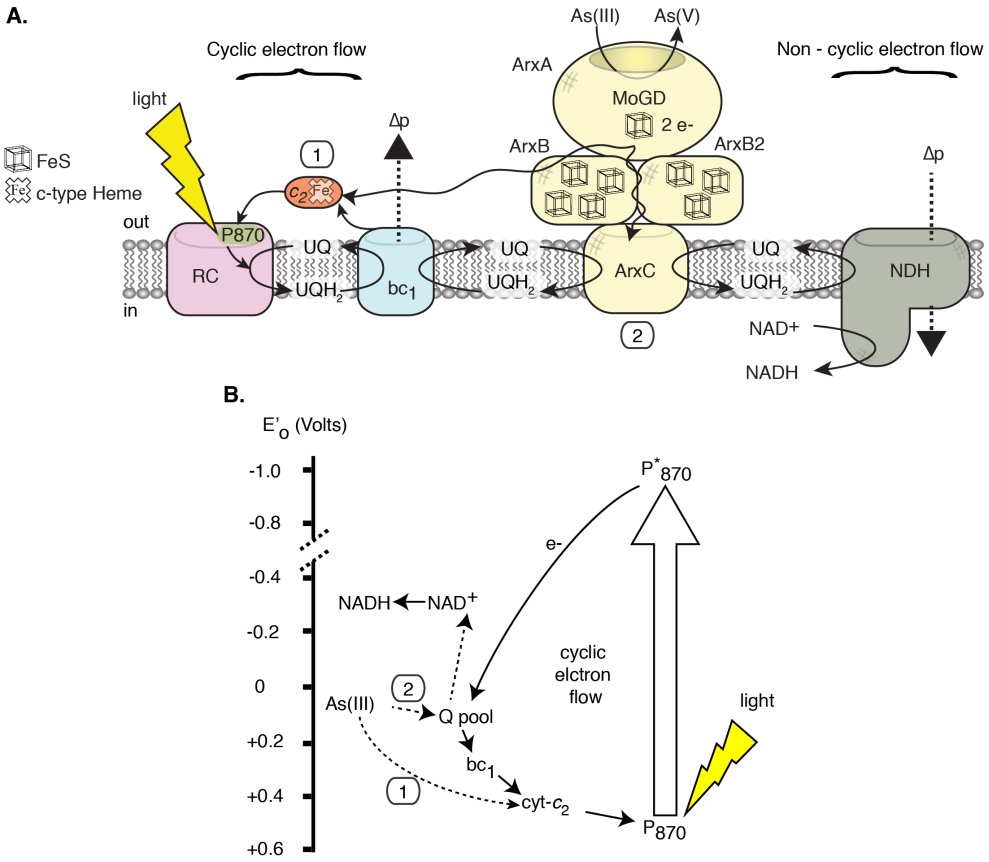

Figure 1. Proposed model for ARX-dependent arsenite oxidation coupled to anoxygenic photosynthesis. (A) Photoarsenotrophy hypothetical molecular mechanism model showing cyclic (1) and non-cyclic electron flow (2), how proton motive force, delta p, is established (dashed lines), and production of NADH by reverse electron transport chain of NADH dehydrogenase (NDH). (B) Electron flow from arsenite oxidation (dashed lines) to the photosynthetic reaction center (RC) may be mediated by a quinone (Q) or a *c*-type cytochrome such as cyt-c_2.

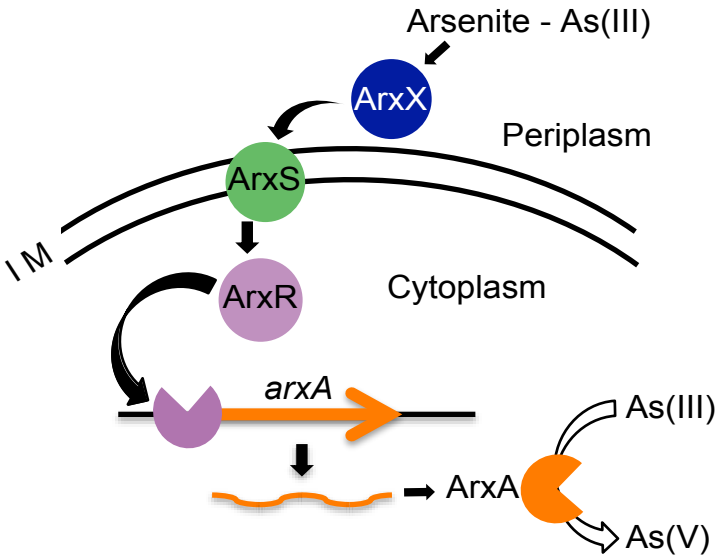

Figure 2. A proposed arsenite oxidation regulation model by ArxXRS. In the presence of arsenite, the periplasmic binding protein, ArxX can bind to arsenite and deliver a signal to the two component regulatory system ArxS/ArxR. The interaction of ArxX with ArxS leads to autophosphorylation in ArxS. A phosphotransfer from ArxS to ArxR can lead to a phosphorylated ArxR. Lastly phosphorylated ArxR can lead to DNA binding downstream of the *arx* gene cluster (*arxB2AB1CD*), potentially leading to transcription activation of *arxB2AB1CD*.

Figure 3. Hypothetical model for photoarsenotorphy activity in arsenic rich anoxic photic zones of alkaline soda environments. (A) Paoha Island, Mono Lake, CA has hot springs dominated by *Ectothiorhodospira* species, photsynthetic purple sulfur bacteria containing the arsenite oxidase, *arxA,* gene. These subsurface anoxic hot springs contain arsenite, where expression of the *arxA* gene was detected, indicating activity of photoarsenotropy. Arsenite may fuel anoxyenic photosynthesis in other environments with similar characteristics.

Section 2.13 References

Acharyya, S.K., Chakraborty, P., Lahiri, S., Raymahashay, B.C., Guha, S., and Bhowmik, A. (1999) Arsenic poisoning in the Ganges delta. *Nature* **401**: 545–545.

Afkar, E., Lisak, J., Saltikov, C., Basu, P., Oremland, R.S., and Stolz, J.F. (2003) The respiratory arsenate reductase from *Bacillus selenitireducens* strain MLS10. *FEMS Microbiology Letters* **226**: 107–112.

Ahmann, D., Roberts, A.L., Krumholz, L.R., and Morel, F.M. (1994) Microbe grows by reducing arsenic. *Nature* **371**: 750–750.

Amend, J.P., Saltikov, C., Lu, G.S., and Hernandez, J. (2014) Microbial arsenic metabolism and reaction energetics. *Reviews in Mineralogy and Geochemistry* **79**: 391–433.

Anderson, G.L., Williams, J., and Hille, R. (1992) The purification and characterization of arsenite oxidase from *Alcaligenes faecalis*, a molybdenum-containing hydroxylase. *Journal of Biological Chemistry* **267**: 23674–23682.

Andres, J. and Bertin, P.N. (2016a) The microbial genomics of arsenic. *FEMS Microbiol. Rev.* **40**: 299–322.

Andres, J. and Bertin, P.N. (2016b) The microbial genomics of arsenic. *FEMS Microbiol. Rev.* **40**: 299–322.

Benner, S.G., Polizzotto, M.L., Kocar, B.D., Ganguly, S., Phan, K., Ouch, K., et al. (2008) Groundwater flow in an arsenic-contaminated aquifer, Mekong Delta, Cambodia. *Applied Geochemistry* **23**: 3072–3087.

Budinoff, C.R. and Hollibaugh, J.T. (2008) Arsenite-dependent photoautotrophy by an *Ectothiorhodospira*-dominated consortium. *The ISME Journal* **2**: 340–343.

Burgess, W.G., Hoque, M.A., Michael, H.A., Voss, C.I., Breit, G.N., and Ahmed, K.M. (2010) Vulnerability of deep groundwater in the Bengal Aquifer System to contamination by arsenic. *Nature Publishing Group* **3**: 83–87.

Cavalca, L., Corsini, A., Zaccheo, P., Andreoni, V., and Muyzer, G. (2013) Microbial transformations of arsenic: perspectives for biological removal of arsenic from water. *Future Microbiology* **8**: 753–768.

Chen, C.J., Chen, C.W., Wu, M.M., and Kuo, T.L. (1992) Cancer potential in liver, lung, bladder and kidney due to ingested inorganic arsenic in drinking water. *British Journal of Cancer* **66**: 888–892.

Cleiss-Arnold, J., Koechler, S., Proux, C., Fardeau, M.-L., Dillies, M.-A., Coppee, J.-Y., et al. (2010) Temporal transcriptomic response during arsenic stress in *Herminiimonas arsenicoxydans*. *BMC Genomics* **11**: 709.

Crane, R.K. and Lipmann, F. (1953) The effect of arsenate on aerobic phosphorylation. *Journal of Biological Chemistry* **201**: 235–243.

Croal, L.R., Gralnick, J.A., Malasarn, D., and Newman, D.K. (2004) The Genetics of geochemistry. *Annu. Rev. Genet.* **38**: 175–202.

Cuzick, J., Sasieni, P., and Evans, S. (1992) Ingested arsenic, keratoses, and bladder cancer. *Am. J. Epidemiol.* **136**: 417–421.

Dhuldhaj, U.P., Yadav, I.C., Singh, S., and Sharma, N.K. (2013) Microbial interactions in the arsenic cycle: adoptive strategies and applications in

environmental management. *Rev Environ Contam Toxicol* **224**: 1–38.

Dowling, C.B., Poreda, R.J., Basu, A.R., Peters, S.L., and Aggarwal, P.K. (2002) Geochemical study of arsenic release mechanisms in the Bengal Basin groundwater. *Water Resour. Res.* **38**: 12–1–12–18.

Ellis, P.J., Conrads, T., Hille, R., and Kuhn, P. (2001) Crystal structure of the 100 kDa arsenite oxidase from *Alcaligenes faecalis* in two crystal forms at 1.64 angstrom and 2.03 angstrom. *Structure* **9**: 125–132.

Engel, A.S., Johnson, L.R., and Porter, M.L. (2012) Arsenite oxidase gene diversity among *Chloroflexi* and *Proteobacteriafrom* El Tatio Geyser Field, Chile. *FEMS Microbiology Ecology* **83**: 745–756.

Fendorf, S., Michael, H.A., and van Geen, A. (2010) Spatial and temporal variations of groundwater arsenic in South and Southeast Asia. *Science* **328**: 1123–1127.

Ferguson, J.F. and Gavis, J. (1972) A review of the arsenic cycle in natural waters. *Water Research* **6**: 1259–1274.

Goldberg, S. (2002) Competitive adsorption of arsenate and arsenite on oxides and clay minerals. *Soil Science Society of America Journal* **66**: 413–421.

Harvey, C.F., Ashfaque, K.N., Yu, W., Badruzzaman, A.B.M., Ali, M.A., Oates, P.M., et al. (2006) Groundwater dynamics and arsenic contamination in Bangladesh. *Chemical Geology* **228**: 112–136.

Harvey, C.F., Swartz, C.H., Badruzzaman, A., Keon-Blute, N., Yu, W., Ali, M.A., et al. (2002) Arsenic mobility and groundwater extraction in Bangladesh. *Science* **298**: 1602–1606.

Harvey, C.F., Swartz, C.H., Badruzzaman, A.B.M., Keon-Blute, N., Yu, W., Ali, M.A., et al. (2005) Groundwater arsenic contamination on the Ganges Delta: biogeochemistry, hydrology, human perturbations, and human suffering on a large scale. *Comptes Rendus Geoscience* **337**: 285–296.

Hernandez-Maldonado, J., Sanchez-Sedillo, B., Stoneburner, B., Boren, A., Miller, L., McCann, S., et al. (2017) The genetic basis of anoxygenic photosynthetic arsenite oxidation. *Environ Microbiol* **19**: 130–141.

Hoeft, S.E., Blum, J.S., Stolz, J.F., Tabita, F.R., Witte, B., King, G.M., et al. (2007) *Alkalilimnicola ehrlichii* sp. nov., a novel, arsenite-oxidizing haloalkaliphilic gammaproteobacterium capable of chemoautotrophic or heterotrophic growth with nitrate or oxygen as the electron acceptor. *Int. J. Syst. Evol. Microbiol* **57**: 504–512.

Hoeft, S.E., Lucas, F.O., Hollibaugh, J.T., and Oremland, R.S. (2010) Characterization of microbial arsenate reduction in the anoxic bottom waters of Mono Lake, California. *Geomicrobiology Journal* **19**: 23–40.

Hohmann-Marriott, M.F. and Blankenship, R.E. (2011) Evolution of photosynthesis. *Annu. Rev. Plant Biol.* **62**: 515–548.

Jung, H.B., Bostick, B.C., and Zheng, Y. (2012) Field, experimental, and modeling study of arsenic partitioning across a redox transition in a Bangladesh Aquifer. *Environ. Sci. Technol.* **46**: 1388–1395.

Kashyap, D.R., Botero, L.M., Franck, W.L., Hassett, D.J., and McDermott, T.R. (2006) Complex regulation of arsenite oxidation in *Agrobacterium tumefaciens*.

Journal of Bacteriology **188**: 1081–1088.

Krafft, T. and Macy, J.M. (1998) Purification and characterization of the respiratory arsenate reductase of *Chrysiogenes arsenatis*. *Eur. J. Biochem.* **255**: 647–653.

Kulp, T.R., Hoeft, S.E., Asao, M., Madigan, M.T., Hollibaugh, J.T., Fisher, J.C., et al. (2008) Arsenic(III) fuels anoxygenic photosynthesis in hot spring biofilms from Mono Lake, California. *Science* **321**: 967–970.

Lai, M.S., Hsueh, Y.M., Chen, C.J., Shyu, M.P., Chen, S.Y., Kuo, T.L., et al. (1994) Ingested inorganic arsenic and prevalence of diabetes-mellitus. *Am. J. Epidemiol.* **139**: 484–492.

Lett, M.C., Muller, D., Lievremont, D., Silver, S., and Santini, J. (2011) Unified nomenclature for genes involved in prokaryotic aerobic arsenite oxidation. *Journal of Bacteriology* **194**: 207–208.

LiEvremont, D., Bertin, P.N., and Lett, M.-C. (2009) Arsenic in contaminated waters: Biogeochemical cycle, microbial metabolism and biotreatment processes. *Biochimie* **91**: 1229–1237.

Lloyd, J.R. and Oremland, R.S. (2006) Microbial transformations of arsenic in the environment: From soda lakes to aquifers. *Elements* **2**: 85–90.

Macy, J.M., Nunan, K., Hagen, K.D., Dixon, D.R., Harbour, P.J., Cahill, M., and Sly, L.I. (1996) *Chrysiogenes arsenatis* gen nov, sp nov; a new arsenate-respiring bacterium isolated from gold mine wastewater. *Int. J. Syst. Bacteriol.* **46**: 1153–1157.

Mailloux, B.J. and Trembath-Reichert, E. (2013) Advection of surface-derived

organic carbon fuels microbial reduction in Bangladesh groundwater.

Malasarn, D., Saltikov, W., Campbell, K.M., Santini, J.M., Hering, J.G., and Newman, D.K. (2004) *arrA* is a reliable marker for As(V) respiration. *Science* **306**: 455–455.

Mandal, B.K. and Suzuki, K.T. (2002) Arsenic round the world: a review. *Talanta* **58**: 201–235.

Masscheleyn, P.H., Delaune, R.D., and Patrick, W.H. (1991) Effect of redox potential and pH on arsenic speciation and solubility in a contaminated soil. *Environ. Sci. Technol.* **25**: 1414–1419.

McEwan, A.G., Ridge, J.P., McDevitt, C.A., and Hugenholtz, P. (2010) The DMSO reductase family of microbial molybdenum enzymes; molecular properties and role in the dissimilatory reduction of toxic elements. *Geomicrobiology Journal* **19**: 3–21.

Meng, Y.L., Liu, Z., and Rosen, B.P. (2004) As(III) and Sb(III) Uptake by GlpF and Efflux by ArsB in *Escherichia coli*. *Journal of Biological Chemistry* **279**: 18334–18341.

Mukhopadhyay, R., Rosen, B.P., Pung, L.T., and Silver, S. (2002) Microbial arsenic: from geocycles to genes and enzymes. *FEMS Microbiol. Rev.* **26**: 311–325.

Muller, D., Lievremont, D., Simeonova, D.D., Hubert, J.C., and Lett, M.C. (2003) Arsenite oxidase *aox* genes from a metal-resistant-*Proteobacterium*. *Journal of Bacteriology* **185**: 135–141.

Murphy, J.N. and Saltikov, C.W. (2009) The ArsR repressor mediates arsenite-

dependent regulation of arsenate respiration and detoxification operons of *Shewanella* sp. strain ANA-3. *Journal of Bacteriology* **191**: 6722–6731.

Murphy, J.N. and Saltikov, C.W. (2007) The *cymA* gene, encoding a tetraheme c-type cytochrome, is required for arsenate respiration in *Shewanella* Species. *Journal of Bacteriology* **189**: 2283–2290.

Murphy, J.N., Durbin, K.J., and Saltikov, C.W. (2009) Functional roles of *arcA*, *etrA*, cyclic AMP (cAMP)-cAMP receptor protein, and cyain the arsenate respiration pathway in *Shewanella* sp. strain ANA-3. *Journal of Bacteriology* **191**: 1035–1043.

Newman, D.K., Ahmann, D., and Morel, F.M.M. (2009) A brief review of microbial arsenate respiration. *Geomicrobiology Journal* **15**: 255–268.

Nickson, R., McArthur, J., Burgess, W., Ahmed, K.M., Ravenscroft, P., and Rahman, M. (1998) Arsenic poisoning of Bangladesh groundwater. *Nature* **395**: 338–338.

Oremland, R.S. (2003) The Ecology of Arsenic. *Science* **300**: 939–944.

Oremland, R.S. and Stolz, J.F. (2005) Arsenic, microbes and contaminated aquifers. *Trends in Microbiology* **13**: 45–49.

Oremland, R.S., Hoeft, S.E., Santini, J.M., Bano, N., Hollibaugh, R.A., and Hollibaugh, J.T. (2002) Anaerobic oxidation of arsenite in mono lake water and by a facultative, arsenite-oxidizing chemoautotroph, strain MLHE-1. *Applied and Environmental Microbiology* **68**: 4795–4802.

Oremland, R.S., Saltikov, C.W., Wolfe-Simon, F., and Stolz, J.F. (2009) Arsenic in the evolution of earth and extraterrestrial ecosystems. *Geomicrobiology Journal*

26: 522–536.

Pallud, C., Kausch, M., Fendorf, S., and Meile, C. (2010) Spatial patterns and modeling of reductive ferrihydrite transformation observed in artificial soil aggregates. *Environ. Sci. Technol.* **44**: 74–79.

Polizzotto, M.L., Harvey, C.F., Li, G., Badruzzman, B., Ali, A., Newville, M., et al. (2006) Solid-phases and desorption processes of arsenic within Bangladesh sediments. *Chemical Geology* **228**: 97–111.

Polizzotto, M.L., Harvey, C.F., Sutton, S.R., and Fendorf, S. (2005) Processes conducive to the release and transport of arsenic into aquifers of Bangladesh. *Proc. Natl. Acad. Sci. U.S.A.* **102**: 18819–18823.

Quiñones, A.R. (2000) Arsenic contamination of groundwater in Bangladesh.

Radloff, K.A., Zheng, Y., Michael, H.A., Stute, M., Bostick, B.C., Mihajlov, I., et al. (2011) Arsenic migration to deep groundwater in Bangladesh influenced by adsorption and water demand. *Nature Geosci* **4**: 793–798.

Rakhunde, R., Jasudkar, D., Deshpande, L., and Juneja, H.D. (2012) Health effects and significance of arsenic speciation in water *Int. Res. J. Environment Sci.*, **1**: 92-96.

Rhine, E.D., Phelps, C.D., and Young, L.Y. (2006) Anaerobic arsenite oxidation by novel denitrifying isolates. *Environ Microbiol* **8**: 899–908.

Rosen, B.P. (2002) Biochemistry of arsenic detoxification. *FEBS Lett.* **529**: 86–92.

Saltikov, C.W. (2011) Regulation of arsenic metabolic pathways in prokaryotes American Society of Microbiology.

Saltikov, C.W. and Newman, D.K. (2003) Genetic identification of a respiratory arsenate reductase. *Proc. Natl. Acad. Sci. U.S.A.* **100**: 10983–10988.

Santini, J.M. and vanden Hoven, R.N. (2004) Molybdenum-containing arsenite oxidase of the chemolithoautotrophic arsenite oxidizer NT-26. *Journal of Bacteriology* **186**: 1614–1619.

Santini, J.M., Sly, L.I., Schnagl, R.D., and Macy, J.M. (2000) A new chemolithoautotrophic arsenite-oxidizing bacterium isolated from a gold mine: Phylogenetic, physiological, and preliminary biochemical studies. *Applied and Environmental Microbiology* **66**: 92–97.

Sharma, P., Rolle, M., Kocar, B., Fendorf, S., and Kappler, A. (2011) Influence of natural organic matter on As transport and retention. *Environ. Sci. Technol.* **45**: 546–553.

Silver, S. and Phung, L.T. (2005) Genes and enzymes involved in bacterial oxidation and reduction of inorganic arsenic. *Applied and Environmental Microbiology* **71**: 599–608.

Smedley, P.L. and Kinniburgh, D.G. (2002) A review of the source, behaviour and distribution of arsenic in natural waters. *Applied Geochemistry* **17**: 517–568.

Smith, A.H., Lingas, E.O., and Rahman, M. (2000) Contamination of drinking-water by arsenic in Bangladesh: a public health emergency. *Bull. World Health Organ.* **78**: 1093–1103.

Stolz, J.F., Basu, P., and Oremland, R.S. (2010) Microbial arsenic metabolism: New twists on an old Poison. *Microbe Magazine* **5**: 53–59.

Tufano, K.J., Benner, S.G., Mayer, K.U., Marcus, M.A., Nico, P.S., and Fendorf, S. (2009) Aggregate-xcale heterogeneity in iron (hydr)oxide reductive Transformations. *Vadose Zone Journal* **8**: 1004–9.

Warelow, T.P. (2013) The respiratory arsenite oxidase: structure and the role of residues surrounding the rieske cluster. *PLoS ONE* **8**: 1–10.

Warelow, T.P., Oke, M., Schoepp-Cothenet, B., Dahl, J.U., Bruselat, N., Sivalingam, G.N., et al. (2013) The respiratory arsenite oxidase: structure and the role of residues surrounding the rieske cluster. *PLoS ONE* **8**: e72535.

Wolfe-Simon, F., Blum, J.S., Kulp, T.R., Gordon, G.W., Hoeft, S.E., Pett-Ridge, J., et al. (2011) A Bacterium that can grow by using arsenic instead of phosphorus. *Science* **332**: 1163–1166.

Zargar, K., Conrad, A., Bernick, D.L., Lowe, T.M., Stolc, V., Hoeft, S., et al. (2012) ArxA, a new clade of arsenite oxidase within the DMSO reductase family of molybdenum oxidoreductases. *Environ Microbiol* **14**: 1635–1645.

Zargar, K., Hoeft, S., Oremland, R., and Saltikov, C.W. (2010) Identification of a novel arsenite oxidase gene, *arxA*, in the haloalkaliphilic, arsenite-oxidizing bacterium *Alkalilimnicola ehrlichii* strain MLHE-1. *Journal of Bacteriology* **192**: 3755–3762.

Chapter 3. Microbial mediated light dependent arsenic cycle

Section 3.01 Abstract

Big Soda Lake (BSL), a stratified meromictic hypersaline alkaline lake contains approximately ~25 μM arsenic, is an ideal environment to investigate photoarsenotrophy. Previous findings demonstrated the presence of a seasonal layer of purple sulfur bacteria lying below the oxycline. I hypothesized that photoarsenotrophy can occur within this anoxic region where light and photosynthetic bacteria co-occur. Based on microcosm experiments with lake water, here I present evidence for light-dependent microbial arsenite oxidation, which shifted to a net arsenate reduction during the dark period of incubation. Arsenite oxidation resumed when the microcosms were shifted back into the light. No arsenic transformations were observed abiotic control samples. Microbial phylogenetic (16S rRNA gene) and functional gene (*arxA*, *aioA*, and *arrA*) diversity were determined for native lake water and the light and the dark incubated microcosms. Similar microbial populations were found between microcosms incubated in the dark and lake water revealing that *Cyanobacteria* and *Deltaproteobacteria* exhibited the highest relative abundance of sequences analyzed for those samples. The light-incubated microcosm exhibited a higher relative abundance of photosynthetic members of the *Gammaproteobacteria* and *Alphaproteobacteria*. *Clostridia* also had a high relative abundance in both lake water and light and dark incubated microcosms. Although *Ectothiorhodospira* species were detected in lake water, the relative abundance of photosynthetic *Alphaprotoeobacteria* (*Rhodobaca*, 22%) exceeded the relative

abundance of photosynthetic *Chromatiales* (i.e. *Ectothiorhodospira* and *Thiocapsa*; <1%*)*. Functional gene analysis for *arxA* revealed one dominant sequence type in light incubated microcosms. The translated product was most similar (97-99%) to *Ectothiorhodospira* sp. strain BSL-9. In contrast, the ArxA-like sequences from lake water and those recovered from the dark incubated microcosms were most similar *Thiocapsa* (~82%) and *Thioalkavibrio* (~80 %). The arsenate respiratory reductase (ArrA) sequences from lake water samples clustered with known ArrA sequences of the Mono Lake arsenate reducers strain MLMS-1 (*Deltaproteobacteria*), *Bacillus selenitireducens* and *Bacillus arseniciselenatis*. There were no amplicons detected for *aioA* genes. An arsenite-oxidizing *Ectothiorhodospira* sp. was isolated from the light incubated microcosm. An *arxA*-like gene sequence was detected by PCR. The translated produced was highly similar (96%) to *Ectothiorhodospira* sp. strain BSL-9. In conclusion, the environmental-functional ecology study demonstrated that Big Soda Lake hosts microbial populations that can carry out anoxygenic photosynthesis coupled to arsenite oxidation. Microcosm incubations demonstrated microbial mediated reversible biogeochemical cycling of arsenite and arsenate where arsenite oxidation dominated the cycle in the light with net arsenate reduction in the dark. These observations open up new possibilities to explore light-dark cycling of arsenic in other arsenic-rich surface water environments.

Section 3.02 Introduction

Arsenic contamination of drinking water aquifers is a major global public health problem (Smith *et al.*, 2000). Arsenite (As^{III}) and arsenate (As^{V}) are the two

most abundant inorganic arsenic species in the environment of most concern to public health (Ferguson and Gavis, 1972; Smith *et al.*, 1992; 2002). Chronic consumption of such water is associated with a disease called "arsenocosis" and increased cancer incidences (Yu *et al.*, 2003). In the environment arsenite is typically stable under anoxic conditions and has a greater hydrological mobility than arsenate, which is more abundant under oxic conditions. The availability of arsenate in solution is controlled by its adsorption to diverse sediments containing iron, aluminum, manganese (hydro) oxides, and sulfide containing minerals (Ferguson and Gavis, 1972; Fendorf *et al.*, 2010; Komorowicz and Barałkiewicz, 2011). In response to arsenic, microorganisms have evolved biochemical mechanisms to cope with this toxic metalloid. In prokaryotes, arsenic detoxification can be mediated by arsenic resistance genes (*ars*), which are wide spread in bacteria and archaea (Rosen, 2002). Microbial metabolisms that harvest metabolic energy by reducing or oxidizing arsenic oxyanions that is coupled to growth are referred to as "arsenotrophy" (Oremland *et al.*, 2009). In the absence of oxygen chemoautotrophic bacteria can carry out anaerobic respiration of arsenate by using a respiratory arsenate reductase ArrA encoded by *arrA* (Saltikov and Newman, 2003). Alternatively, the opposite reaction, where arsenite is used as an electron donor, occurs in photoautotrophic and chemoautotrophic bacteria. There are two known arsenite oxidation pathways encoded by *aio and arx* genes. The former pathway is typically associated with arsenite oxidation coupled to growth with oxygen as a terminal electron acceptor (Santini *et al.*, 2000) or aerobic oxidation for detoxification purposes (Anderson *et*

al., 1992). Recently photosynthetic microbial mats dominated by *Chloroflexus* have reported *aioA*-like sequences within El Tatio Geyser Field in Chile, however the ecological abundance, distribution, and molecular mechanism remains unknown (Engel *et al.*, 2012). The second pathway, *arx*, occurs under anaerobic conditions where, in the absence of oxygen arsenite oxidation is coupled to nitrate respiration and is carried out by the arsenite oxidase *arxA* (Oremland *et al.*, 2002; Hoeft *et al.*, 2007; Zargar *et al.*, 2010). More recently light has been shown to contribute to the arsenic cycle by powering anoxygenic photosynthesis, with arsenite as an electron donor, which is encoded by *arxA* and referred to as "photoarsenotrophy". To fully evaluate the arsenic biogeochemical cycle, light needs to be accounted for when analyzing microbial arsenic metabolisms in the environment.

In 2008, Kulp *et al.* and Budinoff and Hollibaugh (2008) first reported on light-dependent anaerobic arsenite oxidation metabolism. The samples used in Kulp *et al.* (2008) originated from a red-pigmented biofilm located within a hot-spring on Paoha Island, Mono Lake, CA. Whereas Budinoff and Hollibaugh (2008) collected sulfidic sediment samples from the central basin of Mono Lake, CA. In both cases arsenite oxidation linked to anoxygenic photosynthesis was independently described using microcosm studies with environmental samples but provided with synthetic inorganic salts media instead of the local native water sources (i.e,. hot-springs or Mono Lake water). Although biochemical studies with ArxA in photoarsenotrophs were lacking, previous studies with *Alkalilimnicola ehrlichii* strain MLHE-1 demonstrated its ArxA to function *in vitro* as an arsenite oxidase with a reversible

arsenate reductase activity (Richey *et al.*, 2009). An additional molecular genetic study with strain MLHE-1 demonstrated that the *arxA* gene was responsible for arsenite oxidation when coupled to nitrate reduction (Zargar *et al.*, 2010; 2012). This result confirmed that ArxA should be considered a unique clade of arsenite oxidases within the DMSO reductase family molybdenum-containing oxidoreductase. The ArxA of *Alkalilimnicola ehrlichii* strain MLHE-1 would serve as the first reference for this unique clade. Recently, a genetic system was developed in a photosynthetic purple sulfur bacterium, *Ectothiorhodospira* sp. strain BSL-9 where an *arxA* disruption mutant hindered both growth and arsenite oxidation when arsenite was the sole electron donor (Hernandez-Maldonado *et al.*, 2017). This result provided strong evidence for *arxA* being the sole arsenite oxidase in anoxygenic photosynthetic arsenite oxidation. Environmental activity of photoarsenotrophy has also been shown to occur *in situ* in the Paoha Island biofilms noted above. The detection of arsenite oxidase gene transcripts, *arxA,* were attributable to photosynthetic purple sulfur bacteria (Hernandez-Maldonado *et al.*, 2017). The significance of this observation was that the ArxA enzyme could be present and active in a hot spring environment where sulfide, another competing electron donor for anoxygenic photosynthesis, was nearly 60-fold more abundant that arsenite (Kulp *et al*., 2008). From these recent studies, photoarsenotrophy has added an additional layer of complexity to the arsenic biogeochemical cycle. Further environmental studies are needed in order to expand our knowledge regarding how photosynthetic microorganisms may influence the arsenic cycle.

Hypersaline alkaline lakes are known to host diverse photosynthetic and chemotrophic bacterial communities with versatile metabolic properties that are compartmentalized by different depths (Jones *et al.*, 1998; Humayoun *et al.*, 2003; Antony *et al.*, 2012). The water column of meromictic (permanently stratified) soda lakes usually encompasses two layers, separated by a steep saline gradient (pycnocline). The top layer is known as mixolimnion and contains an oxycline that can change throughout the year due to temperature-caused water column mixing, in these instances occurring once a year during winter. Below the mixolimnion the bottom layer is referred to as the monimolimnion, where the hypoxic/anoxic water at depth remains isolated from the mixolimnion. The region between these two layers is referred to as the chemocline, usually coinciding with the pycnocline. These major compartments present a unique opportunity to investigate arsenic cycling as the reduction and oxidation of arsenic may also likely be compartmentalized. Big Soda Lake, located in Fallon, Nevada is a hypersaline alkaline meromictic lake that occupies a volcanic crater. Moreover, the lake water contains approximately 25 μM arsenic. Unique geophysicochemical profiles that favor anoxygenic photosynthetic bacteria are present during summer/autumn, which are likely absent in the winter (Cloern *et al.*, 1987). In summer and autumn, bacteriochlorophyll, which is associated with purple sulfur bacteria, has a peak maximum abundance below the oxycline (~21 m). The photosynthetic bacteria form 1-2 m thick plate and blooms at less than 10% light intensity (Cloern *et al.*, 1987). Within this region the concentration of nitrite and sulfide are low and arsenate is the dominant arsenic

species. This raises the question of what drives arsenite oxidation to arsenate below the oxycline where typically arsenite is predicted to be more thermodynamically stable. One emerging hypothesis is that arsenite oxidation below the oxyline is mediated by anoxygenic photosynthetic arsenite oxidizing bacteria containing *arxA*.

The goal of this study was to address the above hypothesis by characterizing microbial-mediated light dependent arsenite oxidation activity in anoxic Big Soda Lake water, about which little is known. This analysis was done through microcosm experiments and microbial ecology investigations. For the microcosm experiments, anoxic lake water was collected from 21 m depth. The water samples were supplemented with arsenite and subjected to either alternating light-dark-light incubation conditions or dark-light cycling conditions. The microbial diversity was determined by 16S rRNA gene analysis and arsenotrophic functional genes such as *arxA* and *arrA* were analyzed both in lake water as well as in microcosms incubation experiments. The results demonstrated a light-dependent arsenic cycle that can be driven by the coupling of anoxygenic photosynthetic with chemotrophic bacteria containing *arxA* and *arrA* genes, respectively. This opens up the possibilities for a light-dependent arsenic cycle occurring in other surface waters impacted by arsenic.

Section 3.03 Results

(a) Water column vertical profile

Purple sulfur bacterial (PSB) blooms and detection of bacteriochlorophyll have previously been reported within Big Soda Lake (Priscu *et al.*, 1982; Cloern *et al.*, 1983). Nearly 30 years later, we needed to confirm the depth that contained this layer

of photosynthetic bacteria. This was done by determining vertical profiles of the following lake water parameters: temperature [deg °C], conductivity [S/m], salinity [g/kg], oxygen [mg/l], PAR/Irradiance [%], beam transmittance [%], and fluorescence [mg/m^3]. Figure 1 shows the vertical distribution of the physicochemical parameters within the Big Soda Lake water column, collected in September 15, 2016, which correlated with previous reports (Priscu *et al.*, 1982; Cloern *et al.*, 1983; Edwardson, Planer-Friedrich, and Hollibaugh, 2014a). An oxycline was observed by a sharp decrease in oxygen starting near 9 m and depleted near 18 m (Fig. 1). Fluorescence increased from 1 mg/m^3 to approximately 3 mg/m^3 where it was highest near 19 m (Fig. 1). With an attenuation of beam transmittance (decrease from ~90% to 35%) also around 19 m, these observation suggest the occurrence of a layer of photosynthetic microbes in the part of the lake. Overall the measurements around 19-21 m indicated ideal parameters where anoxygenic photosynthetic bacteria might be present.

Next, arsenic speciation and quantification was determined by high performance liquid chromatography-induced coupled plasma-mass spectrometry (HPLC-ICP-MS). Typically, the two dominant arsenic species in the environment are arsenite and arsenate. Where arsenate is typically stable under oxic conditions and arsenite is stable under anoxic conditions provided that there is low sulfide abundance. Alkaline, sulfide-rich waters of soda lakes have been previously shown to induce the formation of thioarsenate compounds in the presence of arsenite and arsenate. However, immediately below the oxycline of Big Soda Lake sulfide levels are low

and the presence of arsenite has been previously reported during earlier in June (Edwardson, Planer-Friedrich, and Hollibaugh, 2014a). At the time of our sampling trip arsenate was the dominant arsenic species below the oxycline (Fig. 2), a finding that was somewhat surprising because arsenate is typically stable under oxic conditions and arsenite under anoxic conditions. It is unclear if arsenite oxidation in Big Soda Lake occurs chemically or is driven by microbial transformations. Parameters below the oxycline may favor anoxygenic photosynthetic arsenite oxidizing bacteria. For this reason, microcosms containing native lake water collected from below the oxycline were supplemented with arsenite and used to investigate light dependent arsenite oxidation.

(b) Microbial arsenic transformation in Big Soda Lake native consortium

To test for microbial arsenite oxidation by anoxygenic photosynthetic bacteria in the water column of Big Soda Lake, we employed the following set-up. Serum bottles containing approximately 50 mL of lake water (21 m) were supplemented with 35-40 μM arsenite. Infrared light (850 nm) was employed as an illumination source to favor photosynthetic bacteria and to eliminate the participation of cyanobacteria (Hernandez-Maldonado *et al.*, 2017). The four different conditions tested for arsenite oxidation where: (i) live incubations under IR containing native Big Soda Lake water (ii) live incubations in the dark (without IR) containing native Big Soda Lake water (iii) incubations under IR containing filtered lake water, where biomass was removed by filtering water through a 0.2 μm filter (iv) incubations in the dark containing filtered lake water. Arsenite and arsenate were quantified and changes in their

concentration were indicative of arsenic transformations (microbial vs. chemical) within microcosms. Heterogeneous arsenic transformations were only present in biotic microcosm samples, where oxidation of arsenite to arsenate occurred in two serum bottles at different time points and had different rates when incubated under IR conditions (Fig. 3AB). Under dark incubations both arsenite oxidation and arsenate reduction occurred (Fig. 3EF). No arsenic transformations occurred in light or dark incubated filtered lake water samples, ruling out chemical arsenite oxidation (Fig. 3CDGH). From these observation, I concluded that photosynthetic arsenite oxidation, although inconsistent among replicate microcosms, was occurring albeit at a slow rate possibly because of the low bacterial abundance in the water. Hence, an alternative approach would be needed to conclusively demonstrate photoarsenotrophy within Big Soda Lake.

(c) Microbial arsenic transformation in Big Soda Lake concentrated consortium

In order to enhance the light-dependent arsenite oxidation in the lake water, native microbial biomass was concentrated 20-fold. A similar microcosm experiment describe above was done using filtered lake water and the addition of the concentrated bacterial fraction according to the following incubation conditions: (i) live cell suspensions incubated under IR, (ii) live cell suspensions incubated in the dark, and (iii) autoclaved killed cell suspensions incubated in IR. All samples were supplemented with approximately 35-40 μM arsenite at the outset but also contained approximately 25 μM arsenate as the starting background lake arsenic concentration.

The starting OD_{600} value for the concentrated samples were approximately 0.060 and did not change over time for the autoclaved control samples. However, the OD_{600} values for the live samples increased to 0.16 and 0.18 for the light and dark incubated microcosms, respectively, indicating cell growth. Interestingly, microcosms given arsenite oxidized under IR, forming arsenate followed by arsenate reduction when shifted to dark conditions. Microcosms resumed arsenite oxidation once they were shifted back into IR (Fig. 4A).

For the dark incubated microcosms, no arsenite oxidation was detected. However, the background arsenate from the lake water was reduced to arsenite within five days of incubation (Fig. 4B). In order to further demonstrate arsenate reduction, approximately 500 μM of arsenate was added at day 17. Within eight days of this additional arsenate was reduced to arsenite (Fig. 4B). To test for light dependent arsenite oxidation, dark incubated microcosms where shifted to IR, but no further arsenic transformations were observed (Fig. 4B), indicating the loss of photosynthetic arsenite oxidizing bacteria. Finally, no abiotic arsenite oxidation or arsenate reduction took place in any of the autoclaved controls (Fig. 4C). These results indicate that Big Soda Lake has the capacity for biologically mediated light-dark cycling of arsenite oxidation and arsenate reduction.

(d) Photoarsenotroph isolation from IR light incubated microcosms

In an attempt to establish a connection between microbes and arsenic cycling in the environment, we pursued bacterial isolations for photoarsenotrophs using light incubated microcosms samples and arsenate respiring chemoautotrophs from dark

incubated microcosm. We obtained a pure culture from the light incubated microcosms by streak platting the consortium in synthetic mineral salts medium (BSM) containing 1 mM arsenite and incubating under anoxic conditions with IR. Single colony forming units (CFUs) were successively re-streaked three times on 1 mM BSM agar plates. The final pure culture was analyzed for *arxA* and 16S rRNA gene by PCR amplification and sequencing. This organism contained an *arxA*-like gene similar (96%) to *Ectothiorhodospira* sp. strain BSL-9. The 16S rRNA gene from this organism was 99% similar also to that of *Ectothiorhodospira sp.* strain BSL-9. The close similarities of the isolate's 16S rRNA and *arxA* genes to those of strain BSL-9 suggest that the isolate may be able to participated in anoxygenic photosynthetic arsenite oxidation in the light incubation microcosms, and potentially below the oxycline of Big Soda Lake.

(e) Microbial population detection by 16S rRNA gene analysis

We characterize the microbial population by sequencing the16S rRNA gene within the microcosms themselves (Fig. 5) and in lake water (Fig 6). Sequences in the light-incubated microcosm were mostly related to *Ectothiorhodospira*-like organisms followed by *Rhodobaca*, *Rhodovulum, Rhodovaculum* like organisms and *Firmicutes*. A lower number of sequences were detected for *Cyanobacteria*, and *Bacteroidetes*. In the dark incubated microcosm, *Cyanobacteria* and *Firmicutes* were most frequently detected. Fewer sequences were detected for *Deltaproteobacteria*, *Tenericutes*, *Bacteroidetes*, *Actinobacteira, Alphaproteobacteria.* No *Ectothiorhodospira* species were detected in the dark incubated microcosms.

Ectothiorhodospira species were present at 21 m in Big Soda Lake. However, their abundance was less than 1% of the total number of sequences analyzed. In contrast, taxa with the greatest relative abundance were those associated with *Synechococcophycideae* (32.1% of the sequences) followed by *Alphaproteobacteria* (~14.3%), *Saprospirae* (~9.1%), *Actinobacteria* (~8.9%), *Nitriliruptoria* (~7.8%), *Flavobacteriia* (7.1%), *Gammaproteobacteria* (~4.7%), *Deltaproteobacteria* (~2.8%), and *Gammaproteobacteria* (unclassified) (~2.2%) (Fig. 7). Overall, detection of 16S rRNA gene sequences in the light incubated microcosms were different from both the lake water and dark-incubated microcosms. Detection of photosynthetic bacteria outside of *Ectothiorhodospira* species questioned the bacteria that may have participated in light dependent arsenite oxidation. For this reason, further analysis for the arsenite oxidase *arxA* and arsenate reductase *arrA* in microcosms and lake water would further elucidate the potential bacteria contributing to the arsenic cycle observed in the microcosm experiments.

(f) Arsenic functional gene analysis, *arrA* and *arxA*

In order to provide further clues as to the microbial arsenic redox reactions that occurred within the microcosm samples, I investigated the presence and sequence diversity of microbial arsenic transforming genes such as arsenite oxidase, *arxA,* and arsenate reductase, *arrA*. Interestingly, *aioA*-type arsenite oxidases were not detected in either of the microcosms or in the lake water samples. Not surprisingly *arxA*-like sequences were detected in the light-incubated microcosms and in the isolate. These sequences were 98% and 96% (translated amino acid sequence) similar to the ArxA-

type arsenite oxidase of *Ectothiorhodospira* sp. strain BSL-9 (Fig. 8), respectively. Alternatively, ArxA-like sequences found in dark incubated microcosms and 21 m lake water samples were distinct from those detected in the light-incubated microcosms (Fig. 8). BLASTx analysis indicated detection of ArxA-like sequences similar to *Thiocapsa* sp. KS1, *Thioalkavibrio* and possibly *Halomonas*. Our data from the light incubated microcosms indicated an *arxA*-like *Ectothiorhodospira* sp. strain BSL-9 which may have been involved in arsenite oxidation with light. However further work is needed to understand the role of other *arxA*-like sequences similar to chemoautotrophic bacteria *Thioalkalivibrio* and *Halomonas* since their relative abundance was higher in microcosms and in lake water samples incubated in the dark. For the lake water *arrA* sequences, BLASTx analysis indicated detection of ArrA-like sequences similar to ArrA found in *Deltaproteobacteria* strain MLMS-1, *Bacillus selenitireducens* and *Bacillus arseniciselenatis* (Fig. 9). It appears that the gene detection of both the arsenite oxidase *arxA* of photoarsenotrophs and arsenate reductase *arrA* of chemoautotrophic bacteria in Big Soda Lake water samples and in the microcosms corroborate the microbial light dependent mediated arsenic cycle observations in the microcosms.

Section 3.04 Discussion

(a) The occurrence of photoarsenotrophy in Big Soda Lake

The goal of this investigation was to determine the impact of anoxygenic photosynthetic arsenite oxidation in the environment by using Big Soda Lake as a model study site. Based on the chemical and physical parameters within the lake, it

was hypothesized that photosynthetic bacteria containing *arxA* could oxidize arsenite to arsenate below the oxycline and in a region with sufficient light. This hypothesis was confirmed through microcosm experiments, which demonstrated a microbial mediated light-dependent arsenite oxidation in lake water supplemented with arsenite. Moreover, the functional gene for arsenite oxidation, *arxA*, was also detected in the lake as well as in the microcosms. These finding provide important evidence the photosynthetic bacteria containing may oxidize arsenite in the lake under the right conditions. This phenomenon could explain why most of the arsenic at this depth is in the form of arsenate and not arsenite; the latter is typically present within anoxic environments.

(b) Microbial arsenic transformation in Big Soda Lake native consortium

In this study we first tested if arsenite oxidation could occur in the anoxic native lake water along the purple sulfur bacterial plate (21 m). Here it was found that the progression of arsenite oxidation was inconsistent over time for the experimental replicates. Dark incubation conditions were variable with demonstrating evidence for arsenate reduction and oxidation (Fig. 3). The variability in arsenic transformations could be attributed to low microbial biomass within the native lake, ~10^6 cells ml^{-1} (Zehr *et al.*, 1987). Each microcosm may have also received an uneven inoculum because of the particulates in the water samples.. Rigorous mixing of the water samples may not have sufficiently dispersed the particulate matter. Because of the inconsistent time course for arsenite oxidation, we decided to follow-up with a high cell suspension experiment that would effectively increase the potential rates of

arsenic transformations in the light vs. dark microcosms. This proved to be a useful approach for investigating photoarsenotrophy by natural microbial assemblages of Big Soda Lake.

(c) Microbial arsenic transformation in Big Soda Lake concentrated consortium

A microbial arsenic cycle was clearly noticeably in microcosms containing concentrated native microbial populations at 20 times the background abundance (Zehr *et al.*, 1987). A similar microcosm experiment indicated an arsenic cycle from biofilm samples collected from Paoha Island hot springs, however the arsenic cycle was examined under synthetic inorganic salt media (Hoeft *et al.*, 2010). In our study we took a similar approach but instead we used native lake water instead of synthetic inorganic salt media. Under these conditions it was more conclusive that the parameters below the oxycline of Big Soda Lake can in fact serve as niches for anoxygenic photosynthetic bacteria and chemoautotrophic bacteria that may participate in light dependent arsenite oxidation coupled to arsenate reduction in the dark (Fig. 4A). The lack of chemical arsenite oxidation in biotic microcosm during our analysis may suggest that microbial mediate arsenite oxidation is more important to convert arsenite to arsenate under anoxic condition. To further validate anoxygenic photosynthetic arsenite oxidation coupled to anaerobic arsenate reduction we identified the microbial population by analyzing the 16S rRNA gene and further attempting to isolate arsenic metabolizing bacteria from the microcosms.

(d) Microbial population detection by 16S rRNA gene analysis

The relative abundance detection of *Ectothiorhodospira* like sequences in light incubated microcosms and in the isolate indicated the potential activity of photoarsenotrophy in Big Soda Lake when arsenite may be available. Under the right circumstances arsenite may fuel photoarsenotrophy. The microbial population in the lake water indicated multiple photosynthetic bacteria, with the most abundant being *Cyanobacteria* and *Alphaproteobacteria*, but less than1% *Ectothiorhodospira*. *Synechococcus* sp. were the relative most abundant *Cyanobacteria* found within 21 m lake water. *Synechococcus* sp. are known to have versatile metabolisms (Paerl, 1991; Ikemoto and Mitsui, 1994; Shen and Bryant, 1995) and have been detected within anoxic environments (Detmer *et al.*, 1993). Detection of an arsenite oxidase *arxA* has yet to detected in *Cyanobacteria* and remains unknown, however it has been reported that arsenite oxidation can occur by *Cyanobacteria* (Zhang *et al.*, 2014). Moreover light dependent arsenite oxidation has also been reported in Paoha Island Mono Lake, CA within hot springs dominated by a green-like biofilms, latter have an absorption spectra peaks at 664 and 638 nm (Kulp *et al.*, 2008). Indicating chlorophyll like pigments which are found in *Cyanobacteria*. These reports along with a maximum peak fluorescence near 21 m lake water, suggest high chlorophyll-like pigments. It may be possible that *Synechococcus* may also contribute to the arsenic cycle below the oxycline of Big Soda Lake. Current preliminary data in our lab indicate *Alphaproteobacteria such as Rhodovaca*, *Rhodovulum* and *Rhodobacter* as potential candidates containing *arxA*, however the similarity of *arxA* in photosynthetic *Alphaproteobacteria* to *Ectothiorhodospira* species remain unknown. During our

environmental sampling, arsenite oxidation may have been completed, due to the presence or arsenate as the dominant arsenite species. It would be interesting to compare the microbial population before, during and after arsenite oxidation took place in Big Sod Lake in order to have a better picture of the microbial dynamic that may occur. We therefor might have missed the snap shoot of the true microbial population that may metabolize arsenite with light. These findings open on going questions regarding the impact of photosynthetic arsenite oxidizing bacteria in the environment.

(e) Arsenic functional gene analysis by *arxA* and *arrA*

The microbial relative abundance detection by 16S rRNA gene analysis raised the question as to which were the major bacterial groups that may have been responsible for anoxic arsenite oxidation in the microcosm and ultimately in the Big Soda Lake water column. The 16S rRNA insights indicate that there were other bacterial types in relative abundance besides *Ectothiorhodospira* species such as *Thiocapsa*, *Thialkalivibrio*, *Halamonas*, present in the lake water. For this reason, a functional arsenic gene analysis of *arxA* and arrA was performed. It has been established that *arxA* is essential for photoarsenotrophy in *Ectothriorhodospira* sp. strain BSL-9 but less is know about the role of *arxA* in *Thioalkalivibrio* and *Halomonas* species (Hamamura N *et al*., 2014). The absence of *arxA*-like similar sequences of *Thiocapsa*, *Thialkalivibrio* and *Halamonas,* in the light incubated microcosms indicates that these organisms are not be the dominant photosynthetic arsenite oxidizers below the oxycline of Big Soda Lake. The same can't be said about

Synechococcus, Rhodovaca, *Rhodovulum* and *Rhodobacter* bacteria since little is known about their *arxA*-like sequences and role in arsenite oxidation, and they were relative abundant in the lake water and were also detected in light incubated microcosms. Together the 16S rRNA gene analysis representing *Ectothiorhodospira* like sequences and *arxA* similar to *Ectothiorhodospira* sp. strain BSL-9 in the isolate and light incubated microcosms indicate the potential activity of photoarsenotrophy in Big Soda Lake when arsenite is available. Perhaps under the right conditions a snap shot of photoarsenotrophy can be detected in Big Soda Lake.

It is likely that the microbial mediated arsenate reduction that occurred in the dark incubated microcosms can be explained by the illumina 16S rRNA and *arrA* like sequence analysis that were detected in the lake water samples. *Firmicutes* and *Deltaproteobacteria* like sequences were detected in lake water (Fig. 6), and relative abundant in both dark and light incubated microcosms (Fig. 5). More interestingly the highest relative abundance *arrA* like sequences within lake water samples clustered similar to ArrA found in *Deltaproteobacteria* strain MLMS-1 (Oremland *et al.*, 2004), *Bacillus selenitireducens* and *Bacillus arseniciselenatis* (Oremland, 2003). All three bacteria have been isolated from Mono Lake, CA (Fig. 9), a hypersaline alkaline environment. However Big Soda Lake near 21 m has low levels of sulfide concentrations, which can provide electrons in order to reduce arsenate anaerobically in MLMS-1.

To better understand the complex interactions that allow these arsenic-metabolizing organisms to thrive in arsenic rich environments future transcriptomic

and metagenomic studies can provide the tools needed to identify the bacteria and genes that contribute to light dependent microbial mediated arsenic cycle within Big Soda Lake when presented with the ideal conditions. During our sampling trip there might have also been a low abundance or lack of anaerobic arsenite oxidazing chemoautotrophic bacteria due to the lack of *aioA*. However, in future studies adding nitrate to dark incubated microcosm, may provide insights into this metabolism. Since anaerobic arsenite oxidation in chemoautotrophic bacteria, depend on nitrate for growth. These microcosm studies served as indications that photosynthetic arsenite oxidation are possible within the euphotic zone below the oxycline of Big Soda Lake. Perhaps under the right conditions a snap shot of photoarsenotrophy can be detected in Big Soda Lake. Future microcosms and physicochemical studies of Big Soda Lake such as the types of compounds (organic and inorganic energy rich) that are found below the oxycline may provide insights to the types of energy sources that fuel microbial biochemical processes. Big Soda Lake has elements such as iron, sulfur and nitrogen, which may impact the activity and regulation of photoarsenotrophy.

Section 3.05 Conclusion

Our work provides evidence for a microbial light dependent arsenite oxidation coupled to arsenate reduction. Our observations indicate that a light dependent microbial arsenic redox cycle may be possible within the oxycline of Big Soda Lake, when optimal parameters are present. These ideal conditions may occur transiently where anoxygenic photosynthetic bacteria could thrive on arsenic temporary. We

present a microbial ecology snapshot of the indigenous bacterial community that may contribute to the arsenic biogeochemical cycle or arsenic in Big Soda Lake by investigating the 16S rRNA microbial fingerprint and functional genes such as *arrA* and *arxA*. Big Soda Lake serves as a model system to study photoarsenotrophy and further ecological studies can expand our understanding of a light dependent arsenic cycle. In order to understand the significance of photoarsenotrophy and accurately assess the complete arsenic biogeochemical cycle by microorganisms there is a need to incorporate light as a driving factor. These studies are the stepping-stones of understating the impact of photosynthetic arsenic based metabolisms on the arsenic biogeochemical cycle that may occur in diverse anoxic euphotic arsenic rich environments. Further work is needed to determine the extent of light-dark arsenic cycling in other environments and if photoarsenotrophy occurs in genera other than *Ectothiorhodospira.*

Section 3.06 Material and methods

(a) Sampling site description

Big Soda Lake occupies a volcanic crater in northwest Nevada in the town of Fallon (39˚31'N118˚52'W). This meromictic hypersaline alkaline environment contains approximately 25 μM arsenic and has ideal physicochemical parameters to inhabit anoxygenic photosynthetic bacteria. Big Soda Lake native water was collected at depth 21 m in September 15th 2015, in order to test for microbial light dependent arsenite oxidation in microcosms supplemented arsenite.

(b) Sample collection

A vertical physicochemical depth profile was acquired by using a Sea-Bird SBE 19 Seacat CTD instrumentation. The physicochemical depth profiles of interests were temperature, salinity, conductivity, photo synthetically active radiation (PAR), oxygen, in vivo fluorescence (WetLabs Wet Star Fluorometer), and Beam transmittance, which previous parameters have been observed in past years (Edwardson, Planer-Friedrich, and Hollibaugh, 2014b). Based on distinct physicochemical parameters detected, Big Soda Lake water was collected with depth from 5, 18, 21, and 30 meters by using a sterile 5 litter Niskin bottle and vacutainers. In order to investigate bacterial diversity by 16S rRNA and the presence of metabolizing arsenic related genes such as *arxA*, *aioA* and *arrA,* genomic DNA was extracted from lake water samples by filtrating two liters of water through 0.22 μM Sterivex GP filters (Cat. No.: SVGP01015). An ideal physicochemical parameters compartment where photosynthetic bacteria were likely to be present was detected approximately between 18-21 meters. Lake water samples were collected by using cubitainers from 21 m depth in order to set-up microcosms back in the laboratory. Approximately 10 mL of filtered water samples from each depth were collected in vacutainers for future arsenic analysis. All samples were stored under dry ice for two days. Upon arrival to UCSC campus Sterivex were stored in -80 freezer and native water samples were stored at 4˚C. Environmental arsenic concentrations were measure by HPLC-ICP-MS as previously reported (Hernandez-Maldonado *et al.*, 2017).

(c) Microcosms set-up: Using anoxic Big Soda Lake water

Two days after collecting lake water samples, microcosms were set-up in quadruplicate inside an anaerobic glove camber, after bubbling 21 m lake water with nitrogen. Sterile serum bottles contain 50 mL of anoxic lake water supplemented with approximately 35 μM arsenite. Four different conditions were tested (i) live unfiltered water stored under infrared (IR) lights (ii) filtered water stored under IR, lacking biomass (iii) live unfiltered water stored in the dark, by covering serum bottles with foil and (iv) filtered water stored in the dark, lacking cell biomass. All serum bottles were incubated under 35˚C and sampled over time for arsenic analysis.

(d) Concentrated microcosms set-up: Using 2L anoxic Big Soda Lake water

Due to the lack of arsenite oxidation and arsenate reduction in a few serum bottles containing live lake water, microcosms experiments were repeated twelve days after sample collection. These samples contained a concentrated consortium which was achieved by filtering 2L of 21 m lake water and suspending the collected biomass from the filters with 100 mL sterile lake water. This was theoretically 20X concentrated of the native microbial population. Due to lack of arsenite oxidation and arsenate reduction in light and dark incubated filtered samples, control samples were heat killed by autoclaving the 20X water sample. All serum bottles were incubated under 35˚C and sampled over time for arsenic analysis.

(e) photoarsenotroph pure culture isolation and growth conditions

Lake water from light incubated microcosm experiments were streak plated on BSM (Hernandez-Maldonado *et al.*, 2017) agar plates containing 1mM arsenite in order to isolate photosynthetic arsenite oxidizing bacteria. Single purple like colony

forming units (CFU) were observed after a week. The CFU's were re-streaked in order to acquire a pure culture. Based on polymerase chain reaction (PCR) analysis isolates 16S rRNA were 97-99% identical to *Ectothiorhodospira* sp. strain BSL-9 and *arxA* was also 95% similar to *Ectothiorhodospira* sp. strain BSL-9. No further arsenite oxidation assays were performed for the strain BSL-9 like isolate.

(f) Environmental genomic DNA extractions

Sterivex filters were used in order to collect environmental native biomass from Big Soda Lake (~ 500mL of water was filtered from the designated depth). The Power Water Sterivex DNA isolation Kit from Mo BIO (cat#14600-50-NF) was used according to protocol in order to extract native Big Soda Lake genomic DNA. DNeasy Qiagen blood and tissue kit was followed according to protocol in order to extract genomic DNA from microcosms after terminating experiment**.**

(g) Functional gene analysis for *arxA* and *arrA*.

In order to determine the presence of arsenic functional genes within Big Soda Lake water column primers for the arsenite oxidase *arxA* (arxA_1824_MS_F and arxA_2380_MS_R) and arsenate reductase *arrA* primers were used (HAArrA_MS_F and HAArrA_MS_R).

(h) Polymerase chain reactions

After extracting DNA from lake water and microcosms, samples were amplified with 16V3V4_MS F and 16V3V4_MS R primer sets in order to analyze the native microbial population. The PCR profile was set-up as followed for one reaction containing 25 µl total volume: 50x Ti Taq (0.5), 50x dNTP mix (0.5), 10x buffer

(2.5), 25mM $MgCl_2$ (3.5), 4µM 16V3V4_MS F (2.5), 4µM 16V3V4_MS R (2.5), 10x enhancer (2.5), DEPC water (9.5), 10 ng DNA (1). The thermal profile was (1) 95˚C for 5 min (2) 95˚C for 30 sec (3) 61˚C for 30 sec (4) 72˚C for 30 sec (5) repeat 2-4 steps for 24 times (6) 72˚C for 5 min (7) 4˚C forever. Amplification of 16S rRNA by this protocol yield approximately a 500 base pair DNA product. The *arxA* PCR profile was set-up as followed for one reaction containing 25 µl total volume: 50x Ti Taq (0.5), 50x dNTP mix (0.5), 10x buffer (2.5), 25mM $MgCl_2$ (3.5), 4µM arxA_1824_MS F (2.5), 4µM arxA_2380_MS R (2.5), 10x enhancer (2.5), DEPC water (8.5), 10 ng DNA (2). The thermal profile was (1) 95˚C for 5 min (2) 95˚C for 30 sec (3) 60˚C for 30 sec (4) 72˚C for 30 sec (5) repeat 2-4 steps for 29 times (6) 72˚C for 5 min (7) 4˚C forever. The PCR products yield approximately a 600 base pair product. The *arrA* PCR profile was set-up as followed for one reaction containing 25 µl total volume: 50x Ti Taq (0.5), 50x dNTP mix (0.5), 10x buffer (2.5), 25mM $MgCl_2$ (3.5), 4µM HAArrA_MS F (2.5), 4µM HAArrA_MS R (2.5), 10x enhancer (2.5), DEPC water (9.5), 10 ng DNA (1). The thermal profile was (1) 95˚C for 5 min (2) 95˚C for 30 sec (3) 57˚C for 30 sec (4) 72˚C for 30 sec (5) repeat 2-4 steps for 29 times (6) 72˚C for 5 min (7) 4˚C forever. The PCR products yield approximately a 480 base pair product.

(i) Clone libraries

The arsenite oxidase gene, *arxA* was amplified as mentioned above, and analyzed on a 1% agarose gel. Restriction Fragment Length Polymorphism (RFLP) was then performed before sending products for Sanger Sequencing. RFLP reactions contain a

10 µl final volume: PCR product (6), restriction enzyme HAEII (0.2), 10X NEB#4 (1), DEPC water (2.8). Samples were then incubated at 37˚C for 60 min. After RFLP samples were analyzed on a 1% agarose gel. Samples with distinct profiles were selected for further analysis and a total of 95 samples were sequenced.

(j) Microcosms microbial diversity by 16S rRNA gene analysis

For the microcosms, a total of 95 sequences were retrieved. Sanger sequences were trimmed and processed before analyzing them through the ribosomal database project online program. Phylogenetic tree was then generated by neighbor-joining method using PAUP, version 4.0b10 for Unix. Well characterized bacteria and environmental detection 16S rRNA gene sequences were included in the analysis in order to compare our environmental DNA and microcosm sequences. A neighbor joining phylogenetic tree illustrates the relative diversity of microbes obtained from clone libraries generated from the light and dark incubated microcosms (Fig. 5).

A further detail analysis of the relative microbial diversity was investigated by analyzing 16S rRNA gene sequences through paired-end illumina next generation sequencing using the primers set x and y, which amplify the variable regions V3 and V4. This high through put sequencing technology allowed us to determine the relative microbial abundance found in 21 m Big Soda Lake water column. Triplicate samples were sequenced. Look over protocol and add the details

(k) Phylogenetic analysis of arsenic oxidoreductases

In order to generate phylogenetic trees for *arrA* and *arxA,* BLASTx was performed for the environmental and microcosms DNA sequences in order to acquire amino acid

sequences. Amino acids were then align with Kalign and phylogenetic tree was then generated by neighbor-joining method using PAUP, version 4.0b10 for Unix. Reference sequences for DMSO family of molybdopterin oxidoreductases such as AioA, ArxA, and ArrA indicated that each enzyme family forms distinct clades that can be used to compare sequences retrieved from the environment through phylogenetic analysis. Illumina paired end sequences were merged by MeFit program and filtered by generating OUT's using USEARCH.

(l) Arsenic analysis

Arsenic analysis was determined by HPLC-ICP-MS as previously described (Hernandez-Maldonado *et al.*, 2017). Approximately 10 mL triplicate samples were filtered in vacutainers from 5 m, 10 m 18 m and 21 m in order to determine arsenic speciation and quantification within Big Soda Lake water column. Samples were stored at 4˚C and analyzed two days after sample collection. Arsenic speciation was also determined for microcosms by filtering (0.2µm sterile filters) approximately 500µL over time. Samples were diluted in 30 mM phosphoric acid mobile phase containing germanium, which served as an internal control.

Section 3.07 Figures

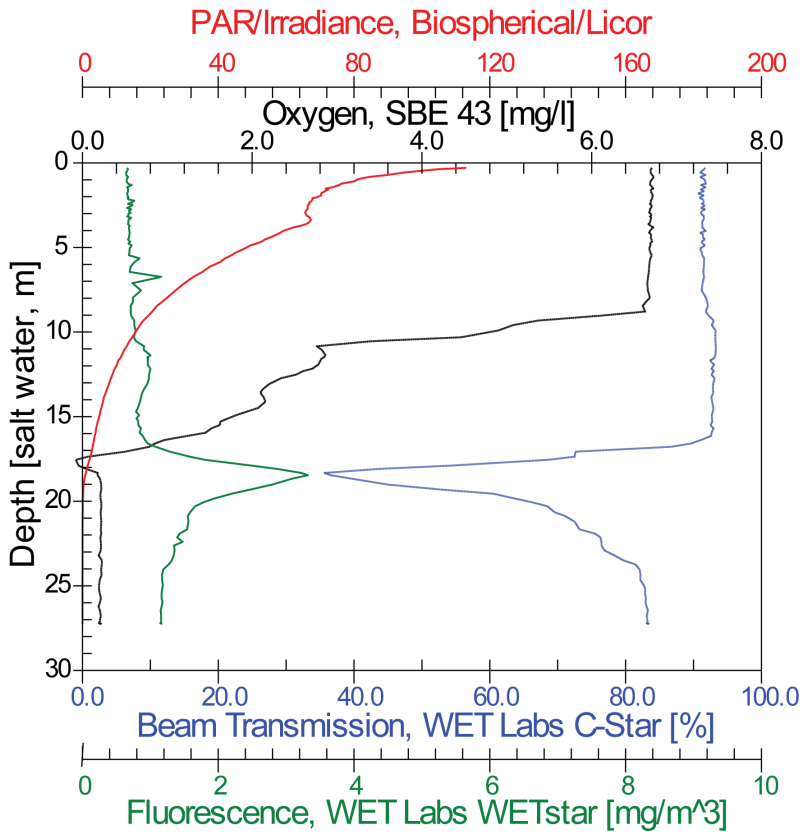

Figure 1. Big Soda Lake water column physicochemical depth profile. Vertical distribution parameters were detected by Seabird conductive, temperature, depth (CTD) instrumentation in September 15th, 2015. Ideal parameters for anoxic light dependent arsenite oxidation where detected below 18 meters indicated by the absence of oxygen (black line), available photosynthetic active radiation (red line), increase in fluorescence (green line), and decrease in beam transmission (blue line). Anoxic water samples were collected from approximately 21 m and in order to set-up microcosms.

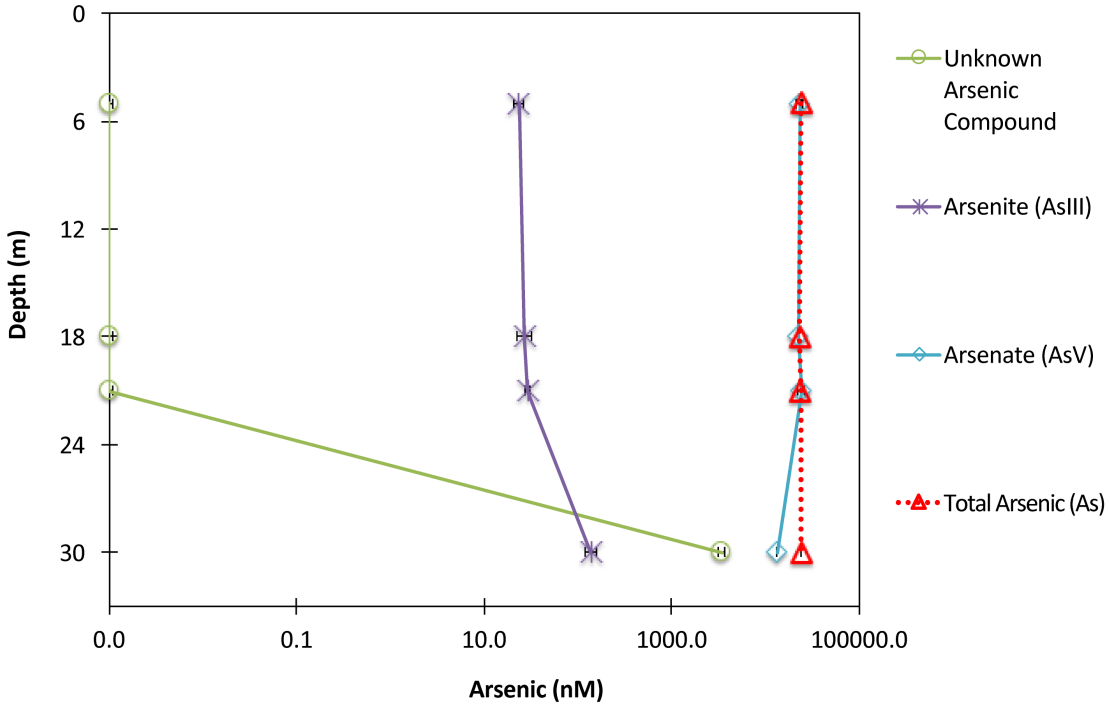

Figure 2. 2015 Big Soda Lake water column arsenic depth profile. Arsenic speciation and concentrations were determined by HPLC-ICP-MS as previously described (Hernandez-Maldonado *et al.*, 2017) from 5, 18, 21 and 30 m. Arsenate is shown in turquoise, arsenite is shown in purple and unknown arsenic species is shown in the green line. Total arsenic concentrations were determined by ICP-MS and shown in red dotted line.

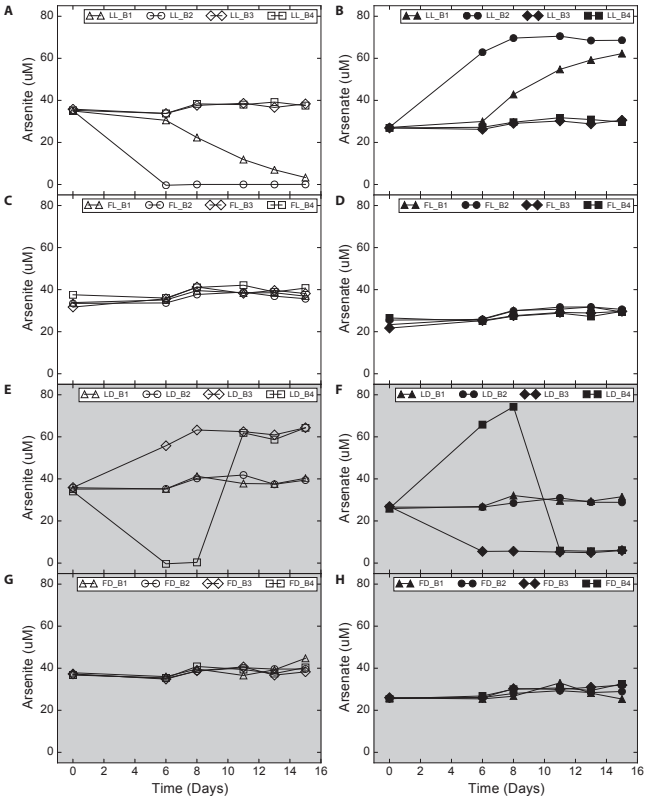

Figure 3. Microbial mediated arsenic cycle within microcosms containing Big Soda Lake native water. Microcosms were supplemented with ~35 μM arsenite and monitored for arsenic over time. (AB) Unfiltered native water incubated in the light are denoted by LL (Live Light) (CD) Abiotic filtered samples incubated in the light are denoted by FL (Filtered Light) (EF) un filtered native water incubated in the dark are denoted by LD (Live Dark) and (GH) abiotic filtered samples incubated in the dark are denoted by FD (Filtered Dark). Arsenite concentrations are denoted with open legends and shown on the left graphs (A, C, E, G) whereas arsenate measurements are denoted with filled legends and shown on the right graphs (B, D, F, H). The triangles, circles, diamonds and squares represent individual serum bottles.

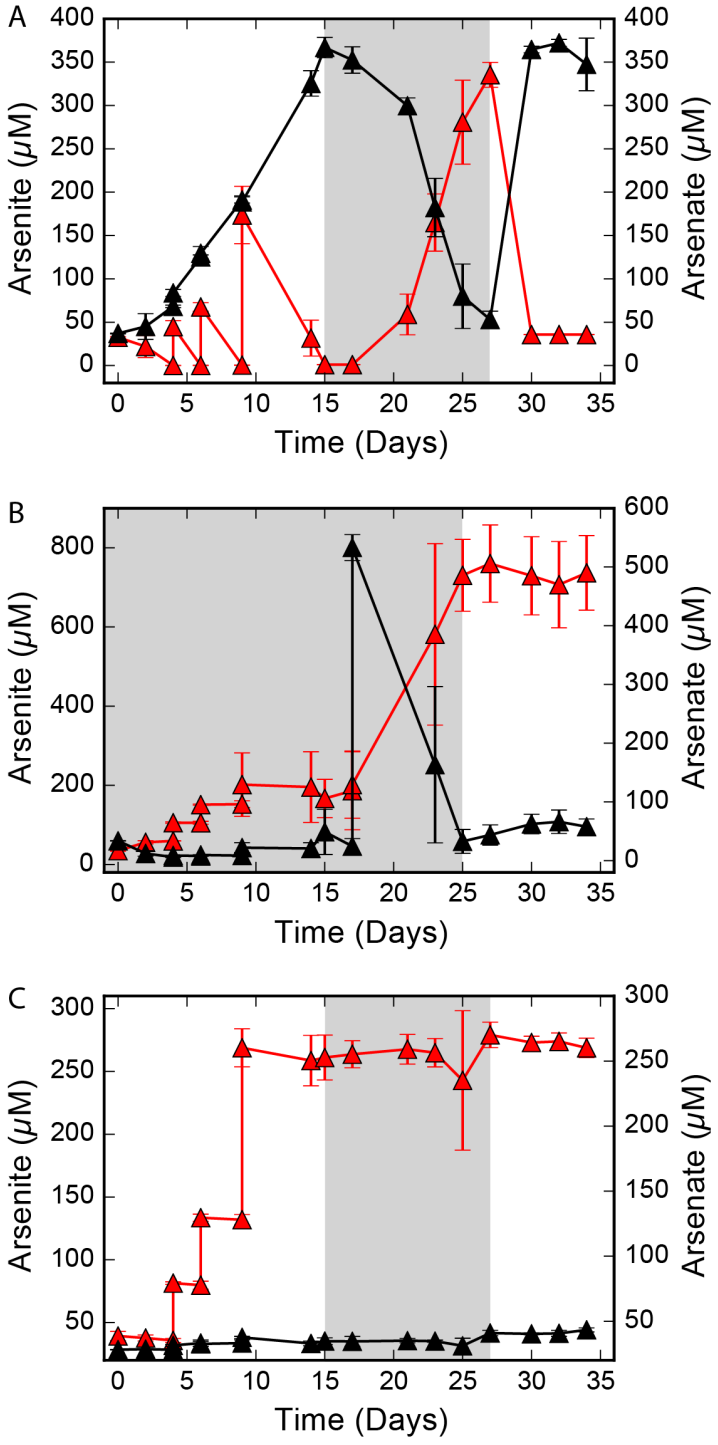
A
Arsenite (μM)
Arsenate (μM)
Time (Days)
B
Arsenite (μM)
Arsenate (μM)
Time (Days)
C
Arsenite (μM)
Arsenate (μM)
Time (Days)

Figure 4. Light dependent microbial mediated arsenic cycle in microcosms containing a concentrated consortium from Big Soda Lake. The dark grey rectangles indicate dark incubations. Microcosms were supplemented with~ 35 μM arsenite. (A) Microcosms incubated under light conditions where a light dependent arsenite oxidation is observed by the decrease of arsenite (red) and increase of arsenate(black) with light (1-15 days and 27-36 days) and arsenate reduction is observed in the absence of light (15-27 days). (B) Microcosms incubated under dark conditions where only arsenate reduction occurred, independent of light (C) Microcosms containing concentrated indigenous microbial biomass that were heat killed by autoclaved prior incubations, where no chemical arsenic transformations were observed.

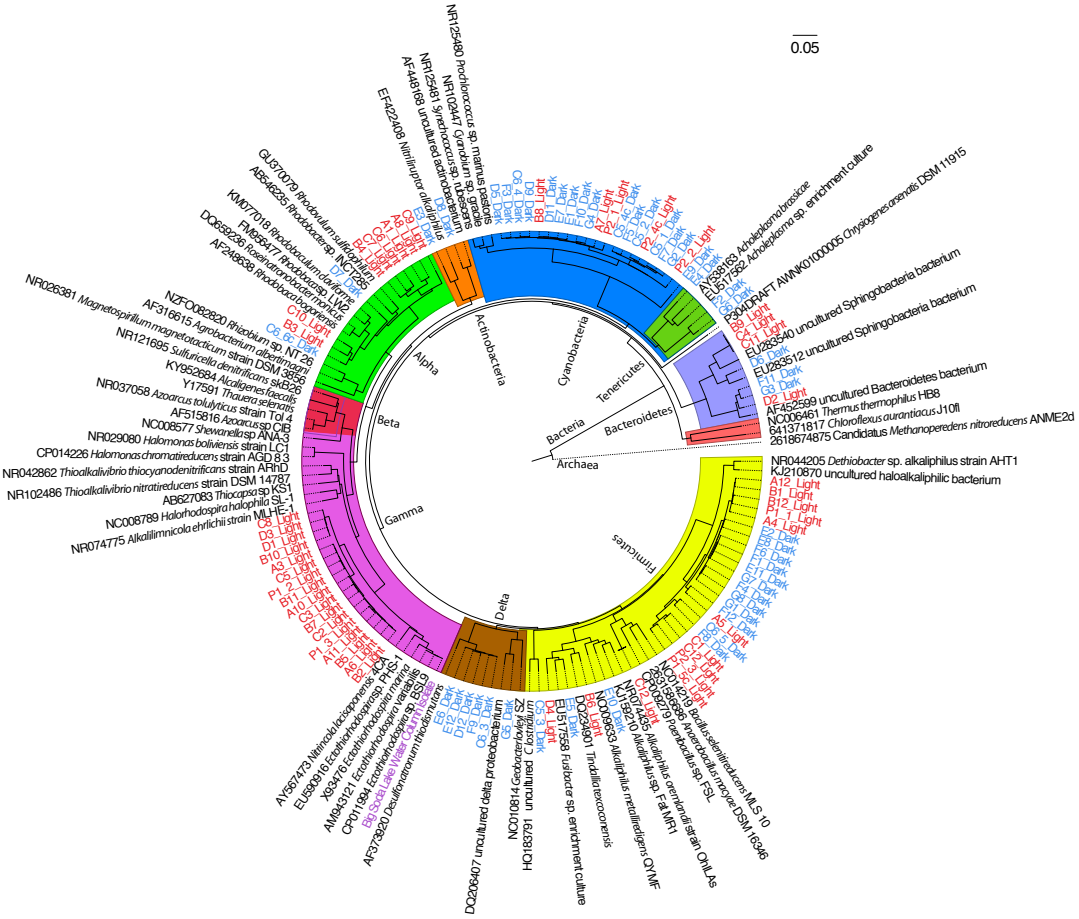

Figure 5. Microcosms microbial diversity by 16S rRNA analysis. The phylogenetic neighbor-joining polar tree is color coded by phylum with the exception of *Proteobacteria*, which is labeled at the "Class" level. The scale bar represents 0.05% sequence differences. 48 16S Sanger sequences originated from light incubated microcosms (red), 47 from dark incubated microcosms (blue) and detection of Big Soda Lake water column (purple).

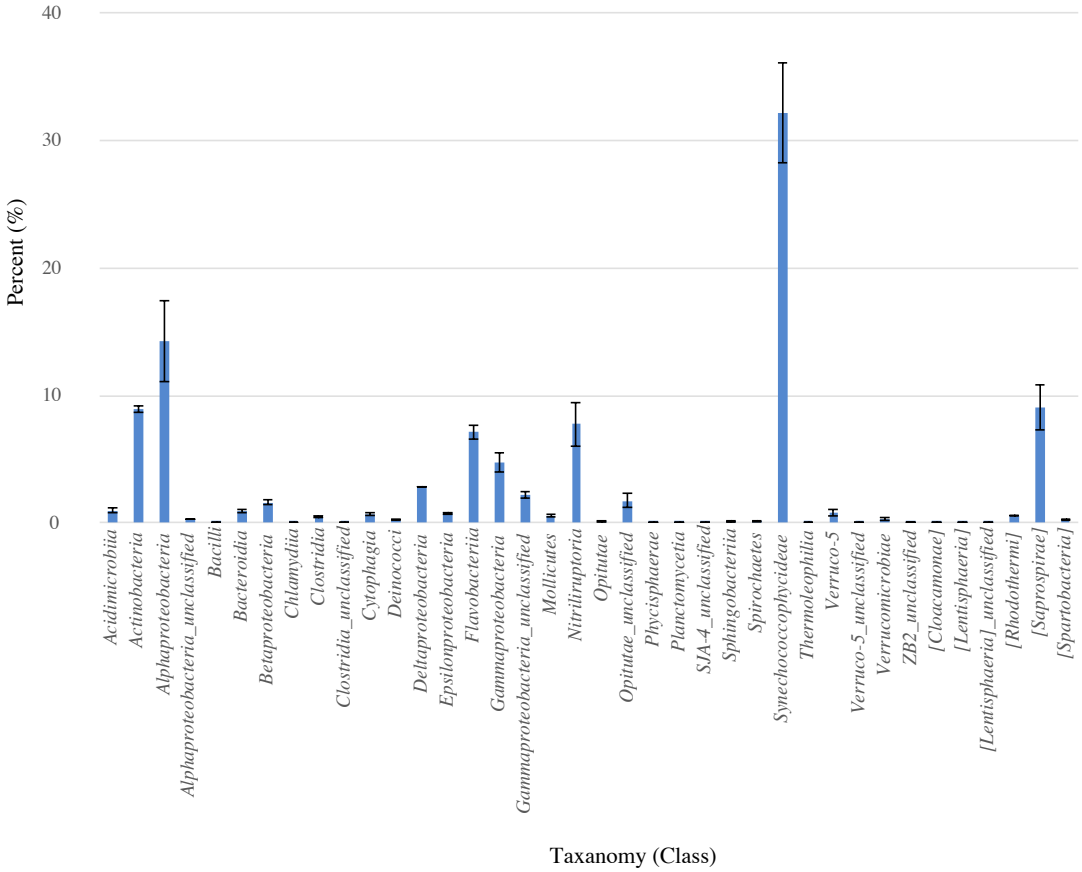

Figure 6. Big Soda Lake microbial relative abundance by class level. The 21 m microbial population was determined by analyzing the average of triplicate samples and their standard deviation. A total of 50,529 16S rRNA illumina DNA sequences were determine for the triplicate samples and plotting the top 0.03% "class" level. The mayor photosynthetic bacterial class were *Cyanobacteria* and *Alphaproteobacteria*. Less than 10% were of *Gammaproteobacteria*.

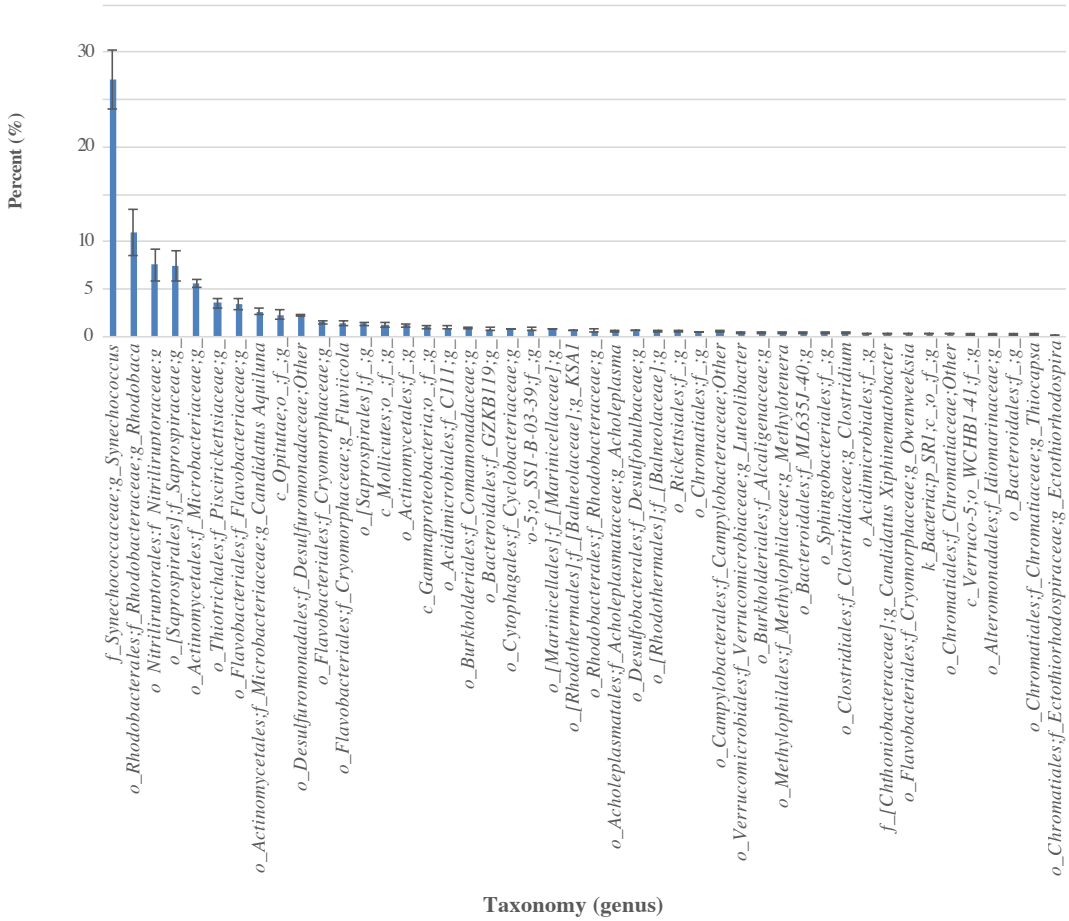

Figure 7. Big Soda Lake microbial relative abundance by genus level. The 21 m microbial population was determined by taking the average and standard deviation of the triplicate samples. A total of 51,540 16S rRNA illumina DNA sequences were analyzed and plotting the top 0.05% "genus" level. The two major photosynthetic bacteria where *Synechococcus* and *Rhodobacca* and less than 1% were *Ectothiorhodospira.*

Figure 8. Phylogenetic analysis of the arsenite oxidase, ArxA in relation to oxidoreductases within the dimethyl sulfoxide (DMSO) reductase family of molybdenum containing enzymes. Bacteria containing ArrA, ArxA and AioA genes were plotted as a reference. Samples in red represent sequences from microcosms in light-incubations, sample in blue represent sequences from microcosm in dark-incubations, the sample in purple represents the Big Soda Lake isolate, and samples in green represent illumina NGS amplicons positive for ArxA. A total of 941583 sequences were analyzed and the average of three replicates were plotted as percent's for the amplicons that texted positive for ArxA.

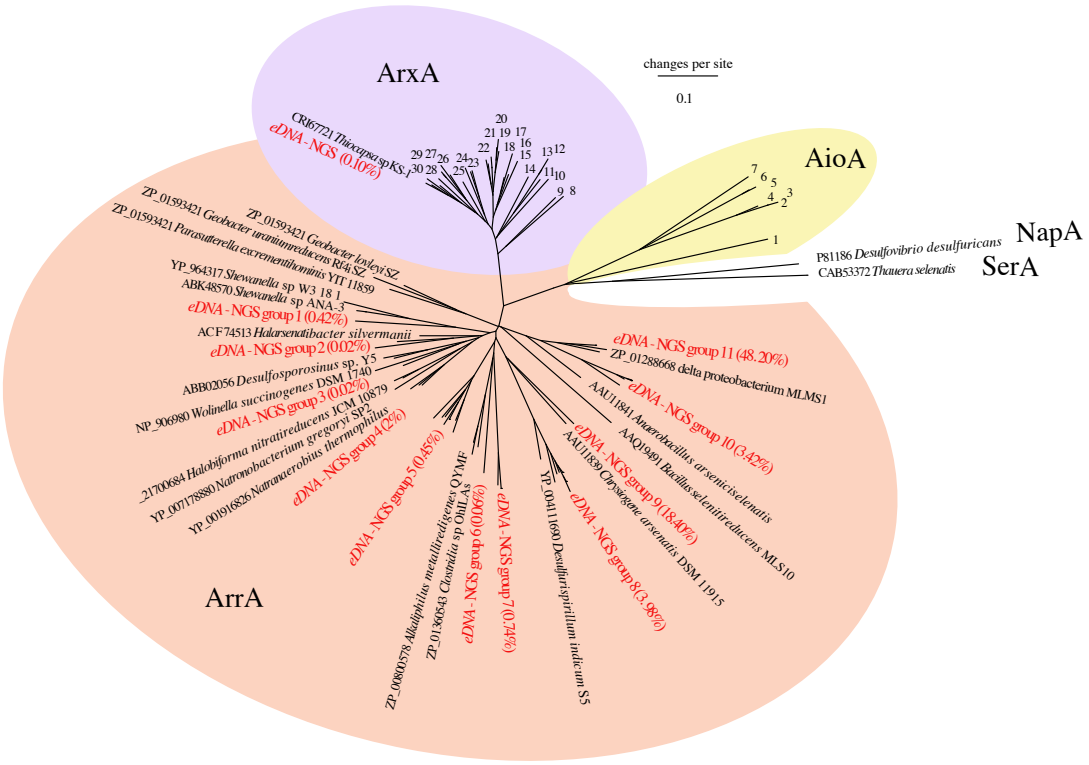

Figure 9. Phylogenetic analysis of the arsenite oxidase, ArrA in relation to oxidoreductases within the dimethyl sulfoxide (DMSO) reductase family of molybdenum containing enzymes. Bacteria containing ArrA, ArxA and AioA genes were plotted as a reference (1-30). Samples in red represent illumina NGS amplicons positive for ArxA. The total sequences analyzed were approximately 443055. The following numbers 1-30 indicate reference bacteria containing AioA an ArxA.

Section 3.08 References

Anderson, G.L., Williams, J., and Hille, R. (1992) The purification and characterization of arsenite oxidase from *Alcaligenes faecalis*, a molybdenum-containing hydroxylase. *Journal of Biological Chemistry* **267**: 23674–23682.

Antony, C.P., Kumaresan, D., Hunger, S., Drake, H.L., Murrell, J.C., and Shouche, Y.S. (2012) Microbiology of Lonar Lake and other soda lakes. *The ISME Journal* **7**: 468–476.

Cloern, J.E., Cole, B.E., and Oremland, R.S. (1983) Autotrophic processes in meromictic Big-Soda Lake, Nevada. *Limnology and Oceanography* **28**: 1049–1061.

Cloern, J.E., Cole, B.E., and Wienke, S.M. (1987) Big Soda Lake (Nevada) .4. Vertical fluxes of particulate matter-seasonality and variations across the chemocline. *Limnology and Oceanography* **32**: 815–824.

Detmer, A.E., Giesenhagen, H.C., and Trenkel, V.M. (1993) Phototrophic and heterotrophic pico-and nanoplankton in anoxic depths of the central Baltic Sea. *Marine Ecology- ...* **99**: 197–203.

Edwardson, C.F., Planer-Friedrich, B., and Hollibaugh, J.T. (2014a) Transformation of monothioarsenate by haloalkaliphilic, anoxygenic photosynthetic purple sulfur bacteria. *FEMS Microbiology Ecology* **90**: 858–868.

Edwardson, C.F., Planer-Friedrich, B., and Hollibaugh, J.T. (2014b) Transformation of monothioarsenate by haloalkaliphilic, anoxygenic photosynthetic purple sulfur bacteria. *FEMS Microbiology Ecology* **90**: 858–868.

Engel, A.S., Johnson, L.R., and Porter, M.L. (2012) Arsenite oxidase gene diversity among *Chloroflexi* and *Proteobacteriafrom* El Tatio Geyser Field, Chile. *FEMS Microbiology Ecology* **83**: 745–756.

Fendorf, S., Nico, P.S., Kocar, B.D., and Masue, Y. (2010) Arsenic chemistry in soils and sediments. *Developments in soil …* **34**: 357–378.

Ferguson, J.F. and Gavis, J. (1972) A review of the arsenic cycle in natural waters. *Water Research* **6**: 1259–1274.

Hernandez-Maldonado, J., Sanchez-Sedillo, B., Stoneburner, B., Boren, A., Miller, L., McCann, S., et al. (2017) The genetic basis of anoxygenic photosynthetic arsenite oxidation. *Environ Microbiol* **19**: 130–141.

Hoeft, S.E., Blum, J.S., Stolz, J.F., Tabita, F.R., Witte, B., King, G.M., et al. (2007) *Alkalilimnicola ehrlichii* sp. nov., a novel, arsenite-oxidizing haloalkaliphilic gammaproteobacterium capable of chemoautotrophic or heterotrophic growth with nitrate or oxygen as the electron acceptor. *Int. J. Syst. Evol. Microbiol* **57**: 504–512.

Hoeft, S.E., Kulp, T.R., Han, S., Lanoil, B., and Oremland, R.S. (2010) Coupled arsenotrophy in a hot spring photosynthetic biofilm at Mono Lake, California. *Applied and Environmental Microbiology* **76**: 4633–4639.

Humayoun, S.B., Bano, N., and Hollibaugh, J.T. (2003) Depth distribution of microbial diversity in Mono Lake, a Meromictic Soda Lake in California. *Applied and Environmental Microbiology* **69**: 1030–1042.

Ikemoto, H. and Mitsui, A. (1994) Diazotrophic synchronous growth of a marine

unicellular cyanobacterium, *Synechococcus* sp. strain Miami BG 043511, under aerobic and microaerobic/anaerobic conditions. *Microbiology* **140**: 2153–2158.

Jones, B.E., GRANT, W.D., Duckworth, A.W., and Owenson, G.G. (1998) Microbial diversity of soda lakes. *Extremophiles* **2**: 191–200.

Komorowicz, I. and Barałkiewicz, D. (2011) Arsenic and its speciation in water samples by high performance liquid chromatography inductively coupled plasma mass spectrometry-Last decade review. *Talanta* **84**: 247–261.

Kulp, T.R., Hoeft, S.E., Asao, M., Madigan, M.T., Hollibaugh, J.T., Fisher, J.C., et al. (2008) Arsenic(III) fuels anoxygenic photosynthesis in hot spring biofilms from Mono Lake, California. *Science* **321**: 967–970.

Oremland, R.S. (2003) The ecology of arsenic. *Science* **300**: 939–944.

Oremland, R.S., Hoeft, S.E., Santini, J.M., Bano, N., Hollibaugh, R.A., and Hollibaugh, J.T. (2002) Anaerobic oxidation of arsenite in Mono Lake Water and by a facultative, arsenite-oxidizing chemoautotroph, strain MLHE-1. *Applied and Environmental Microbiology* **68**: 4795–4802.

Oremland, R.S., Saltikov, C.W., Wolfe-Simon, F., and Stolz, J.F. (2009) Arsenic in the evolution of earth and extraterrestrial ecosystems. *Geomicrobiology Journal* **26**: 522–536.

Oremland, R.S., Stolz, J.F., and Hollibaugh, J.T. (2004) The microbial arsenic cycle in Mono Lake, California. *FEMS Microbiology Ecology* **48**: 15–27.

Paerl, H.W. (1991) Ecophysiological and trophic implications of light-stimulated amino acid utilization in marine picoplankton. *Applied and Environmental*

Microbiology **57**: 473–479.

Priscu, J.C., Axler, R.P., Carlton, R.G., Reuter, J.E., Arneson, P.A., and Goldman, C.R. (1982) Vertical profiles of primary productivity, biomass and physico-chemical properties in meromictic Big Soda Lake, Nevada, U.S.A. *Hydrobiologia* **96**: 113–120.

Richey, C., Chovanec, P., Hoeft, S.E., Oremland, R.S., Basu, P., and Stolz, J.F. (2009) Respiratory arsenate reductase as a bidirectional enzyme. *Biochemical and Biophysical Research Communications* **382**: 298–302.

Rosen, B.P. (2002) Biochemistry of arsenic detoxification. *FEBS Lett.* **529**: 86–92.

Saltikov, C.W. and Newman, D.K. (2003) Genetic identification of a respiratory arsenate reductase. *Proc. Natl. Acad. Sci. U.S.A.* **100**: 10983–10988.

Santini, J.M., Sly, L.I., Schnagl, R.D., and Macy, J.M. (2000) A new chemolithoautotrophic arsenite-oxidizing bacterium isolated from a Gold Mine: Phylogenetic, physiological, and preliminary biochemical studies. *Applied and Environmental Microbiology* **66**: 92–97.

Shen, G.Z. and Bryant, D.A. (1995) Characterization of a *Synechococcus* sp. strain Pcc-7002 mutant lacking photosystem-1. Protein assembly and energy-distribution in the absence of the photosystem-1 reaction-center core complex. *Photosyn. Res.* **44**: 41–53.

Smith, A.H., Hopenhayn-Rich, C., Bates, M.N., Goeden, H.M., Hertz-Picciotto, I., Duggan, H.M., et al. (1992) Cancer risks from arsenic in drinking water. *Environmental Health Perspectives* **97**: 259–267.

Smith, A.H., Lingas, E.O., and Rahman, M. (2000) Contamination of drinking-water by arsenic in Bangladesh: a public health emergency. *Bull. World Health Organ.* **78**: 1093–1103.

Smith, A.H., Lopipero, P.A., Bates, M.N., and Steinmaus, C.M. (2002) Public health-arsenic epidemiology and drinking water standards. *Science* **296**: 2145–2146.

Yu, W.H., Harvey, C.M., and Harvey, C.F. (2003) Arsenic in groundwater in Bangladesh: A geostatistical and epidemiological framework for evaluating health effects and potential remedies. *Water Resour. Res.* **39**:.

Zargar, K., Conrad, A., Bernick, D.L., Lowe, T.M., Stolc, V., Hoeft, S., et al. (2012) ArxA, a new clade of arsenite oxidase within the DMSO reductase family of molybdenum oxidoreductases. *Environ Microbiol* **14**: 1635–1645.

Zargar, K., Hoeft, S., Oremland, R., and Saltikov, C.W. (2010) Identification of a novel arsenite oxidase gene, *arxA*, in the haloalkaliphilic, arsenite-oxidizing bacterium *Alkalilimnicola ehrlichii* strain MLHE-1. *Journal of Bacteriology* **192**: 3755–3762.

Zehr, J.P., Harvey, R.W., and Oremland, R.S. (1987) Big Soda Lake (Nevada). 1. Pelagic bacterial heterotrophy and biomass. *Limnol. Oceanogr*.

Zhang, S., Rensing, C., and Zhu, Y.-G. (2014) *Cyanobacteria*-mediated arsenic redox dynamics is regulated by phosphate in aquatic environments. *Environ. Sci. Technol.* **48**: 994–1000.

Chapter 4. The genetic basis of anoxygenic photosynthetic arsenite oxidation

environmental microbiology

Environmental Microbiology (2017) **19**(1), 130–141 doi:10.1111/1462-2920.13509

The genetic basis of anoxygenic photosynthetic arsenite oxidation

Jaime Hernandez-Maldonado,[1] Benjamin Sanchez-Sedillo,[1] Brendon Stoneburner,[1] Alison Boren,[1] Laurence Miller,[2] Shelley McCann,[2] Michael Rosen,[3] Ronald S. Oremland[2] and Chad W. Saltikov[1]*

[1]*Department of Microbiology and Environmental Toxicology, University of California Santa Cruz, 1156 High Street, Santa Cruz, CA, 95064, USA.*

[2]*US Geological Survey, 345 Middlefield Road Menlo Park, CA, 94025, USA.*

[3]*US Geological Survey, 2730 N. Deer Run Road, Carson City, NV, 89701, USA.*

Summary

'Photoarsenotrophy', the use of arsenite as an electron donor for anoxygenic photosynthesis, is thought to be an ancient form of phototrophy along with the photosynthetic oxidation of Fe(II), H_2S, H_2 and NO_2^-. Photoarsenotrophy was recently identified from Paoha Island's (Mono Lake, CA) arsenic-rich hot springs. The genomes of several photoarsenotrophs revealed a gene cluster, *arxB2AB1CD*, where *arxA* is predicted to encode for the sole arsenite oxidase. The role of *arxA* in photosynthetic arsenite oxidation was confirmed by disrupting the gene in a representative photoarsenotrophic bacterium, resulting in the loss of light-dependent arsenite oxidation. *In situ* evidence of active photoarsenotrophic microbes was supported by *arxA* mRNA detection for the first time, in red-pigmented microbial mats within the hot springs of Paoha Island. This work expands on the genetics for photosynthesis coupled to new electron donors and elaborates on known mechanisms for arsenic metabolism, thereby highlighting the complexities of arsenic biogeochemical cycling.

Introduction

Despite its toxicity arsenic can be used by certain organisms in a favourable way to generate cellular energy (Oremland, 2003; Saltikov, 2011; Amend *et al.*, 2014). Oremland *et al.* (2009). More specifically, this has been defined as 'arsenotrophy' or the coupling of arsenic reduction or oxidation to cellular energy production (Oremland *et al.*, 2009). The interconversion of arsenate and arsenite also has environmental consequences that are known to affect arsenic contamination of water (Harvey, 2002; Kocar *et al.*, 2008; Fendorf *et al.*, 2010). Arsenotrophic microbes oxidize/reduce two major forms of arsenic oxyanions: arsenite [As(III)] and arsenate [As(V)] (Islam *et al.*, 2004; Oremland and Stolz, 2005; Dhar *et al.*, 2011; Stuckey *et al.*, 2015). The latter arsenical is typically less toxic, more stable under oxidizing conditions, and favours sequestration within minerals (O'Day *et al.*, 2004; Kocar and Fendorf, 2009). However, the onset of reducing conditions stimulates microbial arsenate reduction to arsenite. Relative to arsenate, arsenite is more toxic, has a greater partitioning into the aqueous phase, and is more readily transported within anoxic groundwater (Masscheleyn and Delaune, 1991; Kocar *et al.*, 2008). South Asian countries have the most serious arsenic groundwater problems that are manifested by high prevalence of arsenic-related illnesses such as arsenicosis and cancer (Nickson *et al.*, 1998; Acharyya *et al.*, 1999; Smith *et al.*, 2000). The underlying microbial genes responsible for microbial reactions relevant to public health likely developed on the early Earth when the prevailing environmental conditions were reducing (Sessions *et al.*, 2009). During this time, arsenic may have been more abundant in aquatic environments because of increased volcanic activity, which would have brought arsenite and other chalcophilic compounds to the Earth's surface (Oremland *et al.*, 2009). This would have provided the necessary niche that allowed the evolution of autotrophic pathways for arsenite oxidation. Finally, recent discoveries where both arsenic and microbial organic signatures found in a Mars (Wallis and Wickramasinghe, 2015) meteoroid and within stromatolites (Sforna *et al.*, 2014) from 2.7 b.y.a., raises the question on how arsenite may have played a role in arsenotrophy as life evolved within arsenic rich environments.

Most of the well-known pathways of arsenotrophy have been determined in chemotrophic microbes. However, arsenotrophic reactions now include a light-dependent pathway involving anoxygenic photosynthesis with arsenite

Received 17 June, 2016; accepted 17 August, 2016. *For correspondence to: E-mail: saltikov@ucsc.edu; Tel. +831-459-5520.

as an electron donor (Budinoff and Hollibaugh, 2008; Kulp *et al.*, 2008; Hoeft *et al.*, 2010), or 'photoarsenotrophy'. Kulp *et al.* (2008) first described the occurrence of photosynthetic arsenite oxidation in red-pigmented biofilms found within the hot springs of Paoha Island Mono Lake, CA, an arsenic-rich, hypersaline, alkaline lake. Moreover, the first photoarsenotroph, *Ectothiorhodospira* sp. str. PHS-1, was isolated from the Paoha Island hot spring milieu. The genome of PHS-1 lacked evidence for an *aioA*-type arsenite oxidase, which at the time was the prevailing consensus pathway for arsenite oxidation (Lett *et al.*, 2011). Instead, Zargar *et al.* (2010) revealed a potentially 'new' genetic pathway for arsenite oxidation based on a recently identified anaerobic arsenite oxidase gene, *arxA*, in a related chemoautotroph, *Alkalilimnicola ehrlichii* str. MLHE-1. The predicted ArxA enzyme was shown to form a new clade within the DMSO reductase family of oxidoreductases (Zargar *et al.*, 2012), which also contains a separate clade for the AioA enzyme. In the absence of a genetic system in a photoarsenotroph, circumstantial evidence suggested that the *arxA*-like gene of PHS-1 encodes for a molybdenum-containing arsenite oxidase. Furthermore arsenite exposure resulted in the induction of the *arxA* gene in PHS-1. However, the function of ArxA in PHS-1 could not be ascertained because of difficulties in establishing a genetic system in this organism. Ultimately, the ArxA phylogenetic analysis and molecular genetic studies with the chemotrophic, MLHE-1 (Zargar *et al.*, 2010) bacterium suggested that ArxA is the most plausible pathway for photoarsenotrophy in PHS-1.

The ecological distribution and environmental impact of photoarsenotrophs to the arsenic cycle remains unknown. Currently our awareness of the distribution of photoarsenotrophy is limited to the environment where PHS-1 was isolated. Molecular detection of *arxA* from other extreme environments such as Yellowstone, WY, and other hot spring environments around the Mono Lake, CA (Zargar *et al.*, 2012) area, suggest the possibility of anaerobic arsenite oxidation. These extreme, arsenic-rich environments are analogous to the conditions of early Earth (Kempe and Kazmierczak, 1997) that may have favoured the evolution of anoxygenic photosynthetic pathways that are present in green and purple (non-) sulfur bacteria. In order to comprehend the evolutionary trajectory of these metabolisms and to better appreciate the past, present, and future impact of photoarsenotrophs on arsenic geochemistry, additional work is needed to confirm that *arxA* is responsible for arsenite oxidation in photoarsenotrophs.

The purpose of this report is to present the first genetic evidence that *arxA* is the main arsenite oxidase in a photoarsenotroph. We demonstrated that the *arxA* gene is also expressed in the Paoha Island red-mat biofilms where PHS-1 was first isolated. These results not only validate the function of the *arx* gene pathway but also further expand on the complexities of the arsenic biogeochemical cycle by adding 'light' as a considering factor. Investigating the geochemical impact of photoarsenotrophy will depend on further understanding the regulation and kinetic parameters of *arx* genes and protein products, and developing molecular tools to track the genetic signatures of *arx*-containing microorganisms. Thus, this work lays the critical groundwork for these future studies.

Results and discussion

Molecular mechanism for photoarsenotrophy

Our current understanding of the molecular mechanism for anoxygenic photosynthesis coupled to arsenite oxidation is based on previous genetic confirmation that the *arx* gene cluster in *Alkalilimnicola ehrlichii* str. MLHE-1 is required for oxidation and anaerobic growth on arsenite coupled to nitrate reduction (Zargar *et al.*, 2010). The identification of *arx* gene homologs in the PHS-1 genome (Zargar *et al.*, 2010; 2012) and the absence of an *aio*-type gene cluster further supports the hypothesis that photosynthetic arsenite oxidation is conferred by *arx* genes. In anoxygenic photosynthesis electrons are required in order to generate a proton motive force, which in turn can drive numerous biological reactions in the cell such as producing ATP and NAD(P)H that are used for anabolic metabolisms. Due to thermodynamic constraints reduced substrates such as sulfide, nitrite (Griffin *et al.*, 2007), iron(II) (Widdel *et al.*, 1993) and arsenite (Budinoff and Hollibaugh, 2008) may be oxidized via cyclic or non-cyclic electron flow within anoxygenic photosynthetic bacteria. During cyclic electron flow, light energizes electrons within the photosynthetic reaction centre (PRC) followed by electron transport through quinone intermediates to cytochrome bc_1, which generates a proton motive force. The cycle is completed when bc_1 transfers its electrons to a periplasmic *c*-type cytochrome (e.g., c_2) and ultimately back to the PRC for another cycle (Lavergne *et al.*, 2009). In non-cyclic electron flow, reduced quinols (e.g., ubiquinol/ubiquinone, +70 to +112 mV) can be used to drive reverse electron transport, which uses the energy from the proton motive force to generate reducing power [e.g., NAD(P)H] to fuel carbon dioxide assimilation and biosynthesis. Based on the $HAsO_4^{2-}/H_3AsO_3$ redox potential of +54 mV (Budinoff and Hollibaugh, 2008), electrons from arsenite could be transferred to either a c_2-like cytochrome (+325 mV) (Cammack *et al.*, 1981) similar to the predicted cyclic electron transfer for photosynthetic Fe(II) oxidation (Ehrenreich and Widdel, 1994) or the quinone pool (ubiquinone, +70 to +112 mV). Although it is not known how arsenite oxidation is coupled to photosynthesis, we hypothesize that arsenite is oxidized to arsenate by ArxA followed by electron transfer to Fe-S containing subunits (either ArxB2 and/or ArxB1). The predicted protein sequences for ArxB2

and ArxB1 share low amino identities/similarities (14.4%/22.8%) however they both have predicted amino acid motifs for 4Fe-4S clusters (based on pfam analysis http://pfam.xfam.org). Their function with respect to the electron transport chain for photosynthesis or in chemoautotrophs remains unknown. Electrons could then either be transferred to c_2 (or similar *c*-type cytochromes) and back to the PRC or to the quinone pool mediated by ArxC, a predicted membrane protein homologous to the polysulfide reductase NrfD-like membrane protein. The latter is known to transfer electrons from the quinone pool during polysulfide reduction (Jormakka *et al.*, 2008). Nonetheless the generation of NAD(P)H is dependent on electrons from arsenite and coupled to the proton motive force, generated by cytochrome bc_1, which together ultimately generate reducing power, NAD(P)H, and ATP for biological reactions within the cell.

To begin testing the photosynthetic reaction mechanism for arsenite oxidation, we first addressed the hypothesis that *arxA* (or genes within the *arxB2AB1CD* cluster) is required for photosynthetic arsenite oxidation. Because no genetic systems were available for a photoarsenotrophic microbe, our first task was to identify a suitable strain for genetic system development. PHS-1 was determined to be intractable for this purpose because of difficulties in reliably culturing the strain on solid media. We then isolated another photoarsenotroph, *Ectothiorhodospira* sp. str. BSL-9 from Big Soda Lake (NV), an arsenic-rich hypersaline alkaline crater lake similar to Mono Lake (Cloern *et al.*, 1983; Zehr *et al.*, 1987). BSL-9 was facile to work with relative to PHS-1 and could be grown under less "extreme" conditions such as pH 8 and 20 g/L salt. Although BSL-9 was unable to grow in the presence of oxygen, the strain was aerotolerant facilitating the preparation of conjugation reactions and streak plates on the bench prior to incubation under anaerobic conditions. Genetic system development was also aided by completing the BSL-9 genome. Analysis of the genome indicated an *arx* gene cluster with 100% synteny to that found in PHS-1 and MLHE-1 genomes. The BSL-9 ArxA was homologous to other ArxA sequences from *Ectothiorhodospira* sp. str. PHS-1 (75% similarities), *Alkalilimnicola ehrlichii* str. MLHE-1 (86% similarities), and other sequences found in NCBI database such as *Halorhodospira halophila str.* SL-1 (78% similarities). As previously described for other PHS-1 and MLHE-1 ArxA sequences, the BSL-9 ArxA clustered within these sequences and formed its own distinct clade within the DMSO reductase family of molybdenum containing enzymes (Fig. 1). The genomic and phylogenetic evidence strongly supported that the *arxB2AB1CD* gene cluster of BSL-9 encoded for an anaerobic arsenite oxidase, *arxA* (ECTOBSL9_2837, locus tag number) and that it was the best candidate gene for further genetic manipulation.

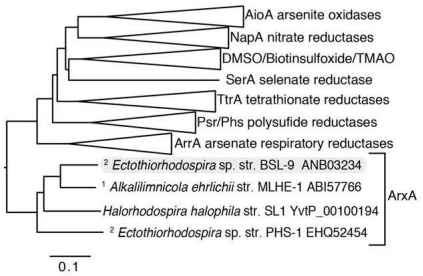

Fig. 1. ArxA forms a clade within the DMSO reductase family of molybdenum containing oxidoreductases. Phylogenetic analysis indicates that ArxA clusters more closely to the arsenate reductase, ArrA than the arsenite oxidase, AioA. However ArxA is distinct from other known DMSO oxidoreductase family enzymes. The ArxA clade contains chemoautorophic[1] and photoautotrophic[2] arsenite oxidizers. The Genbank accession numbers are indicated for the ArxA containing taxa. The scale bar indicates changes per position within the multisequence amino acid alignment.

We were interested in determining if expression of *arxA* in BSL-9 was regulated by arsenite. In our experiments, we used custom-built infrared (IR) LED (850 nm peak centred between 800 and 900 nm) arrays as the primary light source because this was originally used for enrichment cultures to eliminate the growth of oxygenic photoautotrophs, such as cyanobacteria, which absorb light in the visible range spectrum. Oxygen produced by cyanobacteria and algae inhibited the growth of anoxygenic phototrophs in our enrichment cultures. In purple sulfur bacteria the absorbance spectra depends on the bacterial species, light, and growth conditions but most typically have peak absorbance within the near-infrared (~ 800–900 nm) (Overmann, 2008; Robert, 2009). BSL-9 cells grown under IR had comparable absorbance peaks near 800, 860 and 900 nm. To demonstrate the regulation and induction of *arxA* only in the presence of arsenite (Fig. 2, bar graph), BSL-9 cultures were grown on acetate (Fig. 2, solid lines) under infrared (IR) lights to mid-log phase. We then extracted total RNA from BSL-9 cultures spiked with 300 μM arsenite and compared with BSL-9 cultures lacking arsenite over time. After 2 hours of the arsenite spike, *arxA* expression increased ~ 20-fold relative to cultures without arsenite (Fig. 2, bar graph). Peak *arxA* expression (40-fold) was detected after 12 hours following the arsenite spike. This result is consistent with previous reports that showed arsenite also induced *arxA* expression in PHS-1 (Zargar *et al.*, 2012) and MLHE-1 (Zargar *et al.*, 2010). The increased expression of *arxA* by the addition of arsenite is evident of *arxA* being regulated under different growth conditions. The *aio*-type arsenite oxidase is also regulated in response to arsenite (Kashyap *et al.*, 2006; Sardiwal *et al.*,

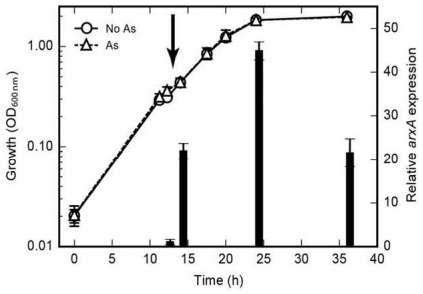

Fig. 2. *arxA* transcription induced by arsenite in *Ectothiorhodospira* sp. str. BSL-9. The wild-type BSL-9 strain was grown photosynthetically with acetate (solid lines) and monitored over time for *arxA* expression (indicated by the bars). After addition of arsenite (300 μM) at 12 hours (bold vertical arrow) *arxA* gene expression increased only in samples receiving the arsenite spike (dashed lines). Relative *arxA* gene expression at each time point was determined using the $\Delta\Delta C_t$ method with the corresponding "no arsenic" condition as the control condition and 16S rRNA gene as the reference gene. Both growth and gene expression data points and error bars represent the averages and standard deviation from triplicate cultures.

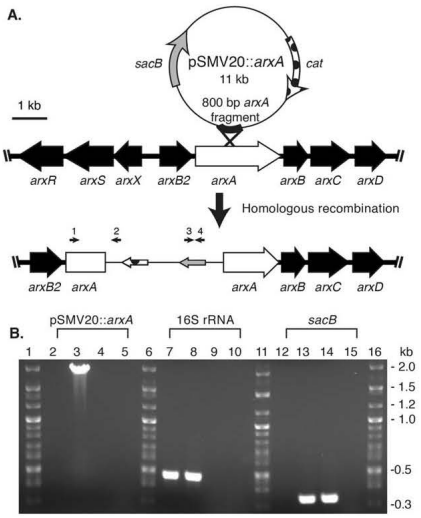

Fig. 3. Development and verification of *arxA* disruption mutation in *Ectothiorhodospira* sp. str. BSL-9. **(A)** The pSMV20 vector was inserted within *arxA* via homologous recombination. **(B)** PCR analysis for DNA from wild-type BLS-9 (2, 7, 12), ARXA1 (3, 8, 13), empty pSMV20 vector (4, 9, 14), and water (5, 10, 15). The following PCR primers were used: (i) *arxA* (Ext_arxA_F, arrow 1) gene and vector-specific (M13_R, arrow 2) (lanes 2-5), (ii) 16S rRNA (0341_16SV3V4-F and 0785_16SV3V4-R) gene (lanes 7-10) and (iii) *sacB* (*sacB*_F and *sacB*_R, arrows 3 and 4) (lanes 12-15). PCRs were analysed by agarose (1%) gel electrophoresis.

2010), which is mediated by a two-component sensor histidine kinase (AioS) and response regulator (AioR). Genes for an analogous regulatory system, *arxXSR* (ArxX, periplasmic binding protein; ArxS, sensor histidine kinase; and ArxR, response regulator), are present immediately upstream and in the reverse direction of the *arxB2AB1CD* gene clusters in BSL-9 (Fig. 3A), PHS-1 and MLHE-1. This gene cluster, *arxXSR*, is of great interest to future studies regarding the regulation of *arxA* in both chemoautotrophic and photoautotrophic organisms. Together with the ArxA phylogenetic analysis, the conservation of *arxXSR* and *arxB2AB1CD* gene clusters within the chemoautotroph, MLHE-1 and photoautotrophs, PHS-1, SL-1 and now BSL-9, paved the way for developing a genetic system in BSL-9 to confirm the function of *arx* genes in photoarsenotrophy.

To begin elucidating the genetics of photoarsenotrophy, we developed a genetic system to test if *arxA* was required for photosynthetic arsenite oxidation. Mutagenesis often requires the use of selectable markers, typically encoded by antibiotic resistance genes. Our analyses indicated that BSL-9 was resistant to kanamycin, ampicillin, gentamycin, but sensitive to chloramphenicol (Cm). A Cm-resistant broad host range plasmid was used to develop a conjugation method for introducing foreign DNA into BSL-9 wild-type strains from an *Escherichia coli* WM3064 donor strain (Saltikov and Newman, 2003), yielding $\sim 10^3$–10^4 Cm^R CFU ml^{-1}. We then took a gene disruption approach to 'knock out' the function of *arxA* gene in BSL-9, using a mutagenesis plasmid, pSMV20 (Zargar *et al.*, 2010), which contains the chloramphenicol acetyltransferase resistance encoding *cat* gene. An 800 base pair internal fragment of *arxA* was cloned into the mutagenesis plasmid (pSMV20). The new plasmid (pSMV20::*arxA*) was introduced into BSL-9 wild-type by conjugation with an *E. coli* donor strain. The partial *arxA* DNA fragment in pSMV20 allowed the disruption of *arxA* within the BSL-9 genome by a single homologous recombination event (Fig. 3A). PCR was utilized to verify that the BLS-9 exconjugants integrated pSMV20::*arxA* within the *arxA* gene. Figure 3B shows positive detection for the recombination event within the *arxA* gene indicated by a ~ 2 kb PCR product in Cm resistant BSL-9 colonies using the primer set Ext_arxA_Forward and M13_Reverse primer, which binds upstream of the *arxA* disruption and within pSMV20 DNA, respectively. As expected, only DNA from BSL-9 Cm resistant colonies produced a 2 kb PCR product (Fig. 3B, lane 3). Furthermore, Fig. 3B shows positive detection for *sacB* gene, which is present in pSMV20 constructs and yields a 200 bp product (lane 14). Detection of *sacB* was observed only in genomic

DNA of a BSL-9 Cm resistance isolates (lane 13) and no detection in BSL-9 wild-type genomic DNA (lane 12) was observed. The data in Fig. 3B confirms that the BSL-9 Cm resistant exconjugant colonies contain a genomic plasmid insertion mutation within *arxA*, potentially disrupting the function of the arsenite oxidase. The BSL-9 *arxA* insertion mutant is referred to as ARXA1.

We performed various physiological growth curve experiments with different electron donors to test for specificity of *arxA* disruption to arsenite. The ARXA1 mutant grew similarly to the wild-type under photoautotrophic (thiosulfate) and photoheterotrophic (acetate) conditions (Supporting Information Fig. S1). Photoheterotrophic growth was also observed with sucrose, lactate, pyruvate, succinate, malate, propionate and photoautotrophic growth with sulfide as electron donors (data not shown). Photoautotrophic growth on arsenite only occurred in BSL-9 wild-type cells with an intact *arxA* gene (Fig. 4A). Moreover, ARXA1 strain was only deficient when grown with arsenite as an electron donor (Fig. 4A). Background arsenate carryover is observed initially when adding arsenite. However there was no indication of arsenite oxidation in ARXA1 even though there was an increase of 33 μM of arsenite and 30 μM of arsenate over time (Fig. 4C). More evidently, arsenite oxidation was observed in BSL-9 wild-type cells with the conversion of ~ 400 μM arsenite to ~ 400 μM arsenate (Fig. 4B). Together the arsenite growth inhibition (Fig. 4A) and no arsenite oxidation (Fig. 4C) in the ARXA1 mutant strongly support the hypothesis that *arxA* is the sole arsenite oxidase within the *arxB2AB1CD* gene cluster. Thus far, our genetic model development system establishes a successful way to disrupt gene functions in *Ectothiorhodospira* sp. str. BSL-9.

Photosynthetic bacteria are unique in evolving diverse strategies to capture light in order to generate biomass. The uniqueness of photoarsenotrophy is how arsenite oxidation can be driven by light energy. We were interested in addressing if arsenite oxidation was light dependent. Figure 5A shows arsenite oxidation in the presence of light in cell suspension experiments (~ 10^8 cells ml^{-1}) by utilizing BSL-9 and ARXA1 mutant. When cell suspensions were initiated in the dark and then shifted to the light, as shown in Fig. 5B, arsenite oxidation was not observed until the suspensions were shifted into the light. Arsenite oxidation promptly occurred in BSL-9 but not in ARXA1. The cell suspension experiments in Fig. 5 confirmed that arsenite oxidation is coupled to light in BSL-9 and dependent on the *arxAB1CD* gene cluster. These findings highlight *arxAB1CD* as the essential genes to carry out photoarsenotrophy.

We then investigated the polar effects of the pSMV20::*arxA* plasmid insertion mutation in ARXA1 to determine if transcription of the downstream *arxBCD* genes were affected by the mutation. To investigate polar

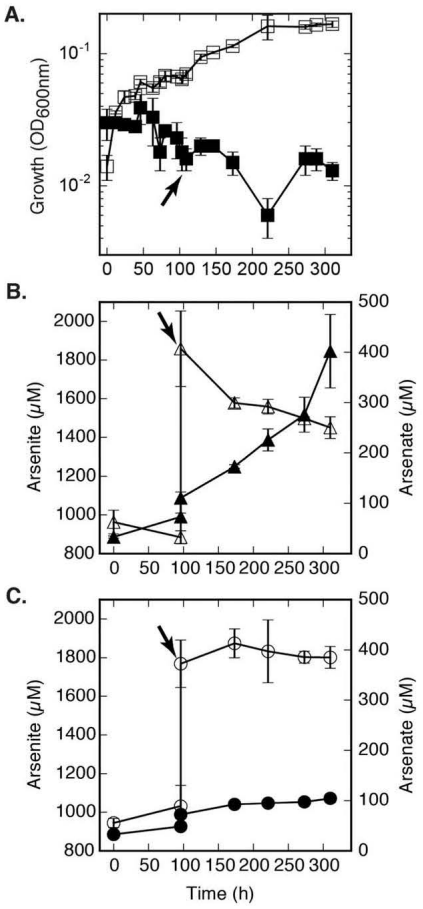

Fig. 4. Effects of ARXA1 mutant on anoxygenic photosynthetic arsenite oxidation. **(A)** Growth of ARXA1 (*arxA* mutant) and BSL-9 wild-type strains were compared under arsenite (600 μM) conditions (■,□). All samples received an additional 1000 μM arsenite spike at the 96th hour, indicated by the arrow. Growth was detected only in BLS-9 wild-type (□) and absent in BSL-9 ARXA1 mutant (■). **(B)** Arsenite (△) oxidation to arsenate (▲) in BSL-9 wild-type cells. **(C)** Arsenite (○) to arsenate (●) oxidation inhibition in ARXA1 cells. No clear indication of arsenic transformations in ARXA1 mutant. Data points and error bars in represent the averages and standard deviation of triplicate cultures, respectively.

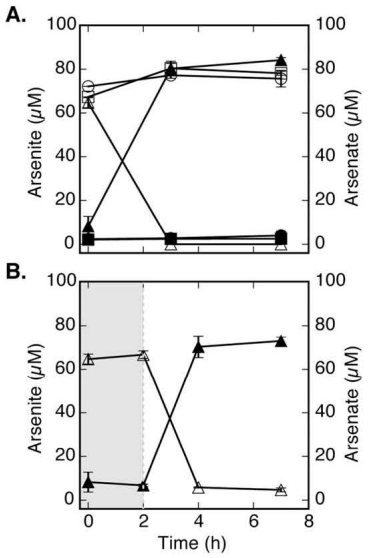

Fig. 5. Light-dependent and growth-independent arsenite oxidation. Cell suspension samples were spiked with approximately 75 μM arsenite and monitored for arsenic speciation. **(A)** Growth independent arsenite oxidation (○) to arsenate (●) was inhibited in ARXA1 (*arxA* mutant). However, wild-type BSL-9 was able to oxidize arsenite (△) to arsenate (▲) within 3 hours. No abiotic arsenic speciation was observed (■/□). **(B)** Light dependent arsenite oxidation (△) to arsenate (▲) was observed only after BSL-9 wild-type cells were shifted to IR light conditions ($t = 2$ hours). Data points and error bars in represent the averages and standard deviation of triplicate samples, respectively.

effects of ARXA1 mutant, we first observed evidence for the induction of the *arxB2AB1CD* gene cluster by the addition of arsenite in the BSL-9 wild-type strain (Supporting Information Fig. S2A, lanes 2, 4, 6, 8, 10 and 12). Arsenite (300 μM) was added to BSL-9 wild-type and ARXA1 mutant cultures grown initially on acetate. The expression of the *arx* gene cluster was determined by reverse transcription-polymerase chain reaction (RT-PCR). For the BSL-9 wild-type strain each gene, *arxB2AB1CD*, was expressed when induced with arsenite (Supporting Information Fig. S2A, lanes 2, 4, 6, 8, 10 and 12). However, only *arxB2* and *arxA* upstream of the mutation were expressed in the ARXA1 strain (Supporting Information Fig. S2A, lanes 3 and 4), and little to no RT-PCR products were observed for *arxA* downstream of the mutation, *arxB1* and *arxC* (Supporting Information Fig. S2A, lanes 7, 9 and 11). RT-PCR without reverse transcriptase enzyme lacked detectable PCR products. These results support the conclusion that the *arxA* insertion mutation caused polar effects on the transcription of the downstream genes. Although we cannot conclusively state that *arxA* is responsible for photosynthetic arsenite oxidation our data strongly suggests that the *arx* gene cluster is responsible for photoarsenotrphy in BSL-9. Future work will be needed to generate gene deletions. However, this has been challenging because of difficulties optimizing sucrose counter selection using *sacB* as a sensitivity marker.

Environmental impact of photoarsenotrophy

Lastly, we were interested in determining if *arxA* transcription could be detected in an arsenic rich environment where previous photoarsenotrophs have been identified. The expression of the *arxA* gene in the environment would signal potential arsenite oxidation activity *in situ*. We therefore sampled the red biofilms within the Paoha Island hot spring microbial mats and analysed the total RNA for the presence of *arxA* mRNA by RT-PCR. As previously reported in 2008, arsenite oxidation was confirmed geochemically in microcosm red mat slurries containing artificial hot spring media. The presence of the *arxA* DNA was also detected within the red mat material (Kulp *et al.*, 2008). Our analysis of the total RNA extracts from these same hot spring biofilms demonstrated *in situ arxA* expression (Fig. 6A) and indirectly arsenite oxidase activity. As a control we also detected 16S rRNA gene expression within the environmental samples (Fig. 6B). Both *arxA* and 16S rRNA gene DNA and cDNA PCR products from the red mats were cloned and sequenced. All the environmental *arxA* cDNA sequences were nearly 100% identical to the *arxA* of PHS-1. The 16S rRNA gene sequencing data also confirmed that the red mat was dominated by a PHS-1-like bacterium, suggesting that photoarsenotrophy is active in this extreme environment and likely carried out by *Ectothiorhodospria* species similar to PHS-1. Our microbial ecology data when considered in the context of Kulp *et al.* (2008), provides additional evidence that ArxA is active within the environment and likely impacting arsenic cycling in the Paoha Island hot springs.

In conclusion, we provided genetic evidence that supports *arxB2AB1CD* gene cluster as essential genes to the photoarsenotrophy mechanism and potentially ArxA as the sole arsenite oxidase in *Ectothiorhodospira* sp. strain BSL-9. These findings raise new questions about the biochemical mechanism, such as how the pathway is regulated, and what are the potential environmental impacts of ArxA-dependent arsenite fuelled anoxygenic photosynthesis. For the latter question, we cannot rule out the possibility that AioA could be a contributor to photoarsenotrophy. *Chloroflexus* is an abundant phototrophic organism in many hot spring microbial mat environments and Engel *et al.* (2013) identified *Chloroflexus*-like AioA sequences in

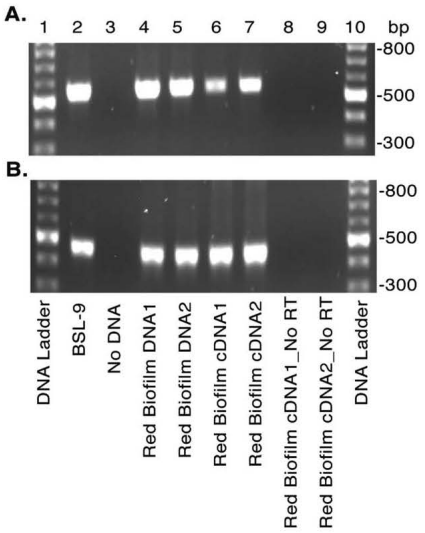

Fig. 6. Evidence for environmental photoarsenotrophy activity determined by *arxA* gene expression *in situ*. Red biofilm-like microbial mats within hot springs of Paoha Island Mono Lake, CA were collected and the RNA and DNA extracts analysed for *arxA* (*arxA*_1824_deg_F and *arxA*_2380_deg_R primers) (A) and 16S rRNA (B) by PCR and RT-PCR respectively. Agarose gel electrophoresis of the PCRs and RT-PCRs are shown in A and B. For both panels lanes correspond to: DNA ladder (1, 10), BSL-9 genomic DNA (2), water negative control (3), environmental DNA (4–5), and cDNA of environmental RNA samples with (6–7) and without (8–9) reverse transcriptase.

microbial mat samples from El Tatio Geyser Field in Chile. Consequently, this opens up the possibility for an AioA-dependent pathway for photoarsenotrophy. In terms of biogeochemical cycling of arsenic, the environmental impacts of photosynthetic arsenite oxidation needs further investigation.

Results from this study for the first time demonstrate that the *arx* gene cluster is responsible for photosynthetic arsenite oxidation. The implications of our microbial ecology survey further builds upon the case that photoarsenotrophs containing *arxA* may be impacting the arsenic geochemical cycle as demonstrated by the *in situ* detection of *arxA* mRNA in Paoha Island hot springs of Mono Lake containing ~ 6 mM sulfide (Kulp *et al.*, 2008). Moreover a broader distribution of *arxA*-like sequences occurs in the environment as observed in non-extreme environmental metagenomic studies (Supporting Information Table S3). It may be plausible that photoarsenotrophs are present within diverse euphotic environment such as freshwater aqueous, sediment, subsurface groundwater and marine sediment microbial communities as indicated by the presence of *arxA*-like metagenomic sequences (Supporting Information Table S3). Detection of *arx* like sequences in groundwater microbial communities may indicate arsenite oxidation coupled to nitrate by chemotroautotrophs (Supporting Information Table S3), which is tremendously relevant in regions such as Bangladesh and other south East Asia countries that suffer from groundwater arsenic contamination. This work is foundational to future studies regarding the potential contribution of photoarsenotrophs in diverse non-extreme environments. Results from this work also have implications for arsenic cycling on the early Earth. Sforna et al. (2014) provided evidence for the occurrence of microbial arsenic metabolism and cycling nearly 2.7 billion years ago. This predates the rise of oxygen. During this time, photoarsenotrophy may have been a key metabolism to providing arsenate as a by-product, which would be used as a terminal electron acceptor for arsenate respiring microbes. This would provide a mechanism for photoarsenotrophy to co-exist with anaerobic arsenate-respiring microbes, as currently occurs in the Paoha Island biofilms (Hoeft et al., 2010). Finally, photosynthetic microorganisms containing *arxA* capable of oxidizing arsenite in anoxic environments are potential candidate metabolisms capable of surviving beyond the Earth, on or within other planet(oid)s such as Mars and Europa. The recent finding of arsenic minerals within the Tissint Martian meteorite raises the possibility of a primitive microbial arsenic metabolism having once occurred on Mars (Wallis and Wickramasinghe, 2015).

Material and methods

Sampling site description

Ectothiorhodospira sp. strain BSL-9 was isolated from Big Soda Lake (BSL), a meromictic hypersaline alkaline crater lake containing approximately 25 μM arsenic located in western Nevada (39°31′N118°52′W) (Cloern *et al.*, 1983; Zehr *et al.*, 1987). Red colored biofilm-like microbial mat samples were collected from Paoha Island hot springs, located (37°59.633 N, 119°01.376 W) in Mono Lake, CA. Paoha Island hot pools are alkaline anoxic hypersaline environments containing ~ 100 μM arsenic and ~ 6 mM sulfide (Kulp *et al.*, 2008).

Media composition and growth conditions for photoarsenotrophs

Strain BSL-9 was grown in Basal Salt Media (BSM) with the following composition (g/L): KH_2PO_4 (0.24), K_2HPO_4 (0.30), $(NH_4)_2SO_4$ (0.23), $MgSO_4$ (0.12), NaCl (20), yeast extract (0.25), SL-10 mineral mix (5 mL/L), Wolfe's vitamin mix (10 mL/L), [2 g/L] Vitamin B-12 (100 μL). Electron donors were added as needed. After autoclaving the basal medium, 1 and 10 mL of filter sterilized stock solutions of $NaCO_3$ (300 g/L) and $NaHCO_2$ (600g/L) were added respectively. The media

was cooled in anaerobic chamber containing 95% nitrogen, 5% hydrogen mixed gas. Typically the pH of the media was 8.0–8.3. Agar (15 g/L) was added to solidify the media when needed. BSL-9 physiology experiments for various growth experiments are typically initiated from frozen (−80°C) 20% glycerol stock cultures by the following method: using a sterile wooden stick, some ice from the glycerol stock is streak plated onto BSM/acetate (20 mM) agar plates in the bench top. Agar plates are then transferred to an anaerobic chamber and incubated at 35°C for 3 days. Single colony forming units (CFU's) are observed within 3 days with continuous illumination of IR (850 nm) LED lights. For liquid cultures, a CFU is picked with a sterile loop and inoculated into an anaerobic Balch tube containing 20 mM acetate BSM media and grown for 2 days at 35°C under IR LED lights. To test other electron donors, cultures grown under acetate were harvested from turbid cultures by centrifugation in the anaerobic chamber. Cell pellets were washed three times in 1× PBS in order to re-suspended to a final optical density (OD) at 600 nanometers (nm) of 0.6 and then inoculated at 1:50 dilution into anaerobic media containing electron donors of choice, all prepared in anaerobic Balch tubes with sterile butyl rubber stoppers. Growth was followed over time using a spectrophotometer (Spectronic 20D+). All growth experiments where done in triplicates unless otherwise stated as similarly reported (Zargar *et al.*, 2012).

Environmental sample collection for RNA extractions

An environmental sample collection trip was conducted in October 2015 to collect red coloured biofilm material from Paoha Island Mono Lake, CA. The red-coloured biofilm-like coatings on the rocks were collected from the Paoha Island hot pools by scraping the material off with a sterile metal spatula. Replicate microbial mat samples were added to RNase/DNase free 1.5 mL microcentrifuge tubes and immediately flash frozen in liquid nitrogen on site. The samples were transported back to the laboratory the next day and stored at −80°C. Total RNA was extracted 2 weeks later and analysed by RT-PCR for 16S rRNA and *arxA* gene expression.

DNA, RNA extractions and cDNA synthesis

In triplicates, environmental microbial genomic DNA and total RNA transcripts were extracted by Qiagen RNeasy Mini kit and Qiagen DNA extraction kit according to the manufacturer's instructions. After RNA extractions, 500 ng of RNA was then treated with RQ1 DNase (Promega) in order to degrade any potential DNA carryover. RNA treated samples were then followed by reverse transcriptase reactions, synthesized by the Applied Biosystems TaqMan kit (Part No. N808-0234) in order to generate cDNA. The extracted DNA and RNA concentrations were quantified by using a NanoDrop spectrophotometer at absorbance 260 nm. All samples were store at −20°C until performing further analysis.

Amplification and detection of genes through PCR

PCR was performed in order to amplify and detect genes of interest in environmental, BSL-9 and ARXA1 mutant DNA and environmental cDNA samples. DNA Sequencing was performed by Sequetech (San Jose, CA). The arxA-1824-deg-F and arxA-2380-deg-R primer set was used as a positive control to detect the presence of *arxA* gene, which yields approximately 500 base pair fragments. As a positive control for bacteria DNA, 16S rRNA gene was amplified with the primer set 0341_16SV3V4-F and 0785_16SV3V4-R. In order to generate the BSL-9 ARXA1 mutant an 800 base pair (bp) BLS-9 *arxA* homology region was amplified with X-*arxA*_Int_F1 and X-*arxA*_ int_R1 primers and cloned into pSMV20. To verify plasmid insertion within *arxA* in BSL-9 chloramphenicol resistant colonies two primer sets were used. First the *arxA*_Ext_F and M13_R primer set was used which bind outside *arxA* and within the pSMV20 DNA, and yields 2 kb DNA fragments. Secondly a partial DNA sequence of *sacB* which encodes for the enzyme levansucrase and is only found in pSMV20 plasmids was amplified with *sacB*_FOR and *sacB*_REV primer set, generating 200 bp DNA products. Amplification of *sacB* was observed in positive control pSMV20 and in genomic DNA of BSL-9 ARXA1 mutant. BSL-9 *arxB2AB1CD* gene cluster was amplified with the following primer sets, *arxB2*_F and *arxB2*_R, *arxA*_UpS_F and *arxA*_UpS_R, *arxA*_DwnS_F and *arxA*_DwnS_R, *arxB1*_F and *arxB1*_R, *arxC*_F and *arxC*_R, *arxD*_F and *arxD*_R. A thermocycler profile was set to 28 cycles of denaturation, annealing, and extension for all PCR reactions. The primer annealing temperature and the extension time varied due to the primer set composition and the product size being amplified. Typically PCR parameters were set to 95°C for 5 min to denature DNA, [28 cycles of 95°C for 30 s to denaturation DNA, primer-specific annealing temperature for 30 s, extension at 72°C and a final extension of 72°C for 5 min. Supporting Information Table S1 shows specific annealing and extension PCR parameters for specific primer sets. Typically PCR reactions contained a final concentration of the following constituents: genomic DNA [10–50 ng] or [10 ug] of plasmid DNA, [0.4 uM] for each primer, 1× PCR Master Mix from Promega containing, buffers, Taq DNA polymerase, dNTP's and Mg^{2+}.

Strains, plasmids and primer sets

Strains and plasmids used in this study are listed in Supporting Information Table S1. Primer composition, annealing and extension times are listed in Supporting Information Table S2.

Isolation of Ectothiorhodospira *sp. str. BSL-9*

Strain BSL-9 was isolated from swamp sediments samples collected in Big Soda Lake, Nevada. Basal salt media containing 2 mM arsenic and trace amounts of yeast (0.01 g/L) was used to set-up enrichment cultures. Enrichment cultures were streaked on BSM agar plates containing 2 mM arsenite in order to isolate single colonies. Purple like single colony forming units (CFU's) were re-streaked in order to acquire a pure culture. Big Soda Lake candidate isolates 1–14 were obtained; however, BSL-9 was selected since it grew well in BSM liquid cultures and in solid agar plates. The BLS-9 genome was sequenced using PacBio technology by UC Davis genome sequencing centre. The genome assembly was done by PacBio HGAP_v2 assembly pipeline within approximate 300× coverage. The annotation was done through NCBI Public

Genome Annotation Pipeline service. The *Ectothiorhodospira* sp. str. BSL-9 can be accessed from NCBI BioProject: PRJNA232800 or using the GeneBank accession number: CP011994.

arxA mutagenesis plasmid (pSMV20::arxA) construct

In order to disrupt *arxA* in BSL-9 wild-type, by single homologous recombination, pSMV20::*arxA* was used. To construct pSMV20::*arxA*, primer Int_*arxA*_F and Int_*arx*_R primers were used to amplified approximately 800 base pair homology region from BSL-9 *arxA* wild-type genomic DNA. The *arxA* homology sequence was ligated to pSMV20 (Zargar *et al.*, 2010) by Gibson Assembly Kit (Gibson *et al.*, 2009) according to the manufacture protocol. The Gibson Assembly reaction was then transformed into *E. coli* DH5 alpha λpir cell line (Saltikov and Newman, 2003). Cells were recovered in LB for an hour at 37°C and then plated on chloramphenicol 25 μg/ml agar plates. CFU's were then picked from chloramphenicol agar plate and grown in chloramphenicol LB liquid media in order to extract plasmid DNA and screen for the presence of pSMV20::*arxA* plasmid. Plasmid DNA was extracted and screened with M13 F/R primer set for 800 bp fragments. The *arx*A homology sequence was approximately 800 bp, which was obtained and verified through sequencing. Plasmid (pSMV20::*arxA*) was then transformed into *E. coli* WM3064 (Saltikov and Newman, 2003) mating strain in order to carry out conjugation reactions with BSL-9 wild-type strain. Diaminopimelic acid (DAP) was added to growth media since *E. coli* WM3064 is an auxotroph DAP mutant strain. BSL-9 exconjugants were then screened with primer *sacB*_FOR and *sacB*_REV and Ext_*arx*A_F and M13_R primer sets in order to detect pSMV20::*arxA* genomic insertion within *arxA* gene in chloramphenicol resistant colonies.

Generation of BSL-9 arxA *gene disruption mutant*

To perform mating reactions *E. coli* strain WM3064 cells containing pSMV20::*arxA* were grown overnight at 37°C in Luria Broth (LB) containing DAP and BSL-9 wild-type strain was grown in BSM for 2 days at 35°C under infrared (IR) lights. About 1 ml of *E. coli* WM3064 containing pSMV20::*arxA* and BSL-9 Wild-type strain liquid cultures were combined and spun down at 10,000 RPM for 1 min. The supernatant was removed (~ 2 ml), and the combined cells were suspended in 1 ml of LB containing DAP. About 60 μl of the combined cells were spot plated on the centre of an LB plate containing acetate and nitrate with approximately pH 8.2. Reactions were incubated for 6 hours at room temperature under IR lights in an anaerobic chamber. After 6 hours the mating reaction was collected with a sterile metal loop and re-suspended in 200 μl BSM liquid media containing 5 μg/ml chloramphenicol. The mixture was then plated on BSM agar plates containing 5 μg/ml chloramphenicol. After a week, BSL-9 chloramphenicol resistant colonies were picked and inoculated into BSM media containing chloramphenicol. Two days thereafter, BSL-9 chloramphenicol resistance cells were harvested by centrifugation and used to extract genomic DNA for analysis of genomic plasmid integration.

arx*A mRNA quantification by qRT-PCR*

BSL-9 wild-type cells were grown in BSM medium with 10 mM acetate to stationary phase then diluted to OD_{600nm} of 0.6. A 1:100 dilution was made into 6 Balch tubes containing BSM media with 10 mM acetate. After cells reached an OD_{600nm} of 0.2, 1 mM arsenite was spiked into each triplicate culture, while three other replicates did not receive arsenite. The tubes were sampled before the arsenite spike and 2, 12 and 24 hours post arsenite addition. Cells were processed for RNA purification and cDNA synthesis as described elsewhere (Saltikov *et al.*, 2005). Briefly 500 ng of DNase treated RNA was conveted to cDNA using Applied Biosystems TaqMan kit (Part No. N808-0234). To quantify *arxA* gene expression relative to the 16S rRNA gene a probe-based real-time PCR assay was used. The IDT PrimeTime qPCR primer probe sets for *arxA* were (*arxA*: Probe 5′-/56-FAM/CCATCGCCA/ZEN/ATGGCAAGCTGTG/3IABkFQ/-3′; Primer 1, 5′-CAAGGTCACCGCCATCTAC-3′; Primer 2, 5′-GTCCGGGTCATACAGGAAATAG-3′), and 16S rRNA gene were (Probe 5′-/56-FAM/CGGTGTAGC/ZEN/GGTGAAATGCGTAGA/3IABkFQ/-3′; Primer 1, 5′-GCATGGCTAGAGTTTGGTAGAG-3′; Primer 2, 5′-CGCACCTCAGTGTCAGTTT-3′). Real-time PCR reactions consisted of 20 μl volume per reaction; 10 μl of 2× Taq Mix (Promega), 1 μl of 20× probe-primer, 5 μl of H_2O and 4 μl of cDNA (diluted 1:4 in H_2O). BSL-9 wild-type DNA was used as a positive control for generating standard curves in order to quantify *arxA* relative expression. The Biorad Real-Time PCR machine was used with the following thermal profile: 95°C for 5 min, 40 cycles of 95°C/30 s, 55°C/30 s and 72°C/30 s. The quantification analysis of *arxA* expression was done using the ΔΔCq method (Livak and Schmittgen, 2001). The ΔCq values were calculated using Cq values of *arxA* and 16S rRNA gene. The no arsenite condition was used as a reference for the ΔΔCq calculation and the fold change corresponded to $2^{-\Delta\Delta Cq}$.

Cell suspension arsenite oxidation assay

To test for growth independent arsenite oxidation, high-density cell suspension assays were examined. BSL-9 wild-type and BSL-9 *arxA* mutant were grown in BSM media containing 10 mM acetate. Cells were washed and transfer to BSM media lacking yeast and acetate but containing approximately 100 μM arsenite. Each growth condition was done in triplicates with an initial OD_{600nm} of 0.5 (~ 4.0×10^8 cells/ml). Anaerobic samples were stored either in the dark or under IR lights at 35°C. In order to analyse and quantify arsenite and arsenate, 500 μl of each sample was taken out over time, every hour and incubated at 4°C. Arsenic analysis was done routinely two days after sampling.

Analytical techniques

Arsenic speciation was analysed and quantified by High Performance Liquid Chromatography (HPLC) coupled to an Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS). Elemental detection was carried out by Thermo X-Series2 ICP-MS equipment. The isocratic mobile phase contained 30 mM phosphoric acid, which was also used in the autosampler flush. The flow rate was 1 min/ml with 100 μl sample injection

loop. An anion exchange column (Hamilton PRP-X-100, particle size 10 μm, size 4.1 × 50 mm) was used with a run time of 3 min per sample. The column temperature was ambient. All samples were diluted in 30 mM phosphoric acid mobile phase containing germanium as an internal control.

Phylogenetic analysis

The phylogenetic analysis of ArxA sequence of *Ectothiorhodospira* sp. str. BSL-9 and other DMSO reductase family molybdenum-containing oxidoreductases was done as previously described (Oremland, 2003).

Acknowledgments

This work was supported by the National Science Foundation (EAR-1349366) award to CWS; UCSC RMI fellowship (NIH-NHGRI 1R25HG006836-01A1) and UCSC IMSD fellowship (NIH/HIGMS 5R25GM058903-14) to Jaime Hernandez-Maldonado; UCSC PREP fellowship (NIH 5R25GM104552) to Benjamin Sedillo-Sanchez. We thank Professor Dianne Newman for constructive advise on sampling red biofilm-like mats in Paoha Island; John Stolz for discussions on earlier versions of the manuscript. We thank Rob Franks (UC Santa Cruz) for tremendous help in developing a rapid automated HPLC-ICP-MS arsenic analysis method. We thank the Sierra Nevada Aquatic Research Laboratory for facilitating fieldwork.

Author contributions

J. Hernandez-Maldonado performed genetic system development, mutant generation, physiology experiments, arsenic analysis, and manuscript writing. B. Sanchez-Sedillo performed qRT-PCR and B. Stoneburner performed cloning and environmental sequencing of *arxA* PCR amplicons. All authors helped with field sampling on Paoha Island Mono Lake, California. A. Boren (Conrad) isolated *Ectothiorhodospira* sp. str. BSL-9. M. Rosen provided access to USGS research vessel on Mono Lake and Big Soda Lake. C.W Saltikov contributed to manuscript writing process and served as PI on the project. R.S. Oremland contributed to manuscript writing, field sampling logistics, and funding USGS Menlo Park personnel in support of field sampling.

Competing financial interests

The author declares no competing financial interests.

References

Acharyya, S.K., Chakraborty, P., Lahiri, S., Raymahashay, B.C., Guha, S., and Bhowmik, A. (1999) Arsenic poisoning in the Ganges Delta. *Nature* **401:** 545–546.

Amend, J.P., Saltikov, C., Lu, G.S., and Hernandez, J. (2014) Microbial arsenic metabolism and reaction energetics. *Rev Miner Geochem* **79:** 391–433.

Budinoff, C.R., and Hollibaugh, J.T. (2008) Arsenite-dependent photoautotrophy by an *Ectothiorhodospira*-dominated consortium. *ISME J* **2:** 340–343.

Cammack, R., Rao, K.K., and Hall, D.O. (1981) Metalloproteins in the evolution of photosynthesis. *Biosystems* **14:** 57–80.

Cloern, J.E., Cole, B.E., and Oremland, R.S. (1983) Autotrophic processes in meromictic Big Soda Lake, Nevada. *Limnol Oceanogr* **28:** 1049–1061.

Dhar, R.K., Zheng, Y., Saltikov, C.W., Radloff, K.A., Mailloux, B.J., Ahmed, K.M., and van Geen, A. (2011) Microbes enhance mobility of arsenic in Pleistocene aquifer sand from Bangladesh. *Environ Sci Technol* **45:** 2648–2654.

Ehrenreich, A., and Widdel, F. (1994) Anaerobic oxidation of ferrous iron by purple bacteria, a new type of phototrophic metabolism. *Appl Environ Microbiol* **60:** 4517–4526.

Engel, AS, Johnson, LR, Porter, ML. (2013) Arsenite Oxidase gene diversity among Chloroflexi and Proteobacteria from El Tatio Geyser Field, Chile. *FEMS Microbiol Ecol* **83:** 745–756.

Fendorf, S., Michael, H.A., and van Geen, A. (2010) Spatial and temporal variations of groundwater arsenic in South and Southeast Asia. *Science* **328:** 1123–1127.

Gibson, D.G., Young, L., Chuang, R.Y., Venter, J.C., Hutchison, C.A., and Smith, H.O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Meth* **6:** 343–345.

Griffin, B.M., Schott, J., and Schink, B. (2007) Nitrite, an electron donor for anoxygenic photosynthesis. *Science* **316:** 1870.

Harvey, C.F. (2002) Groundwater flow in the Ganges Delta. *Science* **296:** 1563.

Hoeft, S.E., Kulp, T.R., Han, S., Lanoil, B., and Oremland, R.S. (2010) Coupled arsenotrophy in a hot spring photosynthetic biofilm at Mono Lake, California. *Appl Environ Microbiol* **76:** 4633–4639.

Islam, F.S., Gault, A.G., Boothman, C., Polya, D.A., Charnock, J.M., Chatterjee, D., and Lloyd, J.R. (2004) Role of metal-reducing bacteria in arsenic release from Bengal Delta sediments. *Nature* **430:** 68–71.

Jormakka, M., Yokoyama, K., Yano, T., Tamakoshi, M., Akimoto, S., Shimamura, T., *et al.* (2008) Molecular mechanism of energy conservation in polysulfide respiration. *Nat Struct Mol Biol* **15:** 730–737.

Kashyap, D.R., Botero, L.M., Franck, W.L., Hassett, D.J., and McDermott, T.R. (2006) Complex regulation of arsenite oxidation in *Agrobacterium tumefaciens*. *J Bacteriol* **188:** 1081–1088.

Kempe, S., and Kazmierczak, J. (1997) A terrestrial model for an alkaline martian hydrosphere. *Planet Space Sci* **45:** 1493–1499.

Kocar, B.D., and Fendorf, S. (2009) Thermodynamic constraints on reductive reactions influencing the biogeochemistry of arsenic in soils and sediments. *Environ Sci Technol* **43:** 4871–4877.

Kocar, B.D., Polizzotto, M.L., Benner, S.G., Ying, S.C., Ung, M., Ouch, K., *et al.* (2008) Integrated biogeochemical and hydrologic processes driving arsenic release from shallow sediments to groundwaters of the Mekong Delta. *Appl Geochem* **23:** 3059–3071.

Kulp, T.R., Hoeft, S.E., Asao, M., Madigan, M.T., Hollibaugh, J.T., Fisher, J.C., *et al.* (2008) Arsenic(III) fuels anoxygenic

photosynthesis in hot spring biofilms from Mono Lake, California. *Science* **321:** 967–970.
Lavergne, J., Verméglio, A., and Joliot, P. (2009) Functional coupling between reaction centers and cytochrome bc1 complexes. In *The Purple Phototrophic Bacteria*. Hunter, C.N., Daldal, F., Thurnauer, M.C., Thomas Beatty, J. (ed.). New York: Springer. pp. 509–536.
Lett, M.C., Muller, D., Lievremont, D., Silver, S., and Santini, J. (2011) Unified nomenclature for genes involved in Prokaryotic aerobic arsenite oxidation. *J Bacteriol* **194:** 207–208.
Livak, K.J., and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2 $^{-\Delta\Delta C}$T method. *Methods* **25:** 402–408.
Masscheleyn, P.H., and Delaune, R.D. (1991) Effect of redox potential and pH on arsenic speciation and solubility in a contaminated soil. *Environ Sci Technol* **25:** 1414–1419.
Nickson, R., McArthur, J., Burgess, W., Ahmed, K.M., Ravenscroft, P., and Rahman, M. (1998) Arsenic poisoning of Bangladesh groundwater. *Nature* **395:** 338.
O'Day, P.A., Vlassopoulos, D., Root, R., and Rivera, N. (2004) The influence of sulfur and iron on dissolved arsenic concentrations in the shallow subsurface under changing redox conditions. *Proc Natl Acad Sci USA* **101:** 13703–13708.
Oremland, R.S. (2003) The ecology of arsenic. *Science* **300:** 939–944.
Oremland, R.S., and Stolz, J.F. (2005) Arsenic, microbes and contaminated aquifers. *Trends Microbiol* **13:** 45–49.
Oremland, R.S., Saltikov, C.W., Wolfe-Simon, F., and Stolz, J.F. (2009) Arsenic in the evolution of earth and extraterrestrial ecosystems. *Geomicrobiol J* **26:** 522–536.
Overmann, J. (2008) Ecology of phototrophic sulfur bacteria. In *Sulfur Metabolism in Phototrophic Organisms*. Hell, R., Dahl, C., Knaff, D.B., Leustek, T. (ed.). New York: Springer. pp. 375–391.
Robert, B. (2009) Spectroscopic properties of antenna complexes from purple bacteria. In *The Purple Phototrophic Bacteria*. Hunter, C.N., Daldal, F., Thurnauer, M.C., Thomas Beatty, J. (ed.). New York: Springer. pp. 199–212.
Saltikov, C.W. (2011) Regulation of arsenic metabolic pathways in prokaryotes. In *Microbial Metal and Metalloid Metabolism*. Stolz, J., Oremland, R. (ed.). Washington, DC: ASM Press. pp. 195–210.
Saltikov, C.W., and Newman, D.K. (2003) Genetic identification of a respiratory arsenate reductase. *Proc Natl Acad Sci USA* **100:** 10983–10988.
Saltikov, C.W., Wildman, R.A., and Newman, D.K. (2005) Expression dynamics of arsenic respiration and detoxification in *Shewanella* sp. strain ANA-3. *J Bacteriol* **187:** 7390–7396.
Sardiwal, S., Santini, J.M., Osborne, T.H., and Djordjevic, S. (2010) Characterization of a two-component signal transduction system that controls arsenite oxidation in the chemolithoautotroph NT-26. *FEMS Microbiol Lett* **313:** 20–28.
Sessions, A.L., Doughty, D.M., and Welander, P.V. (2009) The continuing puzzle of the Great Oxidation Event. *Curr Biol* **19:** 567–574.
Sforna, M.C., Philippot, P., Somogyi, A., van Zuilen, M.A., Medjoubi, K., Schoepp-Cothenet, B., *et al.* (2014) Evidence for arsenic metabolism and cycling by microorganisms 2.7 billion years ago. *Nat Geosci* **7:** 811–815.
Smith, A.H., Lingas, E.O., and Rahman, M. (2000) Contamination of drinking-water by arsenic in Bangladesh: A public health emergency. *Bull World Health Organ* **78:** 1093–1103.
Stuckey, J.W., Schaefer, M.V., Kocar, B.D., Benner, S.G., and Fendorf, S. (2015) Arsenic release metabolically limited to permanently water-saturated soil in Mekong Delta. *Nat Geosci* **9:** 70–76.
Wallis, J., and Wickramasinghe, N.C. (2015) Evidence of ancient microbial activity on Mars. Proceedings of SPIE Optical, Instruments, Methods, and Missions for Astrobiology XVII, 96060V.
Widdel, F., Schnell, S., Heising, S., Ehrenreich, A., Assmus, B., and Schink, B. (1993) Ferrous iron oxidation by anoxygenic phototrophic bacteria. *Nature* **362:** 834–836.
Zargar, K., Hoeft, S., Oremland, R., and Saltikov, C.W. (2010) Identification of a novel arsenite oxidase gene, *arxA*, in the haloalkaliphilic, arsenite-oxidizing bacterium *Alkalilimnicola ehrlichii* strain MLHE-1. *J Bacteriol* **192:** 3755–3762.
Zargar, K., Conrad, A., Bernick, D.L., Lowe, T.M., Stolc, V., Hoeft, S., *et al.* (2012) ArxA, a new clade of arsenite oxidase within the DMSO reductase family of molybdenum oxidoreductases. *Environ Microbiol* **14:** 1635–1645.
Zehr, J.P., Harvey, R.W., and Oremland, R.S. (1987) Big Soda Lake (Nevada). 1. Pelagic bacterial heterotrophy and biomass. *Limnol Oceanogr* **32:** 781–793.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Phototrophic growth curve analysis of BSL-9 wild-type and ARXA1 (*arxA* mutant) with acetate and thiosulfate. Strains were grown under photoautotrophic conditions in the presence of thiosulfate (diamonds) and photoheterotrophic conditions in the presence of acetate (circles) and compared with samples lacking electron donors as a negative control (triangles). No general growth defects were observed in BSL-9 wild-type and ARAX1 when providing thiosulfate (diamonds) or acetate (circles) as electron donors. No growth was observed in the absence of an electron donor (triangles) relative to acetate or thiosulfate. Data points and error bars in corresponding figures represent the averages and standard deviation of replicate samples.
Fig. S2. Transcription analysis of *arx* genes in BSL-9 wild-type and BSL-9 *arxA* mutant strain (ARXA1). To investigate the polar effects and impact of *arxA* gene insertion on downstream genes such as *arxB1CD*, we analyzed activity of the *arxB2AB1CD* gene cluster. BSL-9 ARXA1 and wild-type cells were grown (in triplicates) photosynthetically on 20 mM acetate to early exponential phase (OD_{600nm} 0.2) and induced with 300 μM arsenite for 2 hours. Panels (A) and (B) are agarose gel electrophoresis (1%) images of the RT-PCR analyses for a single representative culture: BSL-9 wild-type (lane 2, 4, 6, 8, 10, 12, 14) and ARXA1 mutants (lane 3, 5, 7, 9, 11, 13, 15). The other two replicates showed identical results. cDNAs were generated with (A) and without (B) reverse transcriptase and analyzed for the presence of *arxB2* (lane 2-3), a region flanking the *arxA* before (lane 4-5) and after (lane 6-7) the plasmid insertion mutation, *arxB1* (8-9), *arxC* (10-11), *arxD* genes (lanes 12-13), 16S rRNA gene as a positive control for bacterial

mRNA expression (lanes 14-15), and No DNA samples as a control for contamination. Genes were down regulated in BSL-9 ARXA1 mutant (lanes 7, 9, 11, 13) relative to BSL-9 wild-type cells (lanes 6, 8, 10, 12). Panel (C) shows PCR results using genomic DNA for BSL-9 wild-type and BSL-9 ARXA1 mutant as a positive control for gene amplification Detection was observed for all genes in the *arxB2AB1CD* gene cluster (same primer sets were used in RT-PCR).

Table S1. Bacterial strains and plasmids used in this study.

Table S2. Primers and probes PCR parameters.

Table S3. Metagenomic *arxA* detection in non-extreme environments. The BSL-9 *arxA* gene was used in a BLAST search of the JGI metagenome data sets. The sequences that were similar where then BLAST searched in NCBI to verify their similarties to BSL-9 wild-type strain and other *arxA*-like sequences. The data reported here are the identities/similarties to the BSL-9 arxA gene (BLASTn) and predict ArxA protein sequence (BLASTx).

See Excel sheet: Table_3S_arxA_metagenomes.xlsx

See Fasta File: Table_3S_arxA_metagenomes.fna

Chapter 5. Comparative genomics of *arxA*

Section 5.01 Abstract

Anaerobic arsenite oxidation by *arxA* adds complexity to the arsenic biogeochemical cycle. Two distinct biochemical microbial anaerobic arsentie oxidation mechanism have been described in non-photosynthetic and photosynthetic bacteria but have yet to be fully characterized. Isolation of prokaryotes containing *arxA* has only been reported in four bacteria belonging to the family of *Ectothiorhodospiraceae*. Three of them are photosynthetic and one of them is a chemoautotrophic bacterium. Out of the three photosynthetic bacteria only *Ectothiorhododospira* sp. strain BSL-9 *and Ectothiorhododospira* sp. strain PHS-1 have been shown to carry out photoarsenotrophy. Latter has yet to be determined in *Halorhodospira halopila* sp. strain SL-1. The chemotrophic bacterium *Alkalilimnicola ehrlichii* sp. strain MLHE-1 is known to oxidize arsenite under anoxic conditions with nitrate as a terminal electron donor during anaerobic respiration. Here we provide an overview of the first photoarsenotrophic fully assembled genome sequence in *Ectothriorhodospira* sp. strain BLS-9.

Section 5.02 *Ecthothiorhodospira* sp. strain BSL-9 genome

AMERICAN SOCIETY FOR MICROBIOLOGY | genomeAnnouncements

Genome Sequence of the Photoarsenotrophic Bacterium *Ectothiorhodospira* sp. Strain BSL-9, Isolated from a Hypersaline Alkaline Arsenic-Rich Extreme Environment

Jaime Hernandez-Maldonado,[a] Brendon Stoneburner,[a] Alison Boren,[a] Laurence Miller,[b] Michael Rosen,[c] Ronald S. Oremland,[b] Chad W. Saltikov[a]

Department of Microbiology and Environmental Toxicology, University of California Santa Cruz, Santa Cruz, California, USA[a]; U.S. Geological Survey, Menlo Park, California, USA[b]; U.S. Geological Survey, Carson City, Nevada, USA[c]

The full genome sequence of *Ectothiorhodospira* sp. strain BSL-9 is reported here. This purple sulfur bacterium encodes an *arxA*-type arsenite oxidase within the *arxB2AB1CD* gene island and is capable of carrying out "photoarsenotrophy" anoxygenic photosynthetic arsenite oxidation. Its genome is composed of 3.5 Mb and has approximately 63% G+C content.

Received 23 August 2016 **Accepted** 25 August 2016 **Published** 13 October 2016

Citation Hernandez-Maldonado J, Stoneburner B, Boren A, Miller L, Rosen M, Oremland RS, Saltikov CW. 2016. Genome sequence of the photoarsenotrophic bacterium *Ectothiorhodospira* sp. strain BSL-9, isolated from a hypersaline alkaline arsenic-rich extreme environment. Genome Announc 4(5):e01139-16. doi:10.1128/genomeA.01139-16.

Address correspondence to Chad W. Saltikov, saltikov@ucsc.edu.

Arsenic-rich soda lakes are ideal environments for culturing microorganisms with unique metabolic capabilities for coupling cellular energy production to arsenic oxidation and reduction (1–6). Here, we report the assembled genome of an anoxygenic photosynthetic arsenite-oxidizing ("photoarsenotrophic") bacterium, *Ectothiorhodospira* sp. strain BSL-9. This microbe was isolated from Big Soda Lake, an arsenic-rich (~25 μM), hypersaline (26 to 88 g/liter total dissolved solids), alkaline (pH 9.7) lake located in Nevada (39°31′N 118°52′W) (7–9). Moreover, this crater lake has a well-defined seasonal bloom of purple sulfur bacteria (*Chromatium* and *Ectothiorhodospira* species) (10) that are proposed to contribute to the arsenic geochemical cycle.

Assessment of the BSL-9 genome revealed an arsenic gene island, *arxB2AB1CD* (11), which is predicted to encode the arsenite oxidase gene *arxA*. Moreover, *arxB2AB1CD* encodes a [4Fe-4S]-containing protein (*arxB2*), a second [4Fe-4S]-containing protein (*arxB1*), a membrane protein (*arxC*), and a TorD-like protein involved in molybdenum enzyme biogenesis (*arxD*). In the chemoautotrophic bacterium *Alkalilimnicola ehrlichii* sp. strain MLHE-1, *arxA* is required for anaerobic arsenite oxidation coupled to nitrate (12). The BSL-9 genome lacks the AioA-type arsenite oxidase. A BSL-9 *arxA* mutant strain shows that *arxA* is the sole arsenite oxidase for photoarsenotrophy (13).

Ectothiorhodospiraceae are common anoxygenic phototrophs with versatile abilities to metabolize inorganic and organic electron donors (14–16), which enables them to occupy distinct euphotic hypersaline alkaline environments. In addition to arsenite, BSL-9 can grow photoautotrophically with sulfide or thiosulfate. This is consistent with the presence of *sox* and *dsr* genes, which are involved in sulfur oxidation (17, 18). Moreover, BSL-9 can also grow as a photoheterotroph with various organic acids (e.g., acetate, malate, propionate, lactate, fumarate, succinate, and pyruvate). BSL-9 is sensitive to chloramphenicol, resistant to kanamycin, carbenicillin, gentamicin, and tetracycline, and grows optimally at 35°C at pH 8, 2% NaCl; these growth patterns are consistent with other *Ectothiorhodospira* species (14–16). Although BSL-9 is an anaerobe, the presence of cytochrome *c* oxidase genes (e.g. cbb_3) found in BSL-9 may explain its tolerance to atmospheric oxygen. For example, cytochrome *c* oxidases (cbb_3) are known for having high oxygen affinity, and cytochrome *c* peroxidases protect cells from reactive oxygen species. The BSL-9 genome also encodes photosynthetic complex genes, such as bacteriochlorophyll *a* synthase, the light-harvesting complex *pucAB*, and two copies of the carbon fixation-related gene *rbcL* (type III RuBisCO). Having the full genome sequence of BSL-9 opens numerous possibilities for studying the metabolic abilities, physiology, and the ecological environmental impact of photoarsenotrophy to the arsenic biogeochemical cycle.

The genome was done at the UC Davis Genome Sequencing Center using PacBio technology. The Hierarchical Genome Assembly Process (HGAP_v2) assembly pipeline (19) was used with ~300× sequence coverage. For annotation, the NCBI Public Genome Annotation Pipeline service was used. The resulting assembly was 3.5 Mb, with 63% G+C content.

Accession number(s). The genome sequence of *Ectothiorhodospira* sp. strain BSL-9 was deposited in the GenBank database under the accession no. CP011994, NCBI BioProject accession no. PRJNA232800, and BioSample accession no. SAMN03795182.

ACKNOWLEDGMENT

We thank the Sierra Nevada Aquatic Research Laboratory for facilitating the fieldwork.

FUNDING INFORMATION

This work, including the efforts of Jaime Hernandez-Maldonado, was funded by NIH-NHGRI (1R25HG006836-01A1 and 5R25GM058903-14). This work, including the efforts of Chad W. Saltikov, was funded by National Science Foundation (NSF) (EAR-1349366) and the Water Mission Area of the U.S. Geological Survey.

REFERENCES

1. Oremland RS, Kulp TR, Blum JS, Hoeft SE, Baesman S, Miller LG, Stolz JF. 2005. A microbial arsenic cycle in a salt-saturated, extreme environment. Science 308:1305–1308. http://dx.doi.org/10.1126/science.1110832.
2. Lloyd JR, Oremland RS. 2006. Microbial transformations of arsenic in the environment: from soda lakes to aquifers. Elements 2:85–90. http://dx.doi.org/10.2113/gselements.2.2.85.
3. Hoeft SE, Blum JS, Stolz JF, Tabita FR, Witte B, King GM, Santini JM, Oremland RS. 2007. *Alkalilimnicola ehrlichii* sp. nov., a novel, arsenite-oxidizing haloalkaliphilic gammaproteobacterium capable of chemoautotrophic or heterotrophic growth with nitrate or oxygen as the electron acceptor. Int J Syst Evol Microbiol 57:504–512. http://dx.doi.org/10.1099/ijs.0.64576-0.
4. Kulp TR, Hoeft SE, Asao M, Madigan MT, Hollibaugh JT, Fisher JC, Stolz JF, Culbertson CW, Miller LG, Oremland RS. 2008. Arsenic(III) fuels anoxygenic photosynthesis in hot spring biofilms from Mono Lake, California. Science 321:967–970. http://dx.doi.org/10.1126/science.1160799.
5. Stolz JF, Basu P, Oremland RS. 2010. Microbial arsenic metabolism: New twists on an old poison. Microbe 5:53–59. http://dx.doi.org/10.1128/microbe.5.53.1.
6. Edwardson CF, Planer-Friedrich B, Hollibaugh JT. 2014. Transformation of monothioarsenate by haloalkaliphilic, anoxygenic photosynthetic purple sulfur bacteria. FEMS Microbiol Ecol 90:858–868. http://dx.doi.org/10.1111/1574-6941.12440.
7. Cloern JE, Cole BE, Oremland RS. 1983. Seasonal changes in the chemistry and biology of a meromictic lake (Big Soda Lake, Nevada, USA). Hydrobiologia 105:195–206. http://dx.doi.org/10.1007/BF00025188.
8. Cloern JE, Cole BE, Oremland RS. 1983. Autotrophic processes in meromictic Big Soda Lake, Nevada. Limnol Oceanogr 28:1049–1061. http://dx.doi.org/10.4319/lo.1983.28.6.1049.
9. Priscu JC, Axler RP, Carlton RG, Reuter JE, Arneson PA, Goldman CR. 1982. Vertical profiles of primary productivity, biomass and physicochemical properties in meromictic Big Soda Lake, Nevada, USA. Hydrobiologia 96:113–120. http://dx.doi.org/10.1007/BF02185426.
10. Zehr JP, Harvey RW, Oremland RS, Cloern JE, George LH, Lane JL. 1987. Big Soda Lake (Nevada). 1. Pelagic bacterial heterotrophy and biomass. Limnol Oceanogr 32:781–793. http://dx.doi.org/10.4319/lo.1987.32.4.0781.
11. Zargar K, Conrad A, Bernick DL, Lowe TM, Stolc V, Hoeft S, Oremland RS, Stolz J, Saltikov CW. 2012. ArxA, a new clade of arsenite oxidase within the DMSO reductase family of molybdenum oxidoreductases. Environ Microbiol 14:1635–1645. http://dx.doi.org/10.1111/j.1462-2920.2012.02722.x.
12. Zargar K, Hoeft S, Oremland R, Saltikov CW. 2010. Identification of a novel arsenite oxidase gene, *arxA*, in the haloalkaliphilic, arsenite-oxidizing bacterium *Alkalilimnicola ehrlichii* strain MLHE-1. J Bacteriol 192:3755–3762. http://dx.doi.org/10.1128/JB.00244-10.
13. Hernandez-Maldonado J, Sanchez-Sedillo B, Stoneburner B, Boren A, Miller L, McCann S, Rosen M, Oremland RS, Saltikov CW. 2016. The genetic basis for anoxygenic photosynthetic arsenite oxidation. Environ Microbiol in press. http://dx.doi.org/10.1111/1462-2920.13509.
14. Trüper HG. 1968. *Ectothiorhodospira mobilis* Pelsh, a photosynthetic sulfur bacterium depositing sulfur outside the cells. J Bacteriol 95:1910–1920.
15. Imhoff JF, Tindall BJ, Grant WD, Trper HG. 1981. *Ectothiorhodospira vacuolata* sp. nov., a new phototrophic bacterium from soda lakes. Arch Microbiol 130:238–242. http://dx.doi.org/10.1007/BF00459526.
16. Gorlenko VM, Bryantseva IA, Rabold S, Tourova TP, Rubtsova D, Smirnova E, Thiel V, Imhoff JF. 2009. *Ectothiorhodospira variabilis* sp. nov., an alkaliphilic and halophilic purple sulfur bacterium from soda lakes. Int J Syst Evol Microbiol 59:658–664. http://dx.doi.org/10.1099/ijs.0.004648-0.
17. Friedrich CG, Bardischewsky F, Rother D, Quentmeier A, Fischer J. 2005. Prokaryotic sulfur oxidation. Curr Opin Microbiol 8:253–259. http://dx.doi.org/10.1016/j.mib.2005.04.005.
18. Frigaard N-U, Dahl C. 2009. Sulfur metabolism in phototrophic sulfur bacteria. Adv Microb Physiol 54:103–200. http://dx.doi.org/10.1016/S0065-2911(08)00002-7.
19. Chin C-S, Alexander DH, Marks P, Klammer AA, Drake J, Heiner C, Clum A, Copeland A, Huddleston J, Eichler EE, Turner SW, Korlach J. 2013. Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data. Nat Methods 10:563–569. http://dx.doi.org/10.1038/nmeth.2474.

Section 5.03 Discussion

Little is known about the distribution, abundance and how widespread *arxA* metabolisms are in prokaryotes. However, the arsenite oxidase *arxA* within in photosynthetic and chemotrophic bacteria is conserved but differences are also observed when performing a NCBI blastp analysis in the currently reported isolates containing *arxA*. The amino acid identities and positives % values for ArxA of PHS-1 to that in BSL-9 is 62 and 75%, respectively, both of which are photosynthetic bacteria. Between photosynthetic and chemoautotrophic bacteria such as PHS-1 and MLHE-1 there is 63 % similarity and 76% identity and between BSL-9 and MLHE-1 there is 78% similarity and 86% identity. Furthermore, an ArxA phylogenetic analysis suggests closer ArxA similarities between BSL-9 and MLHE-1 than that of PHS-1 (chapter 3). The analysis from these three ArxA sequences may suggests that the anaerobic arsenite oxidase *arxA* is diverse among chemoautotrophic and photosynthetic bacteria. If this is the case, future primer development would aid detection of other *arxA* like sequences in other bacteria outside of the family of *Ectothiorhodospiraceae* in the environment as more *arxA* containing bacteria are isolated and characterized. Having degenerate primers for functional genes allows detection for diverse bacteria. This was observed in our *arrA* studies within the Big Soda Lake anoxic water column, where we detected *arrA* in diverse chemoautotrophic bacteria (chapter 3). Lastly it would be interesting to correlate the biochemistry of ArxA found in different bacteria to the biogeochemical cycle of arsenic in the environmental.

To further understand the complexity of these anaerobic arsenite oxidizing bacterial metabolism there is a need to search and isolate more *arxA* containing bacteria from diverse arsenic rich environments. Characterize newly isolated bacteria containing *arxA* can also provide the possibility to generate other genetic model systems to study the molecular mechanism of *arxA*. It is likely that *arxA* metabolisms may be widespread and have been recently reported in metagenomic analysis. Detection of *arxA* has been found in sediment, freshwater aquatic and subsurface groundwater environments (Zargar *et al.*, 2010; 2012; Hernandez-Maldonado *et al.*, 2017). Anaerobic arsenite oxidation metabolisms may be ancient and present in the early earth when oxygen was absent (Sforna *et al.*, 2014; Wallis *et al.*, 2015). In terms of the evolution of arsenic metabolisms, further bacterial isolations may facilitate understanding the evolutionally trajectory of *arxA*, and so it remains to early to raise questions about the evolution of *arxA,* relative to *arrA* or *aioA*.

Through next generation sequencing technologies, computational and bioinformatics analysis we are now are able to explore beyond *arxA*. Further transcriptomic and metagenomic analysis in arsenic rich environments will aid in elucidating the ecology of *arxA*. The molecular mechanisms and regulation of arxA is yet to be fully elucidated. Further molecular genetic studies regarding *arxB2AB1CD* and *arxXSR* may enhance our understanding of anaerobic arsenite oxidation mechanisms and regulatory mechanisms. There are yet numerous questions that have to be answered and so it is exciting and motivating to further study the biogeochemical arsenic cycle which is impacted by anaerobic arsenic metabolisms.

Section 5.04 References

Hernandez-Maldonado, J., Sanchez-Sedillo, B., Stoneburner, B., Boren, A., Miller, L., McCann, S., et al. (2017) The genetic basis of anoxygenic photosynthetic arsenite oxidation. *Environ Microbiol* **19**: 130–141.

Sforna, M.C., Philippot, P., Somogyi, A., van Zuilen, M.A., Medjoubi, K., Schoepp-Cothenet, B., et al. (2014) Evidence for arsenic metabolism and cycling by microorganisms 2.7 billion years ago. *Nature Geosci* **7**: 811–815.

Wallis, J., Wickramasinghe, N.C., Wallis, D.H., Miyake, N., Wallis, M.K., and Hoover, R.B. (2015) Evidence of ancient microbial activity on Mars. In, Hoover,R.B., Levin,G.V., Rozanov,A.Y., and Wickramasinghe,N.C. (eds). SPIE, p. 96060V.

Zargar, K., Conrad, A., Bernick, D.L., Lowe, T.M., Stolc, V., Hoeft, S., et al. (2012) ArxA, a new clade of arsenite oxidase within the DMSO reductase family of molybdenum oxidoreductases. *Environ Microbiol* **14**: 1635–1645.

Zargar, K., Hoeft, S., Oremland, R., and Saltikov, C.W. (2010) Identification of a novel arsenite oxidase gene, *arxA*, in the haloalkaliphilic, arsenite-oxidizing bacterium *Alkalilimnicola ehrlichii* strain MLHE-1. *Journal of Bacteriology* **192**: 3755–3762.

Chapter 6. Conclusions and future directions

Section 6.01 Overview goals

The main goal of this dissertation was to develop a genetic system in order to study the molecular genetics of a photosynthetic arsenite fueled metabolism. Since a genetic system had not been reported our work was crucial to molecular genetic studies regarding photoarsenotrophy. Secondly environmental ecology studies were complementary to our genetic studies and fundamental in order to validation the need to study the genetic basis of anoxygenic photosynthetic arsenite oxidation. These studies provide insights to the biogeochemical cycle of photoarsenotrophy.

Section 6.02 Genetic development in *Ectothiorhodospira* sp. strain BSL-9

The key in developing a genetic system in *Ectothiorhodospira* sp. strain BSL-9 was enabling conjugation reactions between *Ectothiorhodospira* sp. strain BSL-9 and *E. coli* WM3064. This was challenging since both bacteria grew optimally under different conditions. *E. coli* WM3064 is unable to grow under hypersaline alkaline conditions (Jarvis *et al.*, 2001), which are ideal conditions for *Ectothiorhodospira* sp. strain BSL-9 growth. Mating reactions facilitated genetic transfer between *Ectothiorhodospira* sp. strain BSL-9 and *E. coli* WM3064 After satisfactory conditions were meet. The genetic system allowed us to generate an *Ectothiorhodospira* sp. strain BSL-9 ARXA1 mutant in order to validate *arxA* as the sole arsentie oxidase in photoarsenotrophy (Hernandez-Maldonado *et al.*, 2017).

Section 6.03 Photoarsenotrophy molecular mechanism

After generating the an *Ectothiorhodospira* sp. strain BSL-9 ARXA1 mutant, the physiology of ARXA1 mutant strain was compared to BSL-9 WT strain in order to confirm the *arxA* was essential to photoarsenotrophy. The ARXA1 mutant was un able to grow or oxidize arsenite when the strain was grown solely on arsenite relative to the BSL-9 WT strain. However, both the *Ectothiorhodospira* sp. strain BSL-9 WT and *Ectothiorhodospira* sp. strain BSL-9 ARXA1 mutant strain grew well under sulfide, thiosulfate, acetate and sucrose. These observations indicated that growth and arsenite oxidation was specific to the arsenite oxidase disruption mutant (ARXA1). To test external factors that regulate photoarsenotrophy arsenite and light were tested. We speculated that *arxA* would be induce by arsenite, alternatively biochemical arsenite oxidation would be light-dependent. *arxA* induction by arsenite and light-dependent arsenite oxidation confirmed and provided insights to cues that regulate photoarsenotrophy in *Ectothiorhodospira* sp. strain BSL-9. This work provides genetic evidence establishing *arxA* as the sole arsenite oxidase in BSL-9 and clues to the regulation of photoarsenotrophy in the environment by external factors such as, arsenite and light. These findings may facilitate detection and explorations for photoarsenotrophic bacteria outside extreme environments, in anoxic, arsenite rich environments where light is available

Section 6.04 Environmental ecology of *arxA*

Environmental studies from Paoha Island Mono Lake, CA and Big Soda Lake, NV were key in validating the impact of anoxygenic photosynthetic arsenite oxidation. To provide evidence of photoarsenotrophy activity in the environment we

attempted to search for expression the *arxA* gene, which would indicate the production of an active biochemical enzyme with the ability to oxidize arsenite to arsenate. For this reason, we collected environmental red-colored biofilm like samples from hot springs of Paoha Island by scrapping the cobbles covered by red microbial-like mats and analyzed for gene expression (mRNA), specifically *arxA*. Detection of *arxA* within the arsenite rich euphotic hot springs, dominated by *Ectothiorhodospira* species, indirectly indicated photoarsenotrophy in action (Hernandez-Maldonado *et al.*, 2017). Environmental studies from Big Soda Lake, NV also provided indirect evidence of photoarsenotrophy in the water column by studying this phenomenon in microcosms containing native bacteria collected below the oxycline of the water column. Detection of 16S rRNA and *arxA* genes similar to *Ectothiorhodospira*-like species in light incubated microcosms that received arsenite additions, where a light-dependent arsenite oxidation occurred, indicate the possibility of photoarsenotrophy below the oxycline of Big Soda Lake. Detection of the arsenate reductase, *arrA* in lake water provide clues to the arsenic cycle that may also occur below the oxycline of Big Soda Lake. It remains unknown if under ideal conditions photoarsenotrophy is possible within the Big Soda Lake water column. However, it is likely that light dependent arsenite oxidation can occur within the water column of Big Soda Lake, NV.

Section 6.05 Comparative genomics

As we expand our search for *arxA* metabolisms comparative genomics make it possible to identify bacterial genomes containing *arx* gene clusters, which can

provide further evidence of the ecology and distribution of chemoautotrophic and photosynthetic bacteria containing *arxA*. The genome sequence of *Ectothiorhodospira* sp. strain BSL-9 greatly facilitated molecular genetic studies and can serve as a reference DNA blueprint in future ongoing work regarding the metabolism of photoarsenotrophy (Hernandez-Maldonado *et al.*, 2016).

Section 6.06 Implication and future directions

The activity of photoarsenotrophic metabolisms can lead to a decrease in arsenic toxicity and mobility since arsenite is converted to a less toxic arsenic species, arsenate. Perhaps photoarsenotrophy can one day provided arsenic bioremediation tools. However, we have yet to understand how these photosynthetic bacteria coupe with arsenic globally and learn more about their growth limitations under this toxic metal, arsenic. Transcriptomic analysis can provide a globally understanding of molecular genetic mechanisms in photoarsenotrophs. Metagenomic work can provide insights to the complex dynamics or arsenic based metabolism. The photoarsenotrophy molecular genetic regulation has yet to be fully characterized, and it is likely that the two component regulatory systems (*arxXSR*) found downstream of the *arxB2AB1CD* may be responsible for this activity. It is likely that photoarsenotrophy are wide spread and found outside extreme environments since anoxygenic photosynthesis have been present a long time and are know to be ancient metabolisms. As investigations regarding anaerobic arsenite oxidation by *arxA* expands, we may be able to determine the evolution of ArxA relative to the other arsenic oxidoreductases AioA an ArrA.

Section 6.07 References

Hernandez-Maldonado, J., Sanchez-Sedillo, B., Stoneburner, B., Boren, A., Miller, L., McCann, S., et al. (2017) The genetic basis of anoxygenic photosynthetic arsenite oxidation. *Environ Microbiol* **19**: 130–141.

Hernandez-Maldonado, J., Stoneburner, B., Boren, A., Miller, L., Rosen, M., Oremland, R.S., and Saltikov, C.W. (2016) Genome sequence of the photoarsenotrophic bacterium *Ectothiorhodospira* sp. strain BSL-9, isolated from a hypersaline alkaline arsenic-rich extreme environment. *Genome Announc.* **4**: e01139–16–2.

Jarvis, G.N., Fields, M.W., Adamovich, D.A., Arthurs, C.E., and Russell, J.B. (2001) The mechanism of carbonate killing of *Escherichia coli*. *Lett. Appl. Microbiol.* **33**: 196–200.

Appendix A

These experiments are additional data for chapter 4, which was the manuscript that was published with the title: The genetic basis of anoxygenic photosynthetic arsenite oxidation (Hernandez-Maldonado *et al.*, 2017). Figure 3 is preliminary data and was not part of the published work.

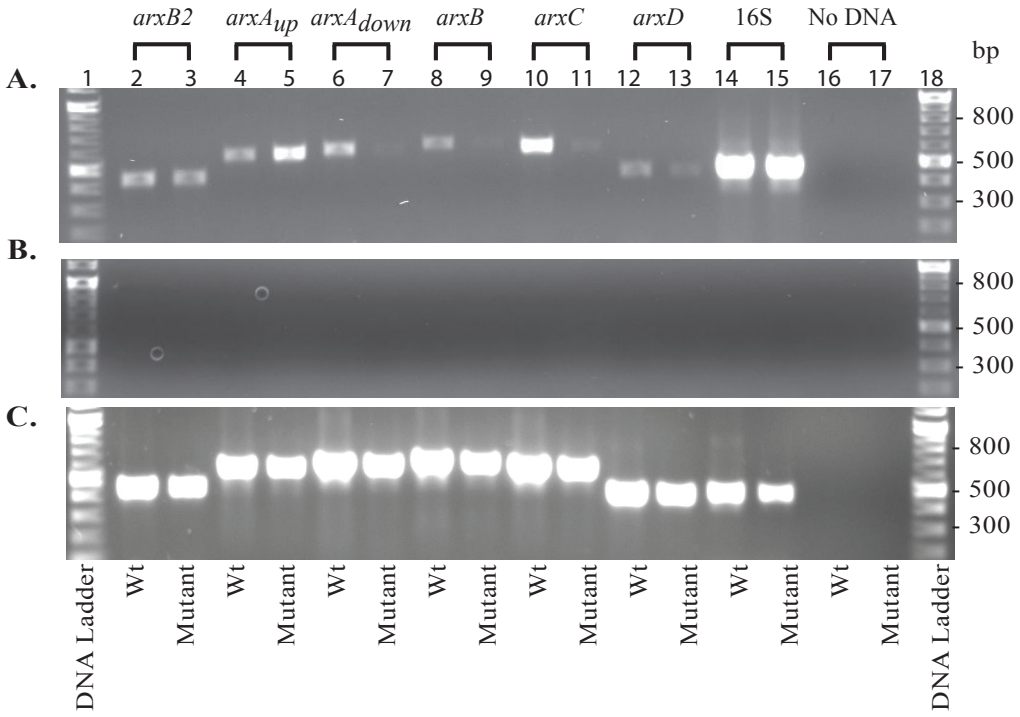

Figure 1. Polar effects of BSL-9 mutant strain (ARXA1). To investigate the impact of *arxA* gene insertion on downstream genes such as *arxB1CD*, we analyzed activity of the *arxB2AB1CD* gene cluster. BSL-9 ARXA1 and wild-type cells were grown (in triplicates) under photosynthetic conditions on 20 mM acetate to early exponential phase (OD_{600nm} 0.2) and induced with 300 μM arsenite for 2 hours. Total RNA was extracted from each replicate and DNase treated prior to RT-PCR analysis. Panels

(A) and **(B)** are agarose gel electrophoresis (1%) images of the RT-PCR analyses for a single representative culture: BSL-9 wild-type (lane 2, 4, 6, 8, 10, 12, 14) and ARXA1 mutants (lane 3, 5, 7, 9, 11, 13, 15). The other two replicates showed identical results. cDNAs were generated with **(A)** and without **(B)** reverse transcriptase and analyzed for the presence of *arxB2* (lane 2-3), a region flanking the *arxA* before (lane 4-5) and after (lane 6-7) the plasmid insertion mutation, *arxB1* (8-9), *arxC* (10-11), *arxD* genes (lanes 12-13), 16S rRNA gene as a positive control for bacterial mRNA expression (lanes 14-15), and No DNA as a control for contamination. Genes were down regulated in BSL-9 ARXA1 mutant (lanes 7, 9, 11, 13) relative to BSL-9 wild-type cells (lanes 6, 8, 10, 12). Panel **(C)** shows PCR results using the same primers sets on genomic DNA for BSL-9 wild-type and BSL-9 ARXA1 mutant, where amplification and detection was observed for all genes in the *arxB2AB1CD* gene cluster.

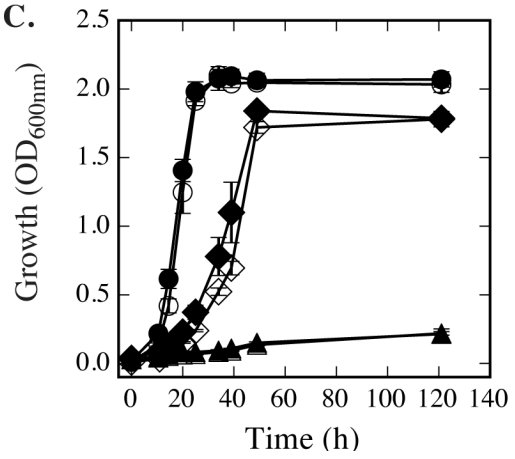

Figure 2. *Ectothiorhodospira* sp. strain BSL-9 ARXA1 physiology. Growth of ARXA1 mutant (clear markers) on acetate (O), thiosulfate (◊) and without electron donors (Δ) was compared relative to *Ectothiorhodospira* sp. strain. BSL-9 WT (solid markers).

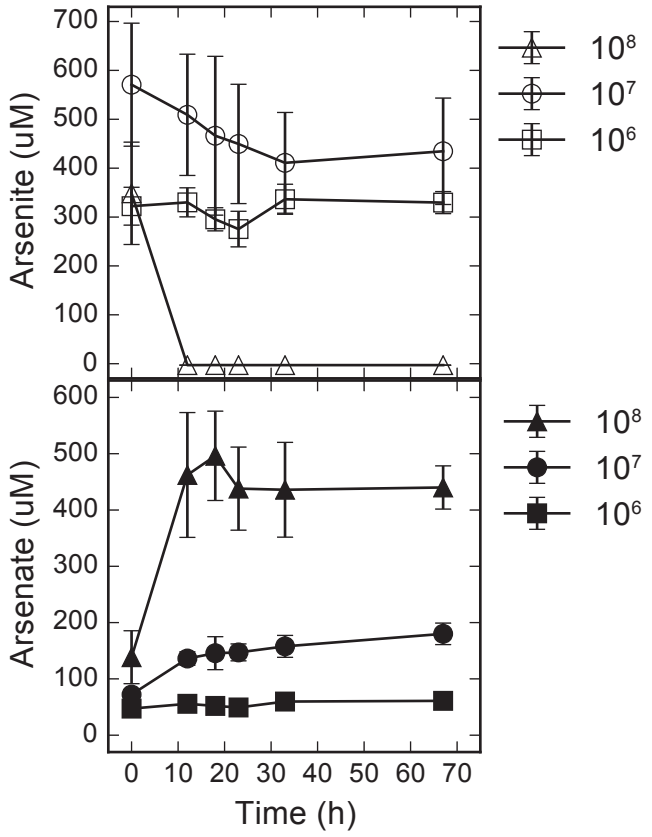

Figure 3. *Ectothiorhodospira* sp. strain BSL-9 high cell suspension, light dependent anaerobic arsenite oxidation using Big Soda Lake 21 m lake water. Clear markers represent arsenite concentrations (top graph) while solid markers (bottom graph) represent arsenate concentrations overtime. The initial cell concentration is approximate 10^8 cells /mL denoted by 10^8, 10^7 cells /mL denoted by 10^7 and 10^6 cells /mL is denoted by 10^6.

Appendix A: References

Hernandez-Maldonado, J., Sanchez-Sedillo, B., Stoneburner, B., Boren, A., Miller, L., McCann, S., et al. (2017) The genetic basis of anoxygenic photosynthetic arsenite oxidation. *Environ Microbiol* **19**: 130–141.