GROWTH, GENES, GENOMES –

INSIGHTS INTO MICROBIAL RESPIRATION

OF ARSENIC AND SELENIUM

by

INES RAUSCHENBACH

A Dissertation submitted to the
Graduate School – New Brunswick
Rutgers, The State University of New Jersey
and
The Graduate School of Biomedical Sciences
University of Medicine and Dentistry
In partial fulfillment of the requirements
For the degree of
Doctor of Philosophy
Graduate Program in Microbiology and Molecular Genetics
Written under the direction of
Dr. Max Häggblom
and approved by

_____

_____

_____

_____

New Brunswick, New Jersey
October 2011

ABSTRACT OF THE DISSERTATION

Growth, Genes, Genomes –

Insights into Microbial Respiration

of Arsenic and Selenium

by INES RAUSCHENBACH

Dissertation Director:

Professor Max Häggblom

Arsenic (As) and selenium (Se) are naturally occurring metalloids in the Earth's crust. Their speciation is governed by the microbial communities in various environments which influences their mobility among the soil, water, and air interface. Microorganisms can utilize As and Se oxyanion as terminal electron acceptors in dissimilatory reduction. These organisms are ubiquitous and phylogenetically diverse. The objectives of the studies in this thesis were to gain an understanding of the metabolism of As and Se respiring bacteria, analyze the genes encoding enzymes involved in respiration and understand how these enzymes are regulated in the presence of various electron acceptors. We were able to isolate two novel As and Se respiring bacteria from different

environments; from a wastewater treatment facility in Verona, NJ and an estuarine canal from Chennai, India. Based on 16S rRNA gene analysis, strain S4 was classified as a novel genus and species, *Selenovibrio woodruffi* and strain S5 as a novel species, *Desulfurispirillum indicum*. We analyzed the genome of *D. indicum* and examined the expression of putative reductases to further understand respiratory metabolism of As and Se oxyanions. Five molybdoenzyme genes were identified in the genome of strain S5, three of which we were annotated to encode for a respiratory arsenate reductase *arr*, periplasmic nitrate reductase *nar*, and respiratory nitrate reductase *nap*. Also, an arsenate resistance system, *ars*, was identified. We were not able to positively identify a selenate reductase gene. Gene expression studies revealed that *arr* was an inducible gene and the only gene highly expressed during arsenate respiration. Growth studies showed that selenate respiration was inhibited by nitrate. Lastly, we also enriched activated sludge samples for tellurium oxyanion respiring bacteria. Thus, we not only added novel, phylogenetically different organisms to the ever-increasing list of As and Se respiring microbes, we also provided insights into the genes and enzymes involved in As and Se respiration and how they are regulated.

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to my dissertation advisor Dr. Max Häggblom for his guidance and advice and my committee members Dr. Nathan Yee and Dr. Elisabetta Bini for their endless support throughout my research, and Dr. Lee Kerkhof for his support and help in successfully completing this work.

I thank the H. Boyd Woodruff Family for endowing the H. Boyd and Jeanette I. Woodruff fellowship fund in Microbiology which provided me with support for the first two years of my PhD studies. I thank the Department of Biochemistry and Microbiology for funding me for the duration of my dissertation research. I also thank the New Jersey Water Resource Institute and the National Science Foundation for funding my research.

The following people have helped make this research possible and I thank them all. Dr. Priya Narasingarao for helping with the isolation of *Desulfurispirillum indicum* strain S5, Dr. Jessica McCormick for the activated sludge samples, Madhavi Parikh for all her life-saving help with ion chromatography, Isabel Gray and Nora Lopez for their help with the HPLC machine after I forgot my password and logged myself out of the system, and my two wonderful undergraduate students Pasqual Cantarella and Valeriya Posternak that helped me with countless transfers, characterization of strains S4 and S5 and molecular work. Also, thank you for helping in washing hundreds of tubes every month! I wish I had counted the number of tubes and cultures that went into collecting all the data to finishing this thesis.

During my years as a Graduate student at Rutgers University, I met wonderful people and made many friends. Thank you to all past and present Haggblom Lab members, especially Dr. Young Beom Ahn, Dr. Joong Wook Park, Dr. Suman Rawat, Isabel Gray, Nora Lopez, Tong Liu, Tiffany Louie, Joanne Ahn, and Jennifer McConnell. Thank you Maryam for being an awesome office mate, and Dave for your help with my

work in Dr. Elisabetta Bini's Lab. I also thank Dr. Diane Davis for helping me become a great instructor and her support during my years as a Teaching Assistant.

Thank you to my wonderful family. My mom, even though she lives far away in Germany, was always at my side to cheer for me. Denise Palombo who collected soil samples for me in Colorado and got me started on my research and Mark and Lauren Ross, who always believed in me. This thesis would have not been possible without them.

Last but not least, thank you to my husband Brian. He stuck with me (…even though I came smelling like butyric acid on our third date!) and was always by my side, supporting me during this endeavor and helping me deal with all my ups and downs. I love you!

I dedicate this work to my dad, Thomas Rauschenbach, who is not with us anymore. He was always proud of my accomplishments and encouraged me to strive for the best. My dad will always be in my heart and is smiling at me from heaven every day.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Metalloids are found in virtually every environment (Silver and Phung 2005). Anthropogenic activities, such as industrial refinement, waste production and management, and agricultural processes have also influenced their accumulation in certain environments, consequently impacting many ecosystems (Silver and Phung 2005, Zannoni et al. 2008). Even though transformation and bioavailability of these elements are influenced by abiotic processes (Myneny et al. 1997), these transformations are mainly carried out by microorganisms (Tucker et al. 1961; Lloyd 2003; Stolz et al. 2006). At low concentrations, metalloids play a key role in maintaining biological activity acting as cofactors for a range of proteins. At higher concentrations, they are utilized as substrates for aerobic or anaerobic respiration acting as electron donors or acceptors (Lloyd 2003). The beneficial roles of metalloids in microbial metabolism have to be balanced against potential toxicity at high intracellular concentrations. Microbes have thus acquired diverse and surprisingly sophisticated detoxification mechanisms for removing these toxic substrates from cells.

Microorganisms influence the biotransformation of many elements, which form the core of many biochemical cycles. Arsenic (As) and selenium (Se) are two elements that can have substantially high, toxic effects on many biological systems because their chemistry is very similar to that of phosphorus and sulfur, respectively. The deaths and deformities in fish, water-fowl and other wildlife caused by very small amounts of selenium at the Kesterson reservoir and recently, the extensive groundwater contamination with arsenic, threatening tens of millions of people in Bangladesh emphasize the need to understand the processes and microorganisms involved in the transformation and mobilization of these two elements.

CHEMISTRY OF SELENIUM AND ARSENIC

Se is the 34th element of the periodic table in Group VIa (with oxygen, sulfur, polonium, tellurium) with an atomic mass of 76.96. This metalloid, belonging to the chalcogen family, was first discovered by John Berzelius in 1817 and is named after the Greek word “selene” meaning moon (Wilber 1980). Se has four oxidation states. The water soluble forms selenate (Se(VI)) and selenite (Se(IV)) are the primary forms of Se in oxic environments and both are toxic. Elemental, water-insoluble Se is the dominant species in anoxic sediments and is non-toxic. Selenide (-II) is the highly toxic gaseous form of Se, readily oxidized in the presence of oxygen, thus seldom causing a major threat to most biological systems. The following redox processes are possible:

$$SeO_4^{2-} \longleftrightarrow SeO_3^{2-} \longleftrightarrow Se^0 \longleftrightarrow Se^{2+}$$

Organic Se compounds (e.g., selenomethionine and selenocysteine) are incorporated into plants and account for the major portion of Se found in vegetables and cereals (Wilber 1980).

In the environment, Se mainly exists in three allotropic forms. The most stable, crystalline hexagonal Se, is metallic gray; crystalline monoclinic Se has a deep red color; and amorphous Se is red in powder form and is black in vitreous form (Figure 1.1).

Se has many commercial uses and finds application in electronics, glass, ceramics, steel, and pigment manufacturing. Se dioxide is the most common industrial Se compound. It is used in the production of photographic cells, as vulcanizing agent for rubber, and an alloy for copper and steel (Wilber 1980; Casteen and Bentley 2003). It also has medicinal uses as Se sulfide for the treatment of dermatitis and tinea versicolor (Chasteen and Bentley 2003).

Arsenic (As), with the atomic number 33 and atomic mass of 74.92, belongs to group 15 along with nitrogen, phosphorus, antimony, and bismuth. As has been recognized as a poison as early as 2000 BC and is sometimes called “inheritance powder” (Stolz et al. 2006). Hippocrates and Aristotle wrote about As in medicinal applications and Albertus Magnus is thought to be the first person to produce pure arsenic. As comes from the Greek word “arsenikon,” meaning “potent” or “yellow orpiment” (Joliffe 1993). The metalloid has four oxidation states, with As(V) and As(III) being the most prevalent in nature.

$$AsO_4^{3-} \longleftrightarrow AsO_2^{-} \longleftrightarrow As^{0} \longleftrightarrow As^{3-}$$

Arsenate [As(V)] is ubiquitous in nature and found in various concentrations in many environments. It is mainly associated with aerobic environments and is readily adsorbed to the surface of several common minerals, which reduces its watersolubitlity and consequently its toxicity. Arsenite (As(III)) is prevalent unde anaerobic conditions. It adsorbs less strongly and to fewer minerals and thus is more mobile and more toxic. Even though there are many allotropes of arsenic, it mainly occurs in only two types: yellow and gray arsenic. Gray arsenic is a very brittle semi-metallic solid. It is steel-gray in color, crystalline, tarnishes readily in air, and is rapidly oxidized (Karttunnen et al. 2007).

Historical uses of As were pharmaceutical, medicinal, as poison, and in the manufacturing of glass (Joliffe 1993). Current applications of As includes use as additive to pesticides and animal feed and in the manufacture of wood preservatives, glass, alloys and electronics (George 2006, Chasteen and Bentley 2003).

SELENIUM AND ARSENIC IN THE ENVIRONMENT

Se is ubiquitous in nature, although the global distribution of Se varies greatly. It is only the 69th most abundant element with average abundance of about 30 to 80 µg/L in the Earth's crust (Wilber 1980, Fishbein 1983). Table 1.1a summarizes the concentration of Se found in food and various other sources in the environment.

Se in water is a result of both wet and dry deposition from the atmosphere and surface and subsurface drainage. Its concentration in seawater is generally low (0.04 – 0.12 µg Se/L) and varies in ground and surface waters (0.1 – 30 µg Se/L) (Valentine 1997). Concentrations in public water supplies usually do not exceed 10 µg Se/L. To control high concentrations of Se in drinking water, the EPA has set a standard an upper limit of 50 ppb (Barceloux 1999). High concentrations of Se in surface and ground water usually occur in farm areas where irrigation water drains from soils with high Se content (Ohlendorf et al. 1986).

Combustion of fossil fuels and coal are the primary source of Se in ambient air. The burning of fossil fuels produces Se dioxide, which is reduced by sulfur dioxide reduces to elemental Se (Andrén and Klein 1975). Therefore, most of the Se in air is bound to fly ash. The incineration of rubber, municipal waste, and paper also releases Se particles, but in lesser amounts (Barceloux 1999).

Se oxyanions do not readily undergo chemical reduction under physiological conditions of pH, salinity, and temperature (Figure 1.2) (Oremland et al. 1989) and most of the Se oxyanions that are present in soils, sediments and water is the result of biotic transformation (Dowdle and Oremland 1998). Abiotic reduction of Se has been observed but with insignificant impact on the natural environments (Myneni et al. 1997). Surveys of eleven different aquatic environments with varying pHs and salinities found only one environment in which biological reduction of selenate was not present (Steinberg and Oremland 1990).

Weathering and leaching of parent bedrock materials that contain Se oxyanions are usually in association with sulfide minerals (e.g. arsenopyrite, chalcopyrite, galena, marcasite, pyrrhotite, pyrite). In soil, Se is present in the form of elemental Se, calcium selenate, basic ferric selenite, and as organic Se compounds (Fishbein 1983).

Arsenic is the 20th most common element with a natural abundance of about 3.4 ppm in the Earth crust. The greatest concentration of As is found in sulfide minerals, for example pyrite, being the most abundant. As also occurs naturally as trace amounts in marcasite and chalcopyrite (Garelick et al. 2008). Under anaerobic conditions, arsenic is associated with arsenopyrite, orpiment, and realgar. When exposed to the surface these compounds may oxidize and form iron arsenates and calcereous arsenolites (Smedley and Kinniburgh 2002).

The origin of As in groundwater is influenced by local geology, hydrogeology, and the geochemistry of aquifers. Elevated As concentrations may be a result of leaching from geologic materials (e.g. arsenopyrite or realgar) containing As and drainage from thermal springs and geysers. The most common source of As contamination in groundwater is from the dissolution of arsenic-bearing sulfide minerals, sorption from arsenic-rich Fe oxides and subsurface microbial acitivity (Ferguson and Gavis 1972). Hydrothermal and volcanic activities also represent an important natural source of As, caused by interaction with leaching ore deposits. Anthropogenic sources of As include the application of pesticides and insecticides to farmland and produce, wood preservation, and oil/coal burning (Lievremont et al. 2009).

As is commonly associated with sulfurous minerals in wastewaters. After contact with both air and water, sulfuric acid is gradually produced, As is solubilized and released. The water flowing through or collecting in excavated ores containing these sulfides then causes the production of acid mine drainage. A summary of sources of As and their environmental concentrations can be found in Table 1.1b.

IMPACT OF ELEVATED SE AND AS ON THE ENVIRONMENT

Selenium

Mining Activities. One of the primary human activitiy that mobilizes and releases Se into the environment is the mining, processing, and combustion of coal for the production of electric power. Se naturally occurs in coal, especially bituminous coal that is mined and burned in the Eastern United States and China. China is the world's largest coal producer, with 75% of all energy needs supported by coal (Finkelman et al. 1999). There are more than 600 coal-fired plants in the US. In 1999, it was estimated that about 60% of all electricity in the United States was generated from the combustion of domestic coal (Finkelman et al. 1999). Almost all of the solid and liquid waste effluents from mining and the power industry are highly enriched with Se as compared to the Se content of the Earth crust or surface waters (Lemly 2004). Se of mined coal may leach out of storage piles when rainwater circulates through and contaminates nearby streams or groundwater. Solid wastes from coal combustion in the form of fly ash can also be transported to other areas and accumulate.

In order to decrease Se that accumulates in streams, rivers, and lakes in coal mining areas in the United States, the EPA has become stricter in enforcing guidelines established for Se wastes. Recently, in January 2011, the EPA halted the construction of a new "mountain top" coal mine in West Virginia with the concern that the Se enriched waste products would cause irreparable damage to the lakes, streams, and valleys in that region (Silva 2011).

Power Plants. Some wastewater containing high amounts of Se (Lemly 1999) from power plants is stored, reused, or evaporated to a disposable sludge. It may also be treated in a settling pond before release into ground water. The effluent of these ash wastewater ponds then tends to leach into nearby bodies of water or the surrounding soil and groundwater.

In one example, Se-contaminated waster water was discharged into Belews Lake, North Carolina, from a coal burning electricity-generating facility over a period of ten years. The bioaccumulation in the aquatic food web caused severe reproductive failure and teratogenic deformities in fish (Lemly 1999; Lemly 2002). In addition, almost all the fish species originally resident in the lake were killed over time by the high concentration of Se (Lemly 1997; Lemly 1999; Lemly 2002). The same problems were reported at Martin Reservoir in Texas (Lemly 1999). In contrast to Belews Lake, the period at which Se entered the water was very short and within only eight months the ecosystem was permanently changed and the physiological effects on fish were severe (Sorensen et al. 1982).

Agriculture. Agricultural irrigation practices may also cause Se accumulation. Usually, irrigation water is applied in far excess to what is needed to sustain plant growth. The extra water must be drained via subsurface canals into surface ponds or creeks. The alkaline nature of the irrigation water cause leaching of the naturally occurring trace metals present in the soil, such as Se, and their accumulation in the drainage water.

A well-known example of contamination caused by agricultural wastewater enriched with Se was the Kesterson Reservoir in San Joaquin Valley, CA (Presser and Ohlendorf 1987; Ohlendorf et al. 1990; Presser et al. 1994). The Reservoir, completed in 1971, consisted of a series of twelve evaporation ponds within the Kesterson National Wildlife Refuge. The refuge was home to various species of waterfowl, plants, and fish, such as bass, catfish, carp, and mosquito fish. The purpose of these ponds was to function as control and storage facility for the subsurface agricultural drain, called the San Luis Drain. From 1971 to 1978, the Kesterson Reservoir first only received fresh water. Funding limitations and growing environmental concerns that the San Luis Drain would destroy the Wildlife Refuge, building was stopped and its extension to the San

Francisco Bay halted. Dwindeling water levels were replenished with saline agricultural runoff (Lemly 2004).

By 1981, all incoming water into the Kesterson Reservoir came from the San Luis Drain. By then, changes to the habitat were noticed, including the declining use of the reservoir by waterfowl; algal blooms; dying cattails; and soon after the salt tolerant mosquito fish was the only species remaining in the habitat (Presser and Ohlendorf 1987). In 1986, scientist attributed the damage to the wildlife refuge to Se poisoning caused by the leaching of selenate and selenite from seleniferous soils, caused by the agricultural runoff. Average concentrations of Se were 300 µg/L, some exceeding 1.4 mg/L (Presser and Ohlendorf 1987). To stop damage and prevent further leaching of Se oxyanions, the Kesterson Reservoir was closed. The storage ponds were graded and filled to prevent future development of a wetland. The USGS is continuing to monitor the land other areas in the Western United States so similar problems will not re-emerge.

Arsenic

Arsenic is one of the most feared contaminants in the environment because of its high toxicity. The origin and mobility of arsenic in groundwater are influenced by local accumulation of the element in the environment causing a significant global human health problem. The extent of high arsenic concentrations in the environment around the world is summarized in Table 1.3.

Groundwater. Exposure to high levels of arsenic can cause arsenicosis, a chronic disease resulting mainly from long-term exposure to contaminated groundwater (For more information about arsenicosis also see the section “Arsenic and Selenium in Biological Systems”).

A number of large aquifers with high levels of As have been identified around the world. Well-known examples include Bangladesh, India, China, Chile, Argentina, and Hungary (Table 1.2). The As contaminated aquifers in Bangladesh represent the most serious global health and environmental concern with about 90% of the wells affected and about 30 million people exposed to the contaminated water in that area (Smith et al. 2000; Smedley and Kinniburgh 2002). As concentrations of up to 2,500 µg/L have been found in well water (Table 1.2). The source of As is geological in nature. The affected aquifers are shallow and composed of sands, silts, and clays, which are washed from the Himalayan Mountains and deposited by the Ganges River. In most cases, the uppermost layers of the aquifer were composed of clay or silt, thus effectively restricting entry of air to the aquifer. Anoxic conditions and the natural presence of organic matter resulted in highly reducing conditions favoring the mobility of As. In addition, the microbial community and/or Fe and As-respiring microorganisms may have also influenced the reducing conditions in that area (Ahmann et al 1997). As a result, solid-phase As was reduced to As(III), desorpted from the Fe-oxides and dissolving Fe-oxides themselves (Smedley and Kinniburgh 2002). The World Health Organization guidelines for As in drinking water are 10 µg/L (WHO, 2004). This limit was also established in the USA and many other countries in the world. In India, China, and most South American countries the maximum allowable As limit in water is 50 µg/L (Mondal et al. 2006).

Agriculture. Arsenic has also found wide application in a variety of insecticides and pesticides applied to fruit orchards in the forms of lead, calcium, magnesium, and zinc arsenate, zinc arsenite, and Paris Green ($Cu(CH_3COO)_2x3Cu(AsO_2)_2$). Other inorganic arsenicals include sodium arsenite, used as a weed killer and arsenic acid, used to desiccate cotton.

The United States began using arsenic-based pesticides in the second half of the 19th century. Mainly lead, calcium and sodium arsenate were applied to apple, blueberry, and potato crops in New England and fruit orchards, cropland, and golf courses in New Jersey. Murphy and Alcott (1998) estimated that a total of 25 million kg lead arsenate and 9 million kg calcium arsenate were applied to soils in New Jersey. New residential developments now built on these previously treated farmlands are contaminated with arsenic and lead.

By the mid-90s, the EPA banned the use of many inorganic arsenic-based pesticides (Wormell 2006). However, mono- and dimethylarsenic acid are still used in farming. Data is lacking in the literature studying their soil stability and potential breakdown, transformation and release from soils into the groundwater system in the future remains unknown.

Mining Operations. Similar to Se, coal deposits are also enriched with As. Mined and combusted coal, and the fly ash may all contain high levels of As causing serious adverse environmental and health problems. Worldwide, humans regularly come into contact with arsenic-rich coal. In the United States, for example, cases of high concentrations of As due to coal have been reported in Alaska, Idaho, California, and Montana (Garelick 2008). Early mining activities have also led to significant accumulations of As in soils and watersheds in England (Garelick 2008). Metals and metalloids produced by the heat-treatment and processing of As-rich polymetallic ores and arsenopyrite leached into local waterstreams causing significant accumulation of As in these areas (Garelick 2008). Probably the worst case of As poisoning related to mining activity was found in Thailand. Sediments that had been extensively dredged during mining operations, causing the oxidation of arsenopyrite, mobilization of As and

consequently accumulating As in the shallow groundwater. Arsenic concentrations of up to 5000 µg/L were found (Williams 1997).

Because As is also associated with gold deposits, gold mining may also contribute to As accumulation. Gold is an important precious metal and commodity that has been extensively mined in countries, such as Australia, and Brazil (Garelick et al. 2008). High levels of up to 15,000 mg/kg As were mainly found in the actual gold deposits, mining waste disposal areas, and surrounding streams, sediments and surface waters. New constructions of settlements on old gold mines, dispersion of mine tailings by floods, and consumption of the local groundwater may pose additional hazards to humans.

Wood preserving arsenicals. The untreated, cut lumber from most commercially harvested trees is prone to attacks by termites and marine organisms; or decay caused by fungi and bacteria. In order to protect the wood, it was commonly been treated with chromated copper arsenate (CCA) (Wormell 2008) in both in Europe and the USA for the past 60 years. CCA was very effective and an excellent wood preservative, especially in residential applications; e.g, decking and outdoor children play sets. Because of the toxicity of both As and chromium, regulatory and public attention has increasingly focused on the risks associated with CCA-treated wood. Many sites used by the timber industry to treat the wood and those sites associated with waste disposal are known to be the main sources of arsenic contamination (Turpeinen et al. 1999). The well-documented toxicity of CCA-treated wood has recently led the EU Commission to restrict the use of these products (Wormell 2008). Since 2004, CCA-treated wood cannot be used any longer for residential applications and is only permitted for limited use by the industry (Wormell 2008).

SELENIUM AND ARSENIC IN BIOLOGICAL SYSTEMS

Selenium

There is a narrow range for Se as an essential trace element and toxic substance for animals and humans. Both very low and high concentration can cause harm to most organisms. The National Research Council set a U.S. recommended daily allowance of 0.87 µg/kg (55 – 70 µg/person) (National Institute of Health 1989).

The nutritional requirement for Se is a result from the dependency of animals, humans and microorganisms on Se containing enzymes. This dependency involves the Se containing amino acid selenocysteine which is also known as the "21st amino acid." Enzymes containing selenocysteine include formate dehydrogenases, clostridial glycine reductases, hydrogenases, mammalian glutathione peroxidases, and thyroid hormone deiodinases (Chasteen and Bentley 2000). Se has also been identified in thioredoxin reductases, which are metabolic enzymes particularly important in cell growth, acting as an antioxidant, preventing cell damage and apoptosis (Arnér and Holmgren 2000; Mustachich and Powis 2000). In addition, Se has been found in some bacterial tRNA species (Wittwer et al. 1984).

The toxicity of Se is dependent on its chemical speciation and concentration in the environment (Barceloux 1999). Also, in vivo, it forms highly reactive Se-containing species such as selenipersulfides or organoselenide anions. This catalytic process has overall been accounted for Se toxicity (Spallholz 1994).

High amounts of Se cause disease in both animals and humans. Probably the first known case of Se poisoning in animals was reported by Marco Polo during his travels in 1295 where he described a disease as "hoof rot" (Yang et al. 1983; Barceloux 1999). Today, the disease is known as "alkali disease" and symptoms include changes in hoofs and loss of tail hair (Barceloux 1999; O'Toole and Raisbeck 1995). Furthermore,

accumulation of Se in tissues can cause birth defects, malformations, such as edema; craniofacial and finfold malformations in fish; edema, stunted growth, and hydrocephaly in waterfowl (Hoffman and Heinz 1988; Heinz et al. 1988; Lemly 2002; Muscatello et al. 2006; Luoma and Presser 2009).

One well reported case of human selenosis took place in Hubei Province, China, in the 1960s. The soil in the region was naturally high in selenium due to weathering and leaching of coal and the uptake of Se into plants was fostered by the application of lime fertilizers. The selenosis was caused by droughts in the region, which had forced the residents to eat corn with high concentration of Se. The disease symptoms included loss of hair and nails, dermatitis, skin lesions, fatigue, and depression (Yang et al. 1983).

In parts of the world where Se is found in extremely low concentrations (e.g. China), Se deficiency is associated with Keshan disease, a heart muscle disease, afflicting especially young women and children (Cheng et al. 1980). Lack of both iodide and Se may cause Kashin-Beck disease. Symptoms include joint swelling, pain, fatigue, and degeneration of cartilage leading to shortened stature (Barceloux 1999; Holbein and Smith 1999). Low intake of Se has also been linked to increased risk of cancer and oxidative stress of HIV patients; and lowered immune responses in patients with Kidney disease (Holbein and Smith 1999; Chasteen and Bentley 2003).

Arsenic

The toxicity of As to humans largely depends on the chemical form and physical state of the compound involved. Inorganic As(III) is generally regarded as more toxic than inorganic As(V). Elemental As is nontoxic, even if ingested in substantial amounts. Data indicated that As doses of 1 to 3 mg/kg per day are usualy fatal. Prolonged oral exposure of inorganic As at doses of more than 0.1 mg/kg per day is associated with neurological and hematological signs of toxicity (Astolfi et al. 1981).

Drinking of As-contaminated groundwater has been considered the main route of exposure for humans (Pokhrel et al. 2009). The first case of chronic arsenic poisoning from drinking water contaminated with As was reported in the early 1980s in China (Xia and Liu 2004). People living in affected area suffered from gastritis, liver and kidney disease, and abnormal heart rhythms. The most common symptoms were hyperpigmentation and thickening of skin on palms and soles.

As mentioned before, groundwater and wells in Bangladesh and India contain some of the world's highest concentrations of As (Table 1.2) (Das et al. 1996; Nordstrom 2002; McArthur et al. 2001). Recently, rising water demands have increased As leaching from bedrock, due to higher flow rates from the aquifers, rapid oxidation, and dissolution of the As (Anawar et al. 2006). Water is mainly used for crop irrigation, drinking, and cooking and the population is directly exposed to the contaminated water via ingestion of water, crops, livestock, and fish from affected waters. Chronically exposed patients had high concentrations of As in hair, nails, and skin (Das et al. 1996).

ENZYMES CATALYZING THE REDUCTION OF SELENIUM AND ARSENIC OXYANIONS

Molybdenum Uptake

Bacteria require specific transport systems for the uptake of molybdate into the cell. Studies in *Escherichia coli* have shown that Mod, encoded by the *modABCD* operon, guides the efficient uptake of Mo into the cell (Corcuera et al. 1993). ModA is the perimplasmic molybdate binding protein; ModB is the integral transmembrane channel through which Mo is transported; ModC is an ATP-binding protein providing the energy

for Mo transport; and ModD is a molybdenum transport protein (Maupin Furlow et al. 1995; Grunden et al. 199).

A second divergent gene, *modE*, regulates the expression *modABDC*. ModE was found to directly bind two molecules of Mo. The Mo-ModE complex then bound to the transcription start site of *modA,* repressing the expression of the operon. Absence of Mo causes a conformational change, dissociated ModE, allowing the expression of modABCD and intracellular transport of Mo (Grunden et al. 1999; Anderson et al. 2000).

Molybdoenzyme Families

Enzymes containing the molybdenum cofactor, found in virtually every organism, are fundamental for the catalysis of key steps in microbial metabolism (Hille et al. 1999; Schwarz et al. 2009). They are grouped into three families based on sequences similarity, cofactor composition, and catalytic function: sulfite oxidase, xanthine oxidase, and dimethyl sulfoxide (DMSO) reductase (Kisker et al. 1997; Schwarz et al. 2009). The members of the DMSO family of reductases are only found in prokaryotes and include the periplasmic nitrate reductase (Nap), respiratory nitrate reductase (Nar), respiratory arsenate reductase (Arr) and selenate reductase (Ser) (Kisker et al. 1997; Malasarn et al. 2008; Lowe et al. 2010).

The crystal structures of the enzymes of the DMSO family show a high degree of similarity (Kisker et al. 1997). However, there are significant differences in the active sites, contributing to the diversity of functions performed by the DMSO family. The metal atoms in these proteins are coordinated by the thiolates of the pterin cofactor and by a variety of other atoms, for example oxygen, sulfur or selenium atoms (González et al 2006). They may also contain other redox cofactors such as iron sulfur centers, flavin, and hemes, which are believed to be involved in the electron transfer processes (Figure 1.3).

The reaction catalyzed by DMSO reductase may be subdivided into an oxidative and a reductive half cycle (Kisker et al 1997). During the oxidative half-cycle, the reduced Mo(IV) form of the enzyme binds the substrate. Two electrons are transferred from Mo to the substrate which yields the reaction product DMS and the oxygen atom of the substrate bound to the metal. In the reductive half-cycle, two protons and two electrons are transferred to the metal center, yielding H_2O and regenerating the Mo(VI) state. An external electron donor, e.g. water-soluble cytochrome, is required because DMSO reductase does not contain a second cofactor (Kisker et al, 1997).

Targeting signals for translocation of molybdoenzymes

Various molybdoenzymes, such as Ser, Arr and archaeal Nap, are translocated into the periplasm (Richardson et al. 2001; Afkar et al. 2003; Lowe et al. 2010). While these enzymes have conserved signal regions or sequences that aid in their correct localization, their successful translocation entails the completion of a series of steps: synthesis of the precursor protein, insertion into the membrane, translocation, recognition of the signal peptide, cleavage, and final localization of the mature protein (Izard and Kendall 1994).

A vast majority of these enzymes are exported by the secretory (Sec) pathway (Natale et al. 2008). A Sec translocated protein has three characteristic domains, spanning about 20 amino acids (Figure 1.4). The n-region contains a commonly positively charged N-terminal domain that interacts with membrane phospholipids; the h-region, an uncharged hydrophobic core, usually in α-helical conformation; and a more polar C-terminus or c-region. The signal protein then cleaves the signal sequences from the mature protein after translocation into the periplasm (Kendall and Izard 1994; Gross et al. 1999; Cristóbal et al. 1999). The proteins targeted by the Sec pathway do not

contain any other conserved regions, thus the structure alone is sufficient to be recognized by the Sec components as the (Natale et al. 2008).

Some bacteria translocate their enzymes by a Sec-independent pathway, the twin-arginine translocation (Tat) system. Precursor proteins are directed to the Tat pathway bearing a characteristic conserved (S/T)-R-R-x-FLK sequence motif (Berks 1996). Depending on the species, there may be a simpler Tat motif present, φ-R-R-x-θ-θ where phi stands for a polar residue and theta for a nonpolar, hydrophobic residue (Natale et al. 2008). All Tat motifs have the two signature arginines at the n-terminus (Figure 1.5) (Berks 1996; Sargent 2009; Natale et al. 2008). Indirect evidence from studies in *E.coli* have shown that the Tat system transports folded proteins across membranes. Tat mutants accumulated all precursor proteins containing a cofactor, such as an iron-sulfur cluster or molybdopterin (Berks 2000; Weiner et al 1996), demonstrating that cofactor insertion happens before export (Gross et al. 1999). The cofactor was also present in a protein with altered or removed Tat signal sequence, even though export was inhibited (Santini et al. 1998; Gross et al. 1999). When cofactor insertion was blocked, Tat precursors didn't accumulate in the periplasm but the cytoplasm, demonstrating that the cofactor was a crucial component of protein export (Berks 1996). Once the ability to insert a cofactor was restored, the peptide was exported to the periplasm (Santini et al. 1998).

Mechanism of Anaerobic Dissimilatory Reduction

After oxidation of an electron donor, hydorgens are released by dehydrogenases and quinone dehydrogenases across the bacterial membrane producing a proton gradient. In addition, electrons are generated. The electrons are then passed along the membrane to a cytochrome carrier. The cytochrome carrier the relases the electrons that will interact with the reductase that is present in the membrane or in the periplasmic

space, reducing the final electron acceptor. The protons generated by this process are pumped back across the membrane to generate ATP used for cellular metabolism (Figure 1.6).

Dissimilatory Selenate Reduction. Only one respiratory selenate reductase has been isolated and characterized thus far. It was purified from the soluble fraction of *Thauera selenatis* cells that had been grown anaerobically with selenate as electron acceptor (Maher et al. 2004; Schroeder et al. 1997). The selenate reductase Ser is a member of the tat translocated type II molybdoenzyme and composed of three subunits. The reductase is expressed from an operon of four genes, *serABCD* (Lowe et al. 2010). Kinetic studies revealed high affinity of Ser to selenate, while nitrate, nitrite, sulfate, and chlorate did not serve as electron acceptors (Schroeder et al. 1997).

The three subunits alpha (SerA), beta (SerB), and gamma (SerC) have relative molecular weights of 96, 40, and 23 kDa, respectively (Dridge et al. 2007; Maher and Macy 2002; Maher et al. 2004; Schroeder et al. 1997). The total molecular weight was estimated to be ~160 kDa (Maher et al. 2004). The SerA subunit has an N-terminal cysteine-rich motif, coordinating an iron-sulfur cluster (Dridge et al. 2007). It also contains the Mo active site in the form of a b-s-molybdopterin guanine dinucleotide cofactor (Maher et al. 2004; Schroeder et al. 1997). SerD may function as a chaperone protein for the functional assembly of the catalytic subunit SerA (Watts et al. 2004). The SerB subunit also has four cysteine-rich motifs and shows four potential iron-sulfur binding motifs. The SerC subunit is coordinated with a b-type cytochrome that has a high redox potential (Dridge et al. 2007).

Electrons are transferred from the quinol-cytochrome c oxidase and quinol dehydrogenase pool to a di-heme cytochrome of the cytochrome c4 ($cytc_4$) family which then delivers them to SerC. The reduced SerC then donates electrons, via the iron-sulfur

clusters, to SerAB for reduction of selenate to selenite and elemental selenium (Dridge et al. 2007)

A recent study characterized the respiratory selenate reduction in *Bacillus selenatarsenatis* (Kuroda et al. 2011). Selenate reduction deficient mutants were generated and the sequence of a putative selenate reductase encoding operon, *srdBCA*, was identified. This operon was found to catalyze selenate reduction indenpendently of arsenate, nitrate and selenite reduction. The protein complex Srd was suggested to be a membrane-bound molybdenum-containing enzyme of the DMSO family (Kuroda et al. 2011).

Dissimilatory Arsenate Reduction. With the isolation and characterization of novel organisms, such as strains MIT-13, SES-3 and OREX-4, it was shown that arsenate respiring organisms are spread among various phyla and niches. *Sulfurospirillum arsenophilum* strain MIT-13 and *Sulfurospirillum barnesii* strain SES-3 were the first bacterial isolates found to respire arsenate (Ahmann et al. 1994; Oremland et al. 1994; Laverman et al. 1995; Stolz et al. 1999). Strain MIT-13 was able to respire 10 mM of arsenate to arsenite during the oxidation of lactate to CO_2 (Ahmann et al. 1994; Stolz et al. 1999), while strain SES-3 was able to reduce 4.5 mM or arsenate to arsenite within 16 hours (Lavermann et al. 1994). Overall cell density in arsenate growing conditions was higher in strain MIT-13 than SES-3.

Both strains were able to reduce nitrate, but not sulfate. A third isolate, *Desulfotomaculum auripigmentum* strain OREX-4 was also able to respire arsenate but was not able to reduce nitrate. Instead, it grew on sulfate and arsenate, preferring arsenate as the electron acceptor (Newman et al. 1997). The microorganisms known to respire arsenate are summarized in Table 1.2.

Respiratory arsenate reductase enzymes (Arr) have been isolated and characterized in *Chrysiogenes arsenatis*, *Bacillus selenitireducens*, and *Shewanella* sp.

strain ANA-3 (Krafft et al. 1998; Afkar et al. 2003; Malasarn et al. 2008) and identified in many other organisms (see Stolz et al. 2006). They are only functional when expressed as heterodimers, with a larger molybdopterin containing subunit ArrA and a smaller, iron-sulfur containing subunit, ArrB (Table 1.6). Even though the protein sequences of the enzymes are highly conserved, there are differences. Arr in *C. arsenatis* and *Shewanella* sp. ANA-3 were found in the periplasm while it was membrane-associated, oriented toward the periplasm, in *B. selenitireducens* (Krafft et al. 1998; Afkar et al. 2003; Malasarn et al. 2008). The K_m of Arr from C. arsenatis is 300 μM but only 34 μM for *B. selenitireducens* and 5μM for *Shewanella* sp. ANA-3. ArrAB had high affinity for arsenate only in *C. arsenatis* and strain ANA-3 (Krafft et al. 1998; Malasarn et al. 2008), while it also able to reduce small amounts of arsenite, selenite and selenate in *B. selenitireducens* in the presence of methyl viologen (Afkar et al. 2003). The reduction of additional electron acceptors by the purified enzyme may only show a general function of molybdoenzymes and may not be relevant, since *B. selenitireducens* does not respire selenate or arsenite (Switzer Blum et al. 1998).

Intracellular Transport of Se and As

Selenium. Little is known about the specifics of selenate transport, the first step in the microbial metabolism of the trace element. Similar to As(V) transport via ABC transporters (see below), uptake of Se(V) in *E. coli* was also via the sulfate ABC transporter, encoded by the *cysAWTP* operon (Sirko et al. 1990; Turner et al. 1998). The Cys complex was composed of two ATP binding CysS proteins; two transmembrane proteins CysT and CysW; and CysP, the periplasmic sulfate binding protein. *E. coli* was also able to transport selenate into the cell interior via its sulfate permease. However,

selenate transport was still seen after the repression of that ABC transporter, suggesting other, secondary transport systems (Turner et al. 1998).

Arsenic. As oxyanions are toxic substrates with no known nutritional value or metabolic roles and thus, there was no need for organisms to evolve specific uptake systems. As is taken up by existing transport systems, such as phosphate transporters in both prokaryotes and eukaryotes. For example in both *E. coli* and *Saccharomyces cerevisiae*, the phosphate transporters Pit and Pst have shown to be responsible for the arsenate uptake (Rosenberg et al. 1977; Bun-ya et al. 1996; Wysocki et al. 2001).

MICROBIAL INFLUENCES ON THE BIOGEOCHEMICAL CYCLING OF SELENIUM AND ARSENIC

Selenium, arsenic, and many other elements that exist in different oxidation states, undergo biogeochemical transformation that influences their mobility in the environment. Change in oxidation states influence solubility, transport potential in water, soil and air and consequently also toxicity to humans and wildlife. The biogeochemical cycles of Se and As are influenced by microbes that govern the oxidation, reduction, methylation, demethylation, and volatilization of these elements.

Oxidation

Few studies have presented evidence for microbial oxidation of Se. In 1923, Lipman and Waksman found that bacteria were able to grow in culture medium with soil consisting of inorganic materials and selenium as the sole energy source (Lipman and Waksman 1923). A follow-up study with more detailed description of the bacterium was never published. A strain of the soil bacterium *Bacillus megaterium* was also found to oxidize elemental selenium to selenite and selenate in the presence of oxygen

(Sarthchandra and Watkinson 1981). In a third study, Dowdle and Oremland (1998) showed that selenium was reoxidized to selenite and small amounts of selenate by bacteria and fungi. The respiratory inhibitor sodium azide, metabolic uncoupler 2,4-dinitrophenol, microbial and fungal antibiotics, autoclaving, and formalin inhibited oxidation; evidence supporting that it was a biotic process. In addition, two bacterial strains *Leptothrix* MNB-1 and *Thiobacillus* ASN-1 oxidized elemental selenium to selenate (Dowdle and Oremland 1998). Overall, microbially influenced rates of oxidation were extremely slow in comparison to reduction of Se oxyanions. Thus, most of the elemental selenium produced during reductition will remain as such or take a long time to be reoxidized back to Se(IV) or Se(VI) (Zawilanski et al. 2003).

The chemical oxidation of arsenite to arsenate is slow and mostly influenced by microorganisms. Many prokaryotes have been identified capable of this mechanism (Stolz et al. 2006), which are almost all heterotrophic arsenite-oxidizing bacteria that require other organic matter for growth. *Alcaligenes* spp. are the most common strains isolated capable of arsenite oxidation. Osborne and Ehrlich (1976) showed that the oxidation process was induced by the presence of arsenite. Arsenite oxidation is generally considered a detoxification mechanism because arsenite is more toxic than arsenate to cells (Tamaki and Frankenberger 1992).

There are also bacteria, e.g. strain NT-26, that use the energy gained from arsenite oxidation for growth (Santini et al. 2000). The strain grew rapidly (doubling time 7.6 h) in the presence of arsenite as electron donor and CO_2-HCO_3^- as the carbon source and the rate of arsenate formation was proportional to the oxidation of arsenite.

Reduction

Selenium. In soils and sediments, the reduction of Se(VI) and Se(IV) to elemental Se is largely influenced by microorganisms, although some abiotic reduction was also found

when Fe(II) and Fe(III) oxides were present (Myneni et al. 1997). Several species, for example *E. coli* (Turner et al. 1998), *Enterobacter cloacae* (Losi and Frankenberger 1998), *Desulfovibrio desulfuricans* (Zehr and Oremland 1987), and *Stenotrophomonas maltophilia* (Dungan et al. 2003), are also capable of reducing selenate without energy generation. Species such as *Rhodobacter sphaeroides* (van Fleet-Stalder et al. 2000; Bebien et al. 2001) or *Ralstonia metallidurans* (Roux et al. 2001) could only reduce selenite.

Two operons, *ygfKMN* and *ynfEFGHdmsD*, have been proposed to encode for the selenate reductase in *E. coli* (Bébien et al. 2002; Gyumer et al. 2009). Through mutants impaired in selenate reduction, researchers were able to deduce that the *ygfKML* operon encoded three subunits, YgfK, YgfM, and YgfL of a putative selenate reductase in *E. coli*. Mutants unable to transport and process molybdenum were also deficient of selenate reduction, suggesting the selenate reductase to be a molybdenum containing enzyme (Bébien et al. 2002). The operon *ynfEFGHdmsD* also encoded a molybdenum-containing oxidoreductase that was found to be central to selenate reduction in *E. coli* (Gyumer et al. 2009). It was speculated that YnfE alone was the selenate reductase, although YnfF also contributed with low activity to selenate reduction in the cultures. Both contained a tat signal sequence providing evidence for a periplasmic localization of the enzyme (Guymer et al. 2009).

E. cloacae strain SLD 1a-1, a facultative anaerobe, was isolated from the San Luis Drain for its ability to reduce both Se(VI) and Se(IV) under aerobic conditions (Losi and Frakenberger 1998). Under anaerobic conditions, it could not sustain growth with selenate as the sole electron acceptor in the absence of fermentable substrates (Watts et al. 2003). The reduction of selenate was attributed to a membrane-bound molybdoenzyme complex, consisting of three subunits, oriented toward the periplasm. It

was found to be independent of a nitrate reductase similar to Nar, which was also identified in the strain (Watts et al. 2003; Ridley et al. 2006).

Similar to studies in *E. coli, E. cloacae* mutants deficient of selenate reduction were generated to study the regulation of the selenate reductase activity (Yee et al. 2007). Fnr, a fumarate nitrate reduction regulator, was found to be responsible for selenate reduction in this strain as well as in *E. coli*. FNR is a transcriptional regulator that monitors oxygen levels and allows microorganisms to adapt to changing oxygen levels in the environment (Green et al. 2009). In the absence of O_2, FNR activates the expression of genes involved in anaerobic respiration, such as Nar and Nir (nitrite reductase) in *E. coli* (Green et al. 2009).

Arsenic. Arsenate is reduced by the arsenate reductase Ars to its trivalent form arsenite. Ars genes are highly conserved, widespread in nature and can be found on either plasmid or chromosome loci (Rosenstein et al. 1992; Ji and Silver 1992; Carlin *et al.* 1995; Silver and Phung, 1996; Li and Krumholz, 2007).

One of the first reports on resistance to arsenate and arsenite was when Novick and Roth (1968) linked As resistance to three plasmids, pI258, pII147, and pI524 found in *Staphylococcus aureus*. Mutants of *S. aureus* strains carrying these plasmids did not only show loss of resistance to arsenate and arsenite, but suggested the genes to be located close together (Novick and Roth 1968). Later studies demonstrated that the resistance genes were part of one operon, *arsRBC* (Novick and Roth 1968; Novick et al. 1979). Plasmid R733 was found to confer resistance to As oxyanions in *E. coli* (Hedges and Baumberg 1973). The resistance operon on the plasmid contained five genes, *arsRDABC* (Xu et al. 1997). Many other organisms have been found to express the highly conserved Ars locus with variation in their genomic configuration (Páez-Espino et al. 2009; Rosen and Lui 2009).

ArsR is a small regulatory protein, acting as a trans-acting repressor. A membrane complex formed by ArsA and ArsB carries out the energy-dependent efflux of As oxyanions, thus reducing the cellular As concentration (Tisa and Rosen 1990). Specifically, ArsA functions as the catalytic subunit, while ArsB acts as the anion channel. ArsD is a homodimer (Wu and Rosen 1993) and is identified as the arsenic chaperone for the ArsAB pump, transferring As(III) to the ArsA subunit of the pump.

Respiration

Oremland et al. (1989) were the first to confirm the existence of a process now known as dissimilatory selenate respiration. By using sediment core profiles, the researchers observed that selenate respiration was a novel process that occurred independent of sulfate reduction. During the process of respiration, anaerobic microorganisms could utilize Se and As oxyanions as terminal electron acceptors in the presence of suitable electron donors and reduced them further to their elemental states. To date, there have been more than 20 phylogenetically diverse microorganisms isolated that were capable of reducing selenate or selenite (Table 1.4).

The transfer of electrons from a reductant to an oxidant forms the basis of redox reactions. Depending on the electron acceptor, microbial communities become active because microorganisms have a redox potential range at which they can function and make enough energy for survival. Although the transformation of Se and As oxyanions can occur by a number of different mechanisms, the most environmentally significant process is dissimilatory reduction (Baesman et al. 2007; Oremland and Stolz 2003). The reduction of both Se and As are energetically favorable (Table 1.3).

Microbes that can respire Se and As are ubiquitous and phylogenetically diverse. Currently there are more than 30 known microorganisms that respire Se and As (Table 1.4). Their physiological and metabolic characteristics vary greatly and the ability to

respire these oxyanions is usually one of the distinguishing factors from their close relatives.

The first two reports of selenate respiration were published in 1989. Oremland et al. (1989) found a novel dissimilatory selenate reduction process that was independent of sulfate reduction. Porewater profiles showed that selenate and sulfate were present at different depths, indicating that two different processes were responsible for their reduction despite earlier findings that both processes could be linked (Zehr and Oremland 1987). Subsequent reports showed that dissimilatory selenate reduction existed in a variety of sediments, regardless of pH, salt content, of previous exposure to Se (Steinberg and Oremland 1990).

In a second study, Macy et al. (1989) were able to isolate the first selenate respiring organism. Mistaken as a novel species belonging to the genus of *Pseudomonas*, this organism was later classified as *Thauera selenatis* (Macy et al. 1993). It is a facultative anaerobe and a member of the Beta-proteobacteria (Table 1.4). In addition to selenate, it also respired nitrate and oxygen (Macy et al. 1993).

Since the first description of this selenate respiring organism in 1989, many other microorganisms have been isolated that were capable of selenate respiration (Table 1.4). *Sulfurihydrogenibium subterraneum*, *Bacillus selenitireducens*, and *Pyrobaculum aerophilum* were found to respire selenite (Switzer Blum *e*t al. 1998; Huber et al. 2000; Takai et al. 2003).

Dissimilatory As(V) reducing bacteria were a more recent discovery. *Sulfurospirillum arsenophilus* strain MIT-13, *Sulfurospirillum barnesii* strain SES-3, *Chrysiogenes arsenatis* strain BAL-1; and *Desulfotomaculum auripigmentum* were the first four As(V) isolated and characterized As(V) respiring bacteria (Table 1.4) (Ahmann et al. 1994; Oremland et al. 1994; Laverman et al. 1995; Macy et al. 1996; Newman et al. 1997; Stolz et al. 1999). *D. auripigmentum* was the only Gram-positive of the four

strains, all others were Gram-negative. Even though the strains were metabolically diverse, they all reduced fumarate in addition to As(V). Strains MIT-13, SES, and BAL-1 respired both nitrate and arsenate but not sulfate, while *D. auripigmentum* also grew on sulfate but not nitrate (Newman et al. 1997). Additional arsenate respiring microorganisms have been isolated (Table 1.4).

The hyperthermophilic *Pyrobaculum arsenaticum* and *Pyrobaculum aerophilum* (Huber et al 2000) are the only two known Se(V) and As(V) respiring archaea. *P. arsenaticum* is a chemolithoautotroph that used CO_2 as carbon source. In addition to respiring selenate and arsenate, it could also utilize thiosulfate and sulfate. *P. aerophilum* is an organotroph that also respired oxygen, nitrate, nitrite, and thiosulfate.

Methylation and Demethylation.

Selenium. Biomethylation of selenium is an important source of atmospheric selenium and occur in a variety or soils, sediments, and even sludge samples (Reamer and Zoller 1980; Ranjard et al. 2003). Methylation of selenium compounds by microorganisms was recognized in the early part of the 20th century. Various researchers noticed a garlicky odor when animals were treated with selenium compounds. These were later identified as methylated forms of selenate and selenite. Challenger and North (1934) also showed that a fungal organism methylated selenide to DMeS.

Se-methylation has been attributed to many different microorganisms. Bacteria with these capabilities are grouped into two bacterial groups: the Proteobacteria (e.g *E. cloacae*, *Aeromons* sp., *Pseudomons* sp., *Rhodospirillum* spp.) and the Cytophagales (e.g. *Flavobacterium* sp.) (Ranjard et al. 2003; Chasteen and Bentley 2003) and can methylate both selenate and selenite to form volatile dimethyl selenide (DMSe), dimethyl

diselenide (DMDSe) (Reamer and Zoller 1980) and other methylated selenium species (Chasteen and Bentley 2003).

The gene *ubiE* encoding a methyl transferase present in *Geobacter stearothermophilus* not only mediated resistance to tellurium compounds, it was also responsible for the formation of DMeS and DMDSe (Swearingen et al. 2006). To demonstrate that the enzymes encoded by this gene conferred methylating capabilities, it cloned into *E. coli*. DMeS and DMDSe accumulated in the headspace (Swearingen et al. 2006).

Various microorganisms have been identified that are capable of metabolizing DMeS and DMeDS (Doran and Alexander 1977). In that study, the researchers demontstrated that *Corynebacterium* sp. and *Xanthomonas* sp. isolated from soil were able to grow on DMeDS as sole carbon source. Also, *Pseudomonas* spp. were able to metabolizing trimethyl selenium and DMeS (Doran and Alexander 1977).

Arsenic. Some microorganisms have been identified that are able to methylate arsenic. *Methanobacterium bryantii* was identified first in the early 1970s (McBride and Wolfe 1971). *Methanobacterium formicum*, *Clostridium collagenovorans*, *Desulforvibrio gigas*, *Desulfovibrio vulgaris* are also known to methylate arsenic (Michalke et al. 2000)

The work based on the fungus *Scopulariopsis brevicaulis* has led to the proposal of a arsenic methylation pathway now known as the “Challenger Mechanism” (Challenger 1945). This pathway described a series of steps in which arsenate reduction is preceded to oxidative addition of a methyl group, resulting in the formation of different methylated arsenic compounds, such as methyl and dimethyl arsenite, dimethyl arsenate and trimethyl arsine oxide.

Not much is known about demethylation of arsenic. The fungal species *S. bevicaulis* and *Penicillium notatum* were found to demethylate methylated arsenic

species to form trimethyl arsine (Challenger 1945; Lehr et al. 2003). Other species capable of demethylating methylated arsenic species include *Alcaligenes*, *Pseudomonas*, and *Mycobacterium* species (Bentley and Chasteen 2000; Lehr et al. 2003).

GOALS AND OBJECTIVES OF THIS STUDY

The overall goals of this study was to isolate novel As and Se respiring microorganisms and utilize the genome analysis for one of these novel organisms to provide a detailed insight into its energy metabolism and understand the genes and pathways involved in arsenate, selenate, and nitrate respiration.

There were three main objectives set forth for this thesis. The first objective was to isolate and characterize the physiologies and metabolisms of the novel As and Se respiring bacteria. The second objective was to analyze the genome of *D, indicum* strain S5, identify the genes involved in its respiratory metabolism, and evaluate the gene expression and regulation of molybdoenzyme oxidoreductases respiration of arsenate, selenate, and nitrate. The last objective was to extend the knowledge of methods employed to isolate Se and As respiring microorganisms to a second chalcogen, Tellurium, and isolate a Te oxyanion-respiring bacteria.

The following research questions provided the main goals of this study:

1. Can we isolate novel selenate and arsenate respiring bacteria and add to the phylogenetic diversity of this group of organisms?
2. What are the metabolic and physiological characteristics of the novel bacteria, strain $S4^T$ and *D. indicum* strain $S5^T$?

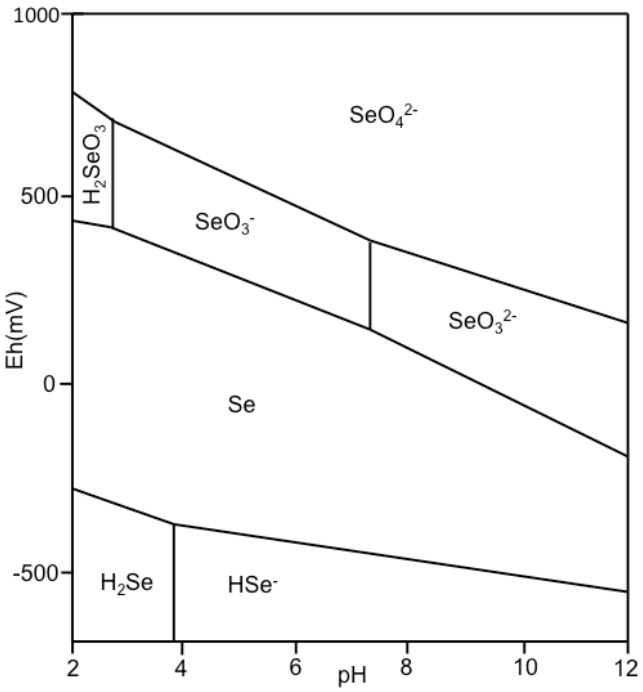
3. What are the genes and enzymes involved in the respiratory capabilities of *D. indicum* strain S5
4. What are the expression patterns of the molybdoenzymes identified in *D. indicum* strain S5?
5. How is selenate and arsenate respiration in strain S5 influenced by alternate electron acceptors?

We hypothesize that the presence of structurally and functionally conserved molybdoenzymes provides metabolic versatility among phylogenetically diverse Se and As respiring bacteria. The expression of these reductase enzymes is influenced by the presence and absence of electron acceptors altering the rate of mobilization and speciation of arsenic and selenium oxyanions in the environment.

Figure 1.1 Bacterial cultures with precipitated amourphous red selenium in the top and black vitrous selenium in the bottom tube.

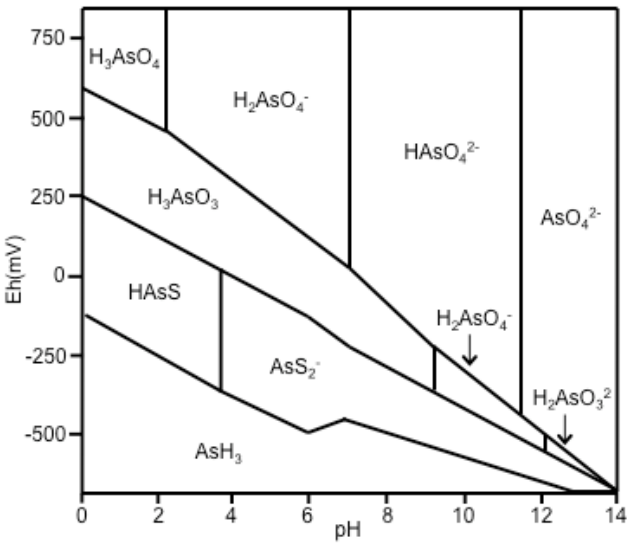
Figure 1.2 Effect of pH and Eh in the environment, at 25°C and 1bar atmospheric pressure, in the speciation of a) Selenium[1] and b) Arsenic[2].

a)



[1]Redrawn from Masscheleyn and Patrick 1993

b)



[2]Adapted from Ferguson and Gavis 1972

Figure 1.3 General structure of a DMSO reductase (adapted from Lowe et al. 2010)

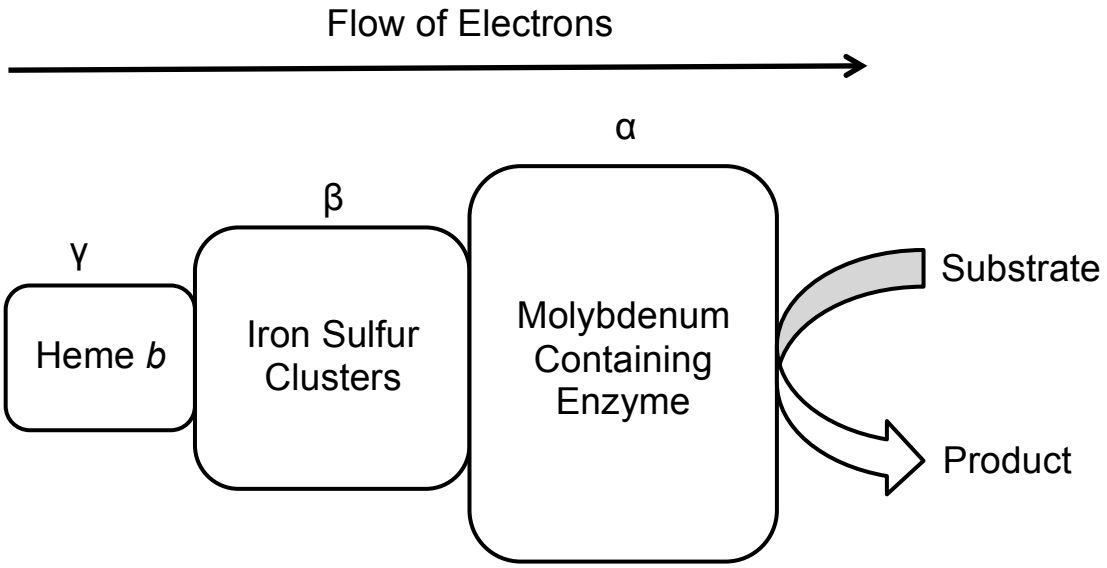


Figure 1.4 Schematic comparison of the signaling regions of the Sec and Tat systems. The location of the conserved signal sequence is indicated for Tat.

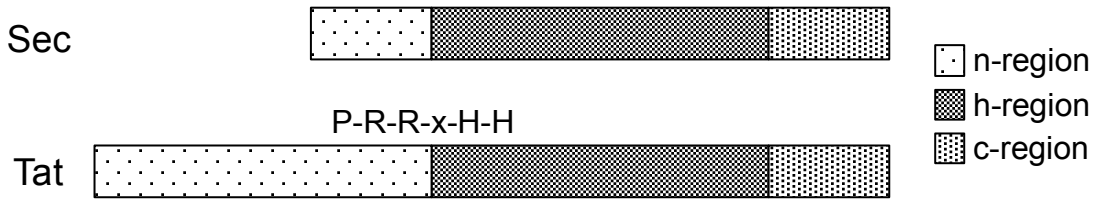


Figure 1.5 Examples of Tat signal peptide n-region consensus sequences S/T-RR-X-FLK or φ-RR-x-θ-θ (φ stands for polar and θ for non-polar residues) found in different species. The consensus sequences are in bold-type face. (NarG, catalytic subunit of the nitrate reductase; ArrA, catalytic subunit of respiratory arsenate reductase; Bis, biotin sulfoxide reductase; YnfE, putative selenate reductase; Psr, polysufide reductase; SerA, catalytic subunit of selenate reductase; Chr, chlorate reductase).

	S/T-RR-x-FLK or **φ-RR-x-θ-θ**
NarG *Pyrobaculum aerophilum*	LKT**TRR**R**ML**AGVATISAA
NarG *Haloferax mediterranei*	SGV**SRR**T**FL**EGIGVASLL
NapA *Escherichia coli*	MKL**SRR**S**FMK**ANAVAAAA
NapA *Shewanella* sp. ANA-3	MSI**SRR**E**FLK**ANAAVAAA
ArrA *Chrysiogenes arsenatis*	MKI**KRR**E**FLK**ASAAVGAV
Bis *Rhodobacter sphaeroides*	--M**NRR**D**FLK**GIASSSFV
YnfE *Escherichia coli*	VGI**SRR**T**LVK**STAIGSLA
Psr *Shewanella oneidensis* MR-1	IEL**NRR**T**FLK**GAGASGAT
SerA *Thauera selenatis*	DGNG**RR**R**FL**QFSMAALAS
Chr *Ideonella dechloratans*	EHNG**RR**R**FL**QFSAAALAS

Figure 1.6 Schematic of bacterial anaerobic respiration.

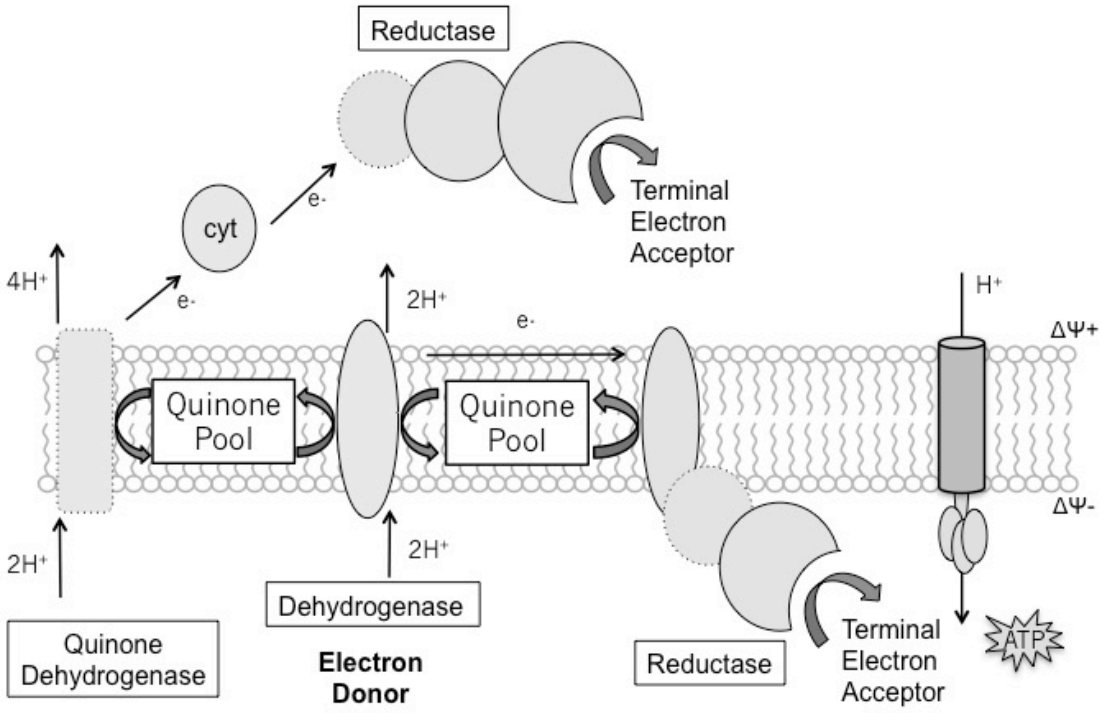


Table 1.1 Sources of a) Selenium and b) Arsenic Oxyanions in the Environment[3,4]

a)

Sources of Se	Se Concentration
Earth's crust	0.2 µg/g
Rain Water	0.003 µg/L
Ocean Water	0.01 µg/L
Surface and Groundwater	0.1 µg/L
River Water	up to 2.4 µg/L
Coal	0.1 – 0.2 µg/g
Seleniferous soils	1 - 10 mg/g
Carbonaceous Shale	0 - 150 µg/g

[3]Information from Wilber 1980; Lemly 1985; Naftz and Rice 1989; and Valentine 1997

b)

Sources of As	As Concentration
Earth's crust	2-5 µg/g
Seawater	1.5 to 5 µg/L
Freshwater	1.7 µg/L
Shales	13 µg/g
Igneous Rock	1.8 µg/g
Sandstone	1 µg/g
Sedimentary Rocks	0.1 – 2900 µg/g
Soil	0.1 - 40ug/g
Air - Northern Hemisphere	0.003 µg/m^3
- Southern Hemisphere	0.001 µg/m^3

[4]Information from Tamaki and Frankenberger 1992

Table 1.2 Overview of Global Groundwater Contamination of As

Continent	Country/Region	Potential Exposed Population	Concentration (µg/Liter)	Environmental Source
Asia	Bangladesh	30,000,000	<1 to > 2,500	Natural; river sediments
	West Bengal, India	6,000,000	<10 to 3,200	Natural; river sediments
	Nepal	3,500,000	10 to >3,000	Natural; rocks, river sediments
	Vietnam	>1,000,000	1 to 3,050	Natural; river sediments
	Thailand	15,000	1 to 5,000	Anthropogenic; mining and dredged rivers
	Taiwan	100,000 to 200,000	10 to 1,820	Natural; coastal zones, black shales
	Inner Mongolia	100,000 to 600,000	<1 to 2,400	Natural; river and lake sediments, alkaline
	Guizhou Province, China	~ 50,000	-	Coal, Air Inhalation, Food
	Xinjiang Province, China	~ 230,000	100 to 1900	Natural; river sediments
Europe	Hungary	450,000	<1 to 180	Natural; river sediments
	Spain	>50,000	<1 to 100	Natural; river sediments
	Cornwall, UK			
	Greece	150,000	-	Natural and anthropogenic; thermal springs and mining
South America	Argentina	~ 2,000,000	<1 to 9,900	Natural; volcanic rocks, thermal springs, sediments; highly alkaline area
	Chile	~ 1,800,000	10 to 1,000	Natural and anthropogenic; volcanic sediments; lakes, thermal springs, mining
	Bolivia	50,000	10 to 970	Natural; similar to Chile and Argentina
	Brazil	-	0.4 to 350	Gold mining
	Mexico	400,000	8 to 740	Natural and anthropogenic; volcanic rocks, mining
North America	USA West/Southwest	13,000,000	<1 to 2000	Natural and anthropogenic; mining, pesticides, thermal springs, river sediments,

Table 1.3 Comparison of approximate Gibbs free energies (ΔG^0 in kJ mol^{-1}) of common electron acceptors.

Electron Acceptors	Reaction	ΔG^0 (kJ mol^{-1})
O_2	1/4 O_2 (g) + 1/2 H_2 → 1/2 H_2O	-28.4
NO_3	1/5 NO_3^- + 1/5 H^++ ½ H_2 → 1/10 N_2 (g) + 3/5 H_2O	-25.5
SeO_4^{2-}	1/2 SeO_4^{2-} + 1/2 H+ + 1/2 H_2 → 1/2 $HSeO_3^-$ + 3/5 H_2O	-23.9
MnO_2	1/2 MnO_2 (s) + H^+ + 1/2 H_2 → 1/2 Mn^{2+} + H_2O	-18.2
NO_3	1/8 NO_3^- + 1/4 H^+ + 1/2 H_2 → 1/8 NH_4^+ + 3/8 H_2O	-17
$HSeO_3^-$	1/4 $HSeO_3^-$ + 2 H^+ + 1/2 H2 → 1/4 Se^0 + 1/2 H_2O	-13.9
$Fe(OH)_3$	$Fe(OH)_3$ (am) + 2 H^+ + 1/2 H2→ Fe^{2+} + 3 H_2O	-12.4
$H_2AsO_4^-$	1/2 $H_2AsO_4^{2-}$ + 1/2 H^+ + 1/2 H_2 → 1/2 $H_2AsO_3^-$ + 1/2 H_2O	-9.5
SO_4^{2-} → HS^-	1/8 SO_4^{2-} + 1/8 H^+ + 1/2 H_2→ 1/8 HS^- + 1/2 H_2O	-4.3

Table 1.4 Diversity of Selenate and Arsenate Respiring Organisms

	Organisms	Phylogeny	Terminal Electron Acceptors	Environment	Reference
Se Respiring Organisms	*Salana multivorans*	Firmicutes	Selenate, Oxygen	River Sediments, Germany	von Wintzingerode *et al.* 2001
	Selenihalanaerobacter shriftii	Halanaerobacteriales	Selenate, Nitrate	Dead Sea sediments	Blum et al. 2001
	Bordetella petrii	Betaproteobacteria	Selenate, Nitrate	River sediments, Germany	von Wintzingerode *et al.* 2001
	Pseudomonas stutzeri strain pn1	Betaproteobacteria	Selenate, Nitrate, Oxygen	Laboratory Contaminant, NJ	Narasingarao and Häggblom 2007b
	Thauera selenatis	Betaproteobacteria	Selenate, Nitrate, Oxygen	Kesterson Reservoir, CA	Macy *et al.* 1993
	Pelobacter seleniigenes	Deltaproteobacteria	Selenate, Fe(III), Nitrate, AQDS, Elemental Sulfur	Kearny Marsh, NJ	Narasingarao and Häggblom 2007a
	Aeromonas hydrophila	Gammaproteobacteria	Selenate, Nitrate, Fe(III), Co(III), Fumarate	Tin of Spoiled Milk	Knight and Blakemore 1998; Stolz and Oremland 1999
	Sedimenticola selenatireducens	Gammaproteobacteria	Selenate, Nitrate, Nitrite	Arthur Kill, NJ	Narasingarao and Häggblom, 2006

	Organisms	Phylogeny	Electron Acceptors	Environment	Reference
As Respiring Organisms	*Chrysiogenes arsenatis*	Chrysiogenes	Arsenate, Nitrate, Nitrite	Gold Mine, Australia	Macy *et al.* 1996
	Deferribacter desulfuricans	Deferribacteres	Arsenate, Nitrate, Elemental Sulfur	Hydrothermal Vent, Japan	Takai *et al.* 2003
	Alkaliphilus oremlandii	Firmicutes	Arsenate, Thiosulfate	River Sediments, PA	Fisher *et al.* 2008
	Desulfotomaculum auripigmentum	Firmicutes	Arsenate, Fumarate, Sulfate, Sulfite, Thiosulfate	Lake Sediments, MA	Newman *et al.* 1997
	Bacillus macyae	Firmicutes	Arsenate, Nitrate	Gold Mine, Australia	Santini *et al.* 2002; Santini *et al.* 2004
	Halarsenatibacter silvermanii	Halanaerobacteriales	Arsenate, Fe(III), Sulfur	Searles Lake, CA	Switzer Blum *et al.* 2009
	Marinobacter santoriniensis	Gammaproteobacteria	Arsenate, nitrate, oxidation of arsenite and Fe(II)	Hydrothermal vent, Greece	Handley et al. 2009
	Shewanella sp. ANA-3	Gammaproteobacteria	Arsenate, Nitrate, Thiosulfate, Fumarate, MnO2, $Fe(OH)_3$, Oxygen, AQDS	Estuarine Sediments, MA	Saltikov *et al.* 2003
	Sulfurospirillum arsenophilum	Epsilonproteobacteria	Arsenate, Nitrate, Nitrite, Elemental Sulfur, Thiosulfate, Oxygen (Microaerophlic)	Sediments, MA	Stolz *et al.* 1999

	Organisms	Phylogeny	Electron Acceptors	Environment	Reference
Se and As Respiring Organisms	*Sulfurihydrogenibium subterraneum*	Aquificales	Selenate, Selenite, Arsenate, Nitrate, Fe(III)	Subsurface Sediments, Japan	Takai *et al.* 2003
	Pyrobaculum aerophilum	Crenarchaeota	Selenate, Selenite, Arsenate, Nitrate, Nitrite, Oxygen (Microaerophilic)	Boiling Water Hole, Italy	Huber *et al.* 2000
	Pyrobaculum arsenaticum	Crenarchaeota	Selenate, Arsenate, Elemental Sulfur	Hot springs, Italy	Huber *et al.* 2000
	Bacillus arseniciselenatis	Firmicutes	Selenate, Arsenate, Nitrate, Fe(III), Fumarate,	Mono Lake, CA	Switzer Blum *et al.* 1998
	Bacillus selenitireducens	Firmicutes	Selenite, Arsenate, Nitrate, Nitrite, Fumarate, TMAO	Mono Lake, CA	Switzer Blum *et al.* 1998
	Desulfitobacterium hafniense	Firmicutes	Selenate, Arsenate, Nitrate, Elemental Sulfur, Sulfite, Thiosulfate, Fe(III), Fe pyrophosphate, Fumarate, MnO2	Stream Sediments, MI	Christiansen and Ahring *et al.* 1996; Niggemeyer *et al.* 2001
	Desulfitobacterium frappieri	Firmicutes	Selenate, Arsenate, Nitrate Elemental Sulfur, Sulfite, Thiosulfate, Fe(III), Fe pyrophosphate, Fumarate, MnO_2	Sludge/Soil Samples, Canada	Bouchard *et al.* 1996; Niggemeyer *et al.* 2001
	Bacillus selenatarsenatis	Firmicutes	Selenate, Arsenate, Nitrate, Oxygen	Effluent Drain, Japan	Yamamura *et al.* 2007
	Wolinella succinogenes R-1	Epsilonproteobacteria	Selenate, Arsenate, Nitrate, Fumarate	Rumen Fluid	Wolin *et al.* 1961; Tomei *et al.* 1992
	Sulfurospirillum barnesii	Epsilonproteobacteria	Selenate, Arsenate, Nitrate, Nitrite, Elemental Sulfur, Thiosulfate, Fe(III), Trimethylamine oxide (TMAO), Oxygen	Selenium Contaminated Freshwater Marsh	Stolz *et al.* 1999
	Sulfurospirillum halorespirans	Epsilonproteobacteria	Selenate, Arsenate, Nitrate, Nitrite, Elemental Sulfur, Fumarate, Perchloroethylene (PCE), Oxygen (Microaerophilic)	PCE Contaminated Soil, The Netherlands	Luijten *et al.* 2003

Table 1.5 Overview of characteristic features of respiratory arsenate reductases.

		Chrysiogenes arsenatis	*Shewanella* sp. strain ANA-3	*Bacillus selenitireducens* strain MLS10
ArrA	Size	~ 87-kDa	~ 95-kDa	~ 110-kDa
	Features	- Molybdenum containing subunit	- Molybdenum-containing subunit - Tat signal	- Molybdenum-containing subunit - Tat signal
ArrB	Size	~29-kDa	~27-kDa	~34-kDa
	Features	Fe-S clusters, Zinc	Fe-S clusters	Fe-S clusters
ArrAB	Localization	Periplasm	Periplasm	Membrane Associated
	K_m (uM)	300	5	34
	V_{max} (U/mg protein)	7,013	11,000	2.5
	Substrate Specificity	Arsenate Only	Arsenate Only	Arsenate, arsenite, selenate, selenite

CHAPTER 2

SELENOVIBRIO WOODRUFFII GEN. NOV., SP. NOV., A SELENATE RESPIRING BACTERIUM

Abstract

The selenate-respiring bacterium, strain S4^T, was isolated from activated sludge from a wastewater treatment plant after enrichment with 10 mM selenate as the sole electron acceptor. In addition to its selenate respiring capability, strain S4^T also respires arsenate with acetate as carbon source and electron acceptor. Fermentative growth was not observed. Phylogenetic analysis of the 16S rRNA gene revealed that strain S4^T is a new member of the Deferribacteraceae with *Denitrovibrio acetiphilus* as its closest cultivated relative with 91% sequence similarity. The 16S rRNA gene phylogeny, cellular fatty acid composition and metabolic capabilities of strain S4^T distinguish it from *D. acetiphilus and Geovibrio* species. Thus, we propose that strain S4^T represents a novel genus and species for which the name *Selenovibrio woodruffii* gen. nov., sp. nov. (Type strain S4^T = DSM 24984 = ATCC BAA-2290^T) is proposed.

During the process of microbial respiration, many different electron acceptors, including arsenate and selenate, can be used to generate cellular energy. Arsenic (As) and selenium (Se) are naturally occurring metalloids in the Earth's crust. The microbial communities in various environments governs their speciation, influencing their mobility in the soil, water, and air interface. Microorganisms that can utilize As and Se oxyanions as terminal electron acceptors in dissimilatory reduction are ubiquitous and phylogenetically diverse (e.g. Stolz et al. 2006; Narasingarao and Häggblom 2007a; Rauschenbach et al. 2011a).

Although microbial reduction of selenium oxyanions was recognized early (Levine 1924; Lipman and Waksman 1923) it was not until 1989 that dissimilatory selenate reduction was first reported in sediment slurries and a selenate respiring organism was isolated (Oremland et al. 1989). Se(VI) was sequentially reduced to selenite (SeIV) and insoluble elemental selenium, while energy was gained (Oremland et al. 1989). The microbial cycling of selenium (Se) oxyanions affects their speciation in various environments thus greatly influencing the biogeochemical cycle of this element in nature. *Sulfurospirillum arsenophilus* strain MIT-13, *Sulfurospirillum barnesii* strain SES-3, *Chrysiogenes arsenatis* strain BAL-1; and *Desulfotomaculum auripigmentum* were the first four As(V) isolated and characterized As(V) respiring bacteria (Ahmann et al. 1994; Oremland et al. 1994; Laverman et al. 1995; Macy et al. 1996; Newman et al. 1997; Stolz et al. 1999). These microorganisms reduced As(V) to As(III) while gaining energy. Here we describe strain S4^T, a novel microorganism that repires both Se(VI) and As(V) and that represents a novel genus and species in the family *Deferribacteriacae*.

Strain S4^T is a strictly anaerobic, dissimilatory selenate and arsenate respiring bacterium isolated from activated sludge, collected from a wastewater treatment plant located in Verona, NJ. The activated sludge sample was enriched in anaerobic minimal

salts medium as described in Fennell et al. 2004 with only 1.17g of salt added and with a combination of 10 mM acetate, lactate, pyruvate as carbon sources and electron donors and 10 mM selenate as electron acceptor under a headspace of N_2. The enrichment cultures were incubated at 28°C. The cultures showed a red precipitate, indicating the formation of elemental selenium, after two weeks. After sequential transfers into soft agar medium (0.4% Noble agar, Difco), a bacterium was isolated in pure culture, and designated strain $S4^T$. Purity of the culture was verified by microscopy and denaturating gradient gel electrophoresis analysis following the protocols described in Muyzer et al. (1993). An 8% acrylamide/bis gel with a 40 to 80 % gradient, which was exposed to 55 Volts for 17.5 hours, was used. The gel was stained in ethidium bromide. Strain $S4^T$ was maintained in minimal salts medium with 10 mM acetate and 10 mM selenate under a headspace of N_2.

Genomic DNA was isolated with phenol-chloroform extraction (Kerkhof and Ward 1999) with the following modifications: 3 mL of selenate respiring cultures of strain $S4^T$ were pelleted and 400 ng/µL of archaeal DNA (*Sulfolobus sulfataricus* strain P2) added to bind excess elemental selenium. The addition of proteinase K was omitted. The 16S rRNA gene was amplified with primers described in Narasingarao and Häggblom (2006) with universal primers 27F and 1535R. The PCR products were sequenced by Genewiz, Inc. (South Plainfield, NJ, USA). The sequence data was manually compiled to obtain a near complete 16S rRNA gene sequence of 1474 bp. This was used to identify related microorganisms using BLAST (Altschul et al.1997). Phylogenetic and molecular evolutionary analyses of selected sequences were conducted using MEGA5 (Tamura et al. 2011), utilizing ClustalW (Chenna et al. 2003) to align the sequences. Various phylogenetic trees were constructed using neighbor-joining, maximum composite, and minimum evolutionary methods (Figure 2.1). The evolutionary distances were computed

using the Maximum Composite Likelihood method with 1000 bootstrap replications (Tamura et al. 2011). The final dataset of the alignment contained 1309 nucleotide positions. Phylogenetic comparison of the 16S rRNA gene sequence revealed that strain S4^T grouped within the family *Deferribacteracaea*. This was supported by high bootstrap values in all phylogenetic tree analyses (Figure 2.1).

There are currently ten described species in the family *Deferribacteraceae*. A *Geovibrio* sp. belonging to this family was also isolated for its ability to respire selenate (Narasingarao and Häggblom 2007a). Strain S4^T was most closely related to *Denitrovibrio acetiphilus* N2460^T (Myhr and Torsvik 2000) with 91% 16S rRNA gene similarity. Other close relatives are *Geovibrio ferrireducens* (86% similarity) (Caccavo et al. 1996) and *Geovibrio thiophilus* (86% similarity) (Janssen et al. 2002). All further physiological and metabolic characterizations of strain S4^T were made in direct comparison to *D. acetiphilus* (Myhr and Torsvik 2000), and in comparison, using published data, to *Geovibrio thiophilus* (Janssen et al. 2002) and *Geovibrio ferrireducens* (Caccavo et al. 1996).

Strain S4^T was grown with 10 mM acetate and 10 mM selenate to examine the cell morphology with transmission electron and light microscopy. Strain S4^T is a Gram negative, highly motile organism. It is a vibrio, approximately 1 to 2 μm long and approximately 0.15 μm wide (Figure 2.2a, b). It has one polar flagellum that was visualized by flagella staining (Hardy Diagnostics, Inc.) and transmission electron microscopy (Figure 2.2c). When grown in anaerobic, soft agar shake tubes (Difco) with 10 mM acetate as carbon source and 10 mM selenate as electron acceptor, bright red colonies developed from the formation of elemental selenium (Figure 2.3).

A range of electron donors and acceptors were used to characterize the metabolic capabilities of strain S4^T (Table 2.1). The utilization of 10 mM each of lactate, acetate, succinate, pyruvate, glucose, propionate, fumarate; and 0.5 g/L yeast extract as carbon sources were tested. Strain S4^T was able to utilize either 10mM selenate or arsenate as electron acceptors, but did not grow with either nitrate, nitrite selenite, arsenite, thiosulfate, sulfate, Fe(III) citrate, DMSO, tellurite or oxygen. Cultures were scored positive for growth after three consecutive transfers. Negative results were evaluated after three inoculations with no growth.

The reduction of selenate to elemental selenium and arsenate to arsenite was analyzed using ion chromatography (IC) (DX 120; Dionex, Sunnyvale, CA) as previously described (Knight et al. 2002). Oxidation of acetate was measured using HPLC (Shimadzu) with a C_{18} column (250 mm by 4.6 mm, 5 μm particle size; Phenomenex Sphereclone) as described in Narasingarao and Häggblom (2007a). Strain S4^T was able to respire selenate and arsenate with acetate as the carbon source and electron donor. Strain S4^T utilized approximately 6 mM of acetate and 6.3 mM selenate within 24 hours (Figure 2.4), which was reduced to elemental selenium within 24 hours. No fermentative growth was observed. Growth of *D. acetiphilus* with acetate as electron donor and 2.5 mM each arsenate, arsenite, selenate, selenite, nitrite as electron acceptor was also determined. In contrast to strain S4^T, *D. acetipihilus* only respired nitrate to ammonium with acetate as carbon source and could not utilize selenite or nitrate. No other electron donors or electron acceptors were utilized for respiration (Table 2.1). *D. acetiphilus* was also capable of fumarate fermentation (Myhr and Torsvik 2000). The generation time for S4^T grown on acetate and selenate was 113 ± 6.7 minutes, determined by monitoring bacterial cell counts using a Petroff Hauser Chamber.

In order to test for optimum pH, NaCl concentrations and temperature, strain S4^T was grown with 10 mM selenate as electron acceptor and 10 mM acetate as carbon source. The pH of the medium was adjusted with HCl and NaOH to a range between 6.6 and 7.4. Strain S4^T grew at a pH range between 6.8 and 7.4, with an optimum pH at 7.0 (Table 2.1). NaCl concentrations were tested between 0 and 100 mM and temperature ranges between 4 to 37 °C. Strain S4^T could grow with salt concentrations between 0 and 100 mM, with an optimum salt concentration at 20 mM, and at temperatures between 20 and 37 °C. The pH, salt and temperature requirements were similar for *D. acetiphilus*, which grew best at a pH between 6.5 and 8.6, with a salt concentration of 0 to 100 mM, and at an optimum temperature between 35 and 37 °C (Table 2.1).

The fatty acid content of strain S4^T was analyzed from cells grown for 24 hours at 37°C with 10 mM acetate and 10 mM selenate and compared to *D. acetiphilus* grown with acetate and nitrate. Cellular fatty acids were methylated and analyzed as previously described (Narasingarao and Häggblom 2006) using an HP 5890 series II gas chromatograph (Hewlett Packard, Palo Alto, CA) with a SHERLOCK Microbial Identification System (MIDI, Inc., Newark, Del.). The identities of the fatty acid methyl esters detected by the MIDI system were confirmed by GC Mass Spectrometry (Agilent GC Series 6890 GC-MS). The fatty acid content of strain S4^T was substantially different from *D. acetiphilus* (Table 2.2). The main fatty acids, about 90%, present in S4^T were the straight-chain fatty acids $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{17:0}$, and $C_{18:0}$. These fatty acids were also detected in *D. acetiphilus*, but in lower amounts, about 50%. The fatty acid $C_{16:0}$ was the most abundant in both strain S4^T (48.7%) and *D. acetiphilus* (28.8%). Another major difference in the fatty acid composition between the species was the abundance (about 35%) of unsaturated fatty acids, $C_{18:1\ \omega 9c}$ and $C_{18:1\ \omega 7c}$, in *D. acetiphilus*, while present at

only approximately 10% in Strain S4^T.

Description of *Selenovibrio* gen. nov. (Se.le.no.vi'bri.o. L.; L.m. *selenium* Selenium; N.L. masc. n. *vibrio* a small curved rod, vibrio; M. L. neut. n. *Selenovibrio*, a vibrio that reduces selenate.)

Gram-negative, motile vibrio. Obligately anaerobic with respiratory metabolism. Utilizes selenate and arsenate as electron donors, and acetate as carbon source and electron donor. Heterotrophic growth only. mesophilic and neutrophilic. Belongs to the family of *Deferribacteriacaea*. The type species is *S. woodruffii*.

Description of *Selenovibrio woodruffii* sp. nov.

Selenovibrio woodruffii (wood.ru'f.fi.i N.L. gen.n. *woodruffii* of Woodruff, named in honor of Dr. H. Boyd Woodruff, a Rutgers University alumnus, for his lifetime dedicated to the advancement of science and his contributions to soil and microbiology and the discovery of natural products important to human and animal health and agriculture). Gram-negative, motile bacterium, curved rod. Obligately anaerobic with respiratory metabolism. Non-fermenting. Respires selenate and arsenate coupled to oxidation of acetate. Mesophilic and neutrophilic with an optimum growth temperature range between 28 °C and 37 °C and optimum pH at 7, respectively. Growth in 0 to100 mM NaCl, with an optimum salt concentration at 20 mM. The predominant fatty acids are saturated fatty acids, $C_{14:0}$ and $C_{16:0}$.

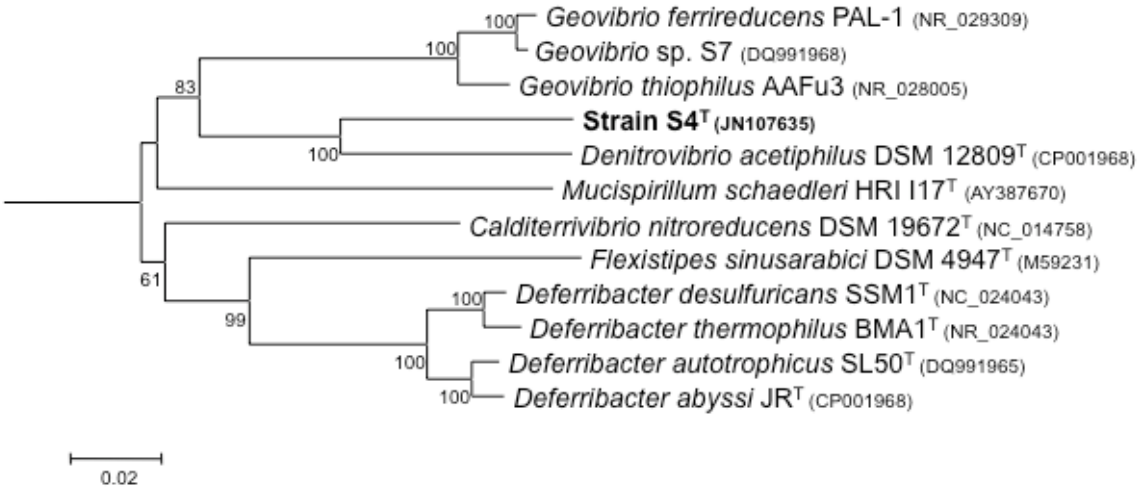
The type strain S4^T (DSM = 24984 = ATCC BAA-2290^T) was isolated for its ability to respire selenate to elemental selenium isolated from activated sludge samples collected from a wastewater treatment facility located in Verona, New Jersey, USA. The Accession Number of the GenBank 16S rRNA gene sequence is JN107635.

Acknowledgements

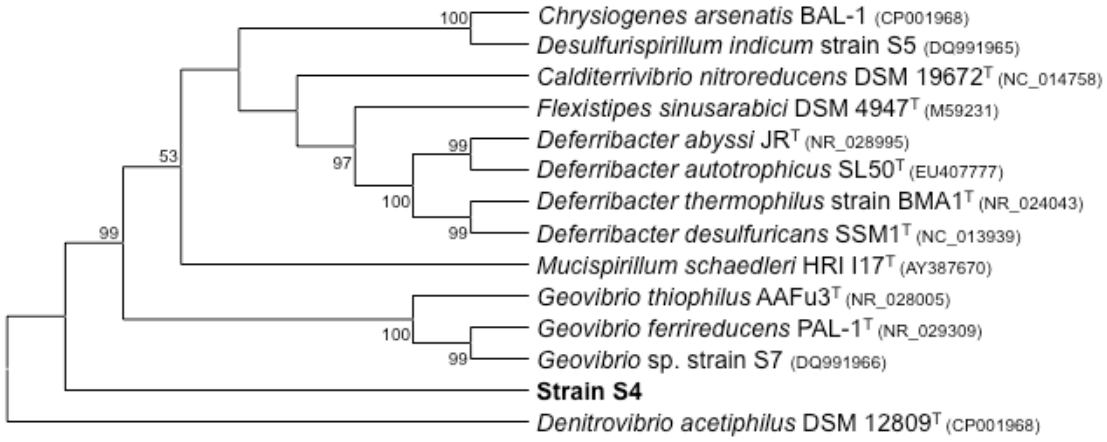
We thank Jessica McCormick for supplying the activated sludge sample and Sharron Crane for help with DGGE analysis. This study was supported in part by a grant from the National Science Foundation (EAR 084329530). IR was the recipient of a H. Boyd and Jeanette I.Woodruff Graduate Fellowship in Microbiology.

Figure 2.1 Phylogenetic trees comparing Strain S4^T with closely related species. a) Neighbor-joining b) Maximum Parsimony and c) Minimum Evolution method. The trees were constructed with aligned 16S rRNA gene sequences. The evolutionary distances were computed using Maximum Composite Likelihood method and bootstrap values above 50 are indicated. There were a total of 1309 positions in the final dataset. *Desulfurispirillum indicum and Chrysiogenes arsenatis* were used as outgroup.

a)



b)



c)

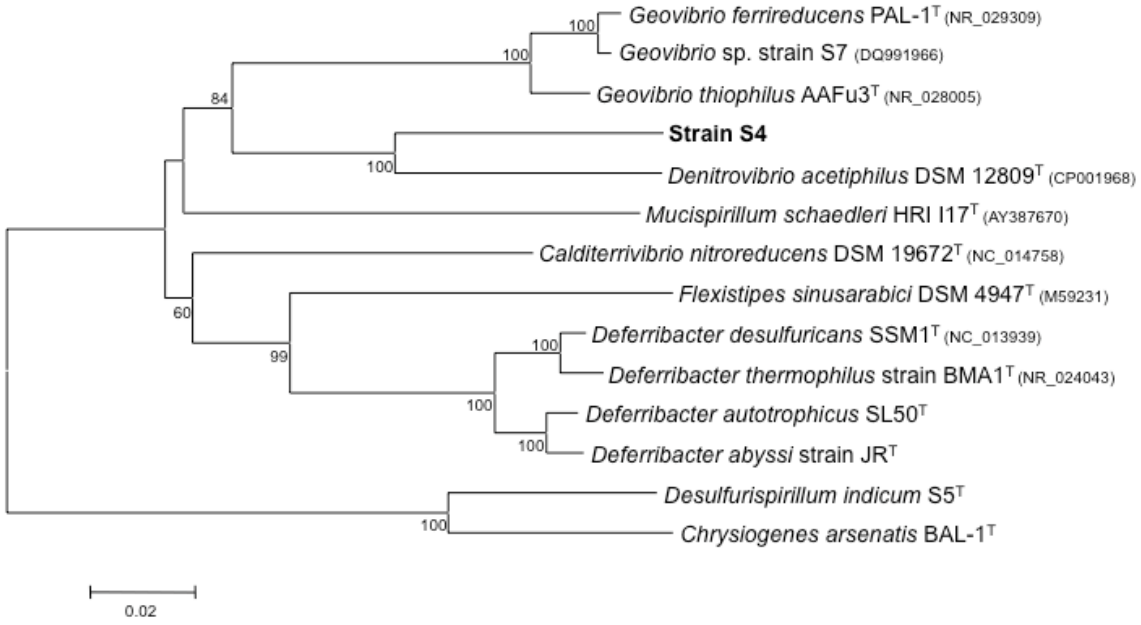

Geovibrio ferrireducens PAL-1T (NR_029309)
Geovibrio sp. strain S7 (DQ991966)
Geovibrio thiophilus AAFu3T (NR_028005)
Strain S4
Denitrovibrio acetiphilus DSM 12809T (CP001968)
Mucispirillum schaedleri HRI I17T (AY387670)
Calditerrivibrio nitroreducens DSM 19672T (NC_014758)
Flexistipes sinusarabici DSM 4947T (M59231)
Deferribacter desulfuricans SSM1T (NC_013939)
Deferribacter thermophilus strain BMA1T (NR_024043)
Deferribacter autotrophicus SL50T
Deferribacter abyssi strain JRT
Desulfurispirillum indicum S5T
Chrysiogenes arsenatis BAL-1T
100
100
84
100
60
99
100
100
100
100
100
0.02

Figure 2.2 Cell morphology of Strain S4^T grown on selenate for 24 hours. a) Transmission electron micrograph with intracellular deposits of elemental selenium granules. Bar, 0.2 µm. b) Phase-contrast micrographs of cells. Bar, 10 µm. c) Transmission electron micrograph strain S4^T with single polar flagellum (Palladium shadow at 30% angle). Bar, 1 µm.

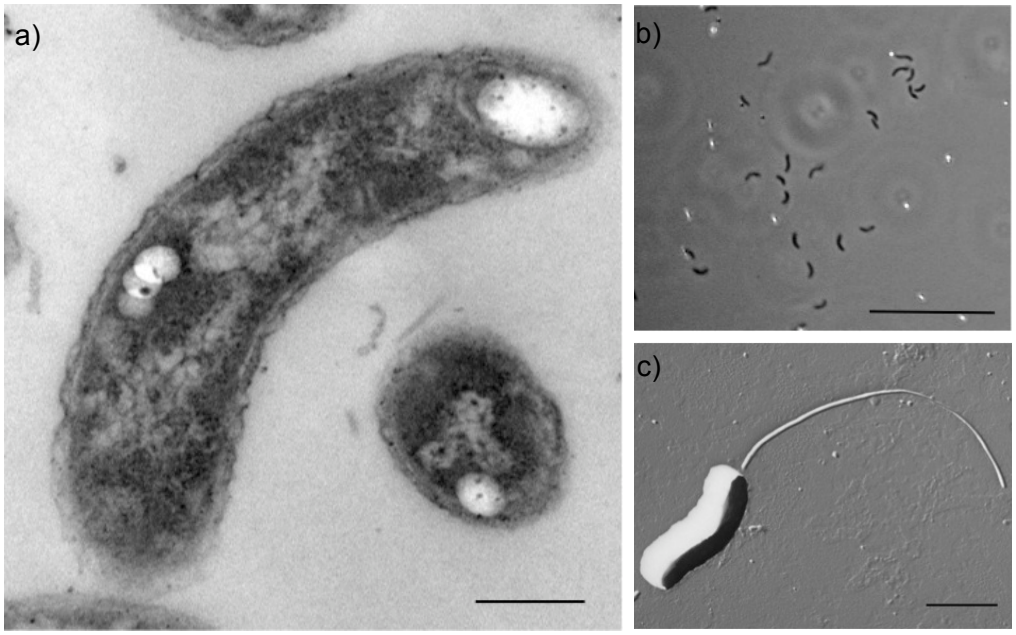


Figure 2.3 Picture of strain S4^T grown in soft agar shake tubes. Visible red colonies indicate the precipitation of elemental selenium.

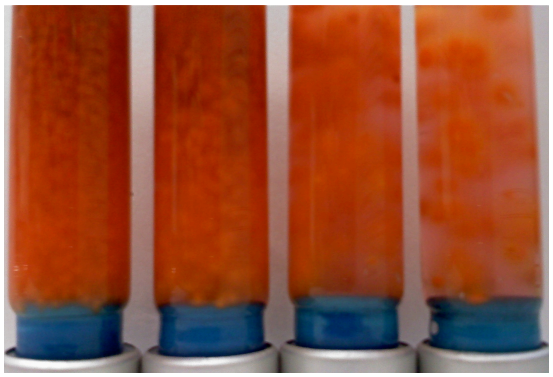


Figure 2.4 Growth of strain S4^T during selenate and arsenate respiration (◆ Se(VI), ■As(V), ▲ No Electron Acceptor)

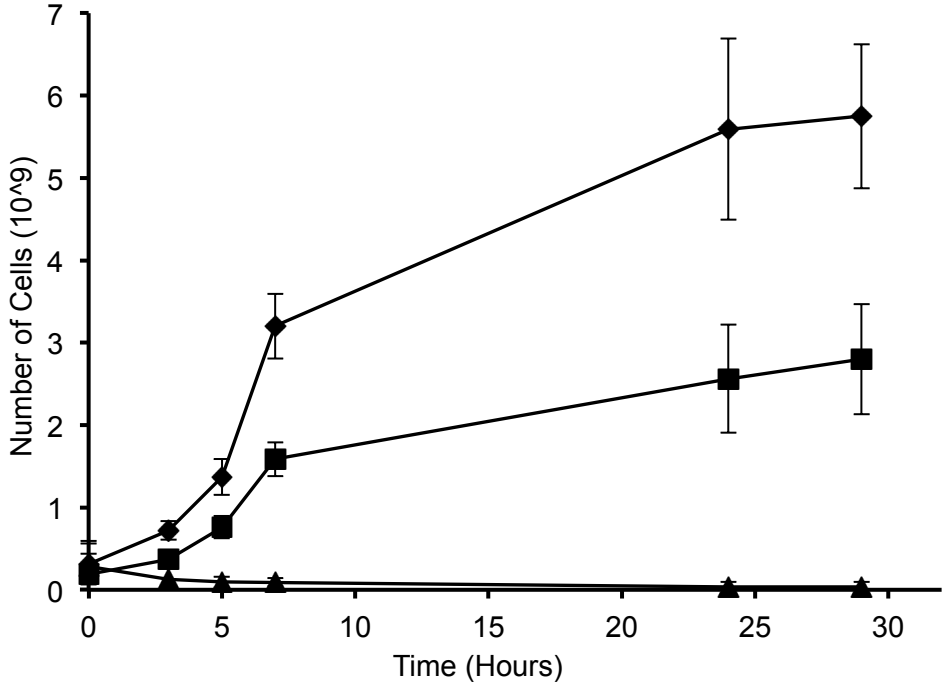
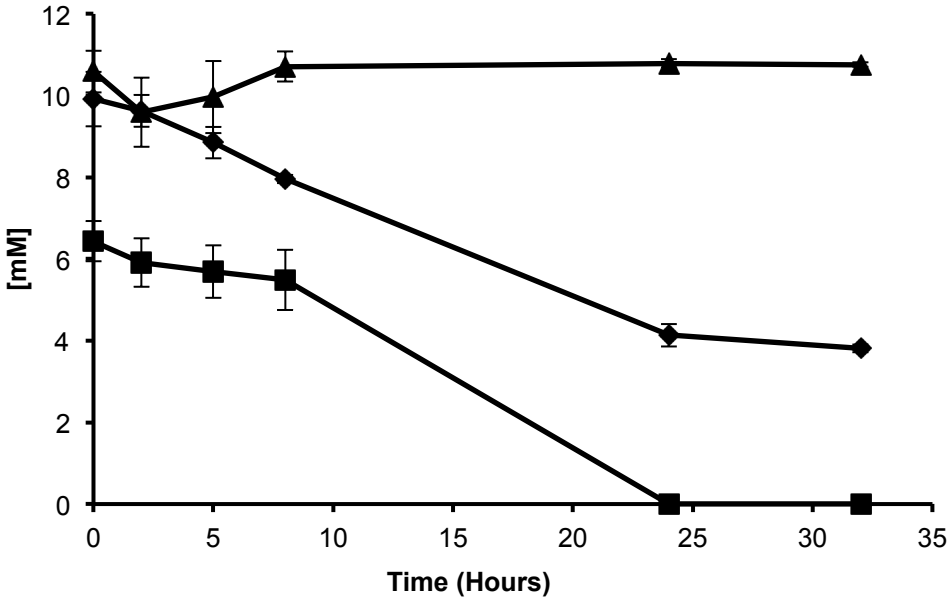


Figure 2.5 Respiration of a) selenate in the presence of acetate (■ Se(VI); ◆ Acetate (with Se(VI)); ▲ No Electron Acceptor) and b) arsenate in the presence of acetate by strain S4^{T} (■ Se(VI); ◆ Acetate (with As(V)); ▲No Electron Acceptor).

a)



b)

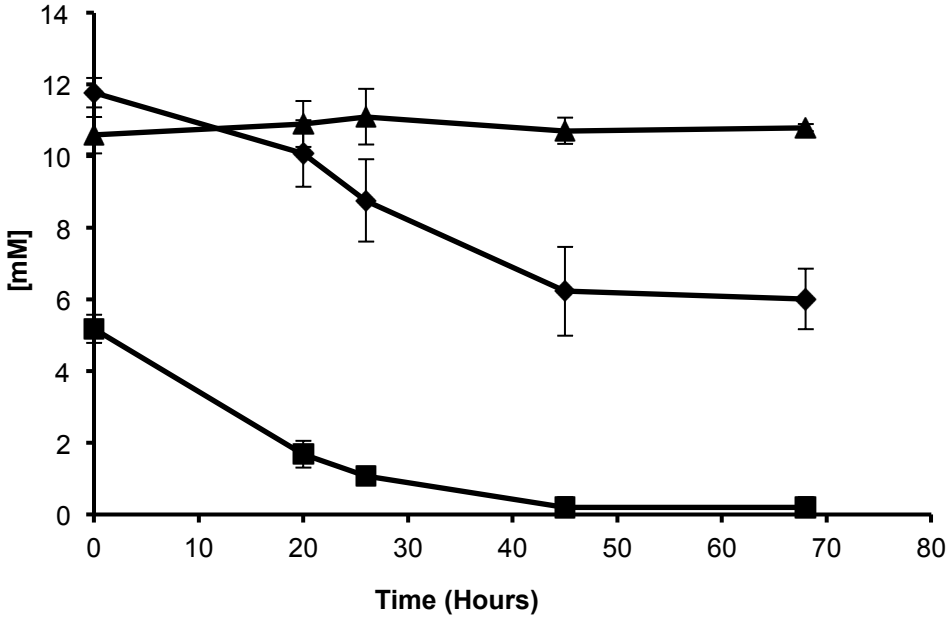


Table 2.1 Characterization of a) physiologies and b) metabolisms of strain S4^T and related organisms (ND, not determined; *, this study.

a)

Characteristics	Strain S4^T	*Denitrovibrio acetiphilus*	*Geovibrio ferrireducens*	*Geovibrio thiophilus*
Environment	Sludge, wastewater treatment plant, NJ	Oil reservoir model column	Sediment of hydrocarbon-contaminated ditch, USA	Sediments from Koblenz, Germany
Gram Reaction	Negative	Negative	Negative	Negative
Morphology	Vibrio	Vibrio	Vibrio	Spirillum
Motility	Motile	Motile	Motile	Motile
Cell Length (μm)	1 – 2	1.7 – 2	2 – 3	2.5 – 6
Cell Diameter (μm)	0.15	0.5-0.7	0.5	0.35
DNA G+C content (mol%)	ND	42.6	42.8	50.2
Growth Temperature (°C)				
Range (Optimum)	20 – 37 (28-37)	4 – 40 (35-37)	35	4 – 40 (37 and 40)
pH Range for Growth				
Range (Optimum)	6.8 – 7.4 (7)	6.5 – 8.6	Neutral	Neutral
NaCl concentration (M Na+)				
Range (Optimum)	0 – 0.1 (0.02)	0 – 1	0.09 – 0.34	0.02 – 0.34

b)

Metabolism	Strain S4^T	*Denitrovibrio acetiphilus*	*Geovibrio ferrireducens*	*Geovibrio thiophilus*
Fermentation	No	Fumurate Only	-	+
Electron Donors				
Lactate	-	-	+	-
Acetate	+	+	+	+
Succinate	-	ND	+	-
Pyruvate	-	-	+	-
Glucose	-	-	-	-
Propionate	-	-	+	-
Fumarate	-	-	+	+
H_2	-	-*	+	+
Yeast Extract	-	-	+	ND
Electron Acceptors				
Oxygen	-	-	-	Microaerophilic
DMSO	-	-*	ND	+
Nitrate	-	+ (to Ammonia)	-	+
Nitrite	-	-*	ND	-
Sulfate	-	-	-	-
Thiosulfate	-	-	ND	ND
Sulfur	-	-	+	+
Selenate	+	-*	ND	ND
Selenite	-	-*	ND	ND
Arsenate	+	-*	ND	ND
Arsenite	-	-*		
Tellurite	-	-*	ND	ND
Fe(III)	-	-*	+	-

Table 2.2 Cellular fatty acid composition of strain S4^T (1) and *Denitrovibrio acetiphilus* N2460^T (2). Both Strain S4^T and *D. acetiphilus* were grown at 37°C (ND, Not Determined).

Fatty Acids	**1**	**2**
$C_{14:0}$	30.8 ± 2.3	4.2 ± 0.9
$C_{15:0}$	3.2 ± 0.2	1.6 ± 0.61
$C_{15:0\ ANTEISO}$	0.9 ± 0.5	ND
$C_{16:1\ \omega 9c}$	0.7 ± 0.3	5.1 ± 1.3
$C_{16:1\ \omega 7c}$	1.1 ± 0.2	2.8 ± 0.9
$C_{16:0}$	48.7 ± 3.6	28.8 ± 4.6
$C_{17:0}$	1 ± 0.04	2.1 ± 1
$C_{18:1\ 1\omega 7c}$	3.5 ± 1.4	13.5 ± 4.6
$C_{18:1\ 1\omega 9c}$	2.5 ± 0.8	21.9 ± 6.6
$C_{18:0}$	6.4 ± 1.1	13.4 ± 3.8
Other	1.2	6.6

CHAPTER 3

DESULFURISPIRILLUM INDICUM SP. NOV., A SELENATE AND SELENITE RESPIRING BACTERIUM ISOLATED FROM AN ESTUARINE CANAL

Published in IJSEM (2011, 61:654-658)

ABSTRACT

Strain S5^T is a novel bacterium that was isolated for its capability to respire selenate to elemental Se. In addition to selenate respiration, it is also capable of dissimilatory selenite, arsenate, and nitrate reduction with organic acids, such as pyruvate, lactate and acetate as the carbon sources and electron donors. The isolate was unable to grow fermentatively. Strain S5^T was isolated from the sediment of an estuarine canal in Chennai, India. Phylogenetic analysis of the 16S rRNA gene of this novel isolate revealed that it belongs to the family *Chrysiogenaceae* with sequence similarities of 92 and 98 %, respectively, with the type strains of *Chrysiogenes arsenatis* and *Desulfurispirillum alkaliphilum*, its closest known relatives. Strain S5^T and *D. alkaliphilum* were closely related by 16S rRNA gene phylogeny; however they varied greatly in their genomic DNA G+C content (56 mol% versus 45 mol%) and cellular fatty acid compositions, as well as many metabolic capabilities. Strain S5^T represents a novel species for which the name *Desulfurispirillum indicum* sp. nov. is proposed; the type strain S5^T (= DSM2283^T =ATCC BAA-1389^T).

Introduction

Selenium (Se), a naturally occurring element, is essential for most organisms (Conde and Sanz Alaejos, 1997; Zannoni et al. 2007). Although considered to be a trace element, its toxicity is highly dependent on its oxidation state and speciation. Se occurs in a variety of oxidation states, water-soluble selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}), insoluble elemental selenium (Se0) and gaseous selenide. Even though redox transformations of selenium oxyanions can occur abiotically (Myneni et al. 1997; Kessi and Hanselmann, 2004), the global selenium cycle is greatly influenced by micro-organisms (Stolz et al. 2006).

Various microbial species have been found to not only reduce selenate and/or selenite to elemental Se, but to also respire selenate oxyanions in order to gain cellular energy (Oremland et al. 1989; Steinberg and Oremland 1990; Narasingarao and Haggblom, 2007b; Stolz and Oremland, 1999; Stolz et al. 2006). These selenium oxyanion-respiring microorganisms display a broad phylogenetic diversity and can be found among the phyla 'Proteobacteria', 'Deferribacteres', 'Firmicutes' and 'Actinobacteria' (Narasingarao and Häggblom, 2006, 2007a, b; Switzer Blum et al. 2001; von Wintzingerode et al. 2001). The ability of bacteria to use selenate and selenite and other alternative terminal electron acceptors, such arsenate, nitrate, sulfate, and/or iron plays a major role in the oxidation of organic carbon in sediments and contributes to the biogeochemical cycling of these elements in nature. In this study, we describe strain $S5^T$ that represents a novel member of the family Chrysiogenetes.

Strain $S5^T$ is a strictly anaerobic, dissimilatory selenate- and selenite-respiring bacterium isolated from an enrichment culture of sediments collected in an estuarine channel in Chepauk in Chennai, located in Southern India (Narasingarao and Häggblom 2007a). Strain $S5^T$ was isolated after sequential transfers into fresh anaerobic medium

with selenate as electron acceptor and pyruvate as carbon source and electron donor, Purity of the culture was verified with Terminal Restriction Fragment Length Polymorphism as described in Fennell et al. (2004). Strain $S5^T$ was maintained in an anaerobic minimal salts medium (Fennell et al. 2004) with 20 mM pyruvate as the carbon source and 10 mM selenate as the electron donor under a headspace of N_2.

Genomic DNA was extracted by phenol-chloroform extraction as previously described (Kerkhof and Ward 1993). The 16S rRNA gene was selectively PCR amplified from the genomic DNA as described in Narasingarao and Häggblom (2006). The PCR products were sequenced by Genewiz, Inc., South Plainfield, NJ, USA. Sequence data were compiled using Contig Express (Vector NTI Suite; Informax). The 16S rRNA gene sequences of related microorganisms were identified by BLAST search (Altschul et al. 1997) and downloaded from GenBank. Phylogenetic and molecular evolutionary analyses of all sequences were conducted using MEGA4 (Tamura et al. 2007), which utilized ClustalW (Chenna et al. 2003) to align the sequences. Neighbor-joining and maximum-parsimony phylogenetic trees with 1000 bootstrap replications were reconstructed using the program package MEGA4 with the maximum composite likelihood moel for nucleotide substitutions (Tamura et al. 2004). Comparative 16S rRNA gene sequence analysis places strain $S5^T$ within the family *Chrysiogenetes,* which, at the time of writing. had only two known members. *Chrysiogenes arsenatis,* the first species to be identified in this class, was isolated for its ability to respire arsenate to arsenite coupled to oxidation of acetate and other short chain fatty acids (Macy et al. 1996). *Desulfurispirillum alkaliphilum*, the second organism, is capable of reducing elemental sulfur to sulfide and nitrate to ammonium (Sorokin et al. 2007). The type strain of *D. alkaliphilum* only shared 91% 16S rRNA gene similarity with *C. arsenatis* and so it was placed with the novel genus *Desulfurispirillum* (Sorokin et al. 2007). The 16S rRNA

gene of strain S5^T shared imilarities of 94% and 98% respectively, with those of the type strains of *C. arsenatis* and *D. alkaliphilum.* Phylogenetic analysis with unambiguously aligned 16S rRNA gene sequences of strain S5^T and other related species (Figure 3.1) indicate that strain S5^T belongs to the family *Chrysiogenetes* and is most closely related to *D. alkaliphilum* and *C. arsenatis,* respectively. This was supported by high bootstrap values in both the neighbor-joining and maximum parsimony analyses. All further physiological and molecular characterizations of strain S5*T* were made in comparison to the two above-named organisms.

Strain S5^T was grown with selenate (10 mM) or nitrate (10 mM) as electron acceptors and pyruvate (20 mM) as carbon source to examine cell morphology via transmission electron microscopy (TEM) (Narasingarao and Häggblom, 2006) and phase contrast microscopy. Strain S5^T is a Gram negative, slender, spiral-shaped bacterium. It is approximately 2 to 7 µm long and 0.1 to 0.15 µm in diameter (Table 3.1, Figure 3.2a, b). Cells grown in the presence of selenate produce a bright red precipitate accumulating in the bottom of the culture tube. The precipitate was further evaluated and confirmed as elemental Se by X-ray absorption near edge structure analysis (XANES, Narasingarao and Häggblom 2007a). Strain S5^T grew both in liquid medium and soft agar shake tubes. It did not form distinct colonies when grown anaerobically in soft agar (0.4% Noble Agar; Difco) shake tubes with 10 mM selenate or 10 mM selenite. The agar first turned completely red and then black over a period of 14 days as the Se oxyanions were reduced to elemental Se.

Rapid motility was observed under the microscope, thus a flagella stain was applied to visualize the presence of flagella (Kodaka et al. 1982). One drop of a liquid culture of strain S5^T was applied to a clean glass slide and allowed to completely air dry.

The flagella stain was then applied for 10 minutes before being washed off with deionized water. Bipolar flagella were observed that extended about 4 to 6 μm at either end of the cell and allowed for rapid movement of strain $S5^T$ (Figure 3.2b).

A wide range of electron donors and acceptors were used to characterize the metabolic capabilities of strain $S5^T$ (Table 3.1). The Se oxyanions, nitrate, and arsenate were analyzed using ion chromatography (Dionex model ICS 1000; Dionex, Sunnyvale, CA) with an AS9-HC column (Dionex) and an eluent of 11.4 mM $NaHCO_3$, with a flow rate of 1.25 mL/min. The organic acids pyruvate, lactate and acetate were measured using HPLC (Shimadzu) equipped with a C_{18} column (250 mm by 4.6 mm, 5 μm particle size; Phenomenex Sphereclone) as described in Narasingarao and Häggblom (2007a). Negative results were evaluated after three consecutive transfers with no growth.

In addition to respiring selenate to elemental Se with pyruvate as the carbon source, strain $S5^T$ also respired selenite, nitrate (to ammonium), and arsenate. Pyruvate, acetate and lactate were its preferred carbon sources. Neither *C. arsenatis* nor *D. alkaliphilum* respired selenate and selenite and *D. alkaliphilum* did not respire arsenate (Table 3.1). In addition to pyruvate, lactate, and acetate, both *C. arsenatis* and *D. alkaliphilum* were also able to use fumarate as carbon sources (Macy et al. 1997; Sorokin et al. 2007). In addition, *D. alkaliphilum* is also capable of using HS-, H_2, and propionate as electron donors and carbon sources (Table 3.1) (Sorokin et al. 2007). The generation times of strain $S5^T$ were calculated from cell counts (with Petroff Hauser cell counter) of cultures grown with 10 mM pyruvate as carbon source and 10 mM each nitrate, arsenate, selenate or selenite. The generation time on nitrate was 19.2 ± 2 hours; arsenate was 4.3 ± 0.2; selenate was 28.6 ± 2.6 (Table 3.2).

In order to test for optimum pH, strain $S5^T$ was grown with10 mM nitrate as

electron acceptor and 20 mM pyruvate as carbon source in anaerobic growth medium. The pH of the medium was adjusted with HCl and NaOH (pH 5 – 9). Strain S5^T grew at a pH range between 6.8 and 7.6, with an optimum pH at 7.4 (Table 3.1). Growth of strain S5^T at different NaCl concentrations (0 to 2.5 M) and temperatures (4 °C to 37 °C) was determined by growing cells in anaerobic medium (pH 7.4) with 10 mM nitrate and 20 mM pyruvate. The organism grew at a temperature range between 20 °C and 37 °C, with an optimum of 28 °C. The strain grew at a NaCl range of 0.1 M up to 0.75 M with an optimum NaCl concentration of 0.4 M.

The G+C (mol%) content of the genomic DNA of strain S5^T was determined using a modified method of Mesbah et al. (1989). A Synergy 4U Fusion-RP 80A C18 reverse-phase column (Phenomenex) was used in an Agilent HPLC 1100 system. The mobile phase consisted of two eluents, 20 mM ammonium acetate (pH 4.5, Eluent A) and acetonitrile (Eluent B) at a flow rate of 1 mL min^{-1}. A gradient of eluent A from 95% to 60% was established over a period of 10 minutes. The nucleosides were detected at a wavelength of 260 nm. Salmon sperm DNA was used for calibration with *Sedimenticola selenatireducens* strain AK4OH1 as control. The G+C content of strain S5^T was found to be 56 mol% which differed greatly from both *C. arsenatis* (49 mol%) and *D. alkaliphilum* (45 mol%) supporting the classification of strain S5^T as a new species of *Desulfurispirillum* (Table 3.1).

The fatty acid content of strain S5^T was analyzed from cells grown at 28 °C with 20 mM pyruvate and 10 mM nitrate. Cellular fatty acids were methylated and analyzed as previously described (Narasingarao and Häggblom 2006) using an HP 5890 series II gas chromatograph (Hewlett Packard, Palo Alto, CA) with a SHERLOCK Microbial

Identification System (MIDI, Inc., Newark, Del.). Fatty acid methyl esters detected by MIDI were further confirmed by gas chromatography-mass spectrometry using an Agilent GC Series 6890 GC-MS. The most common fatty acids found in the organism were $C_{18:1\ \omega7c}$ (38.5%) and $C_{16:0}$ (20.6%). Overall, unsaturated fatty acids, such as $C_{18:1\ \omega7c}$, $C_{16:1\ \omega7c}$, and $C_{16:1\ \omega5c}$ made up about 56% of all fatty acids in strain S5^T. The predominant straight chain fatty acids were $C_{12:0}$, $C_{16:0}$, and $C_{18:0}$ which made up about 25% of all fatty acids (Table 3.3). Strain S5^T also contained 3-hydroxy fatty acids, mainly $C_{12:0\ 3OH}$, $C_{12:1\ 3OH}$, indicative of fatty acids contained in the lipopolysaccharides of the outer membranes of Gram negative bacteria. A fair amount of $C_{17\ iso3OH}$ (7.8%) was also detected. The fatty acid composition of strain S5^T and its closest relative *D. alkaliphilum* were found to be distinctly different (Table 3.3). Overall, the fatty acid composition of strain S5 was made up of 14.3% hydroxy fatty acids compared to only 2.1% detected in *D. alkaliphilum.* In strain S5 and *D. alkaliphilum*, the most prevalent fatty acids were $C_{18:1\ \omega7c}$ and $C_{16:0}$, however, the abundance of C16:0 differed (15% in *D. alkaliphilum* vs. 21% in strain S5^T). Straight chain fatty acids ($C_{12:0}$, $C_{16:0}$, $C_{14:0}$, and $C_{18:0}$) made up only 22% of all fatty acids.

Description of *Desulfurispirillum indicum* sp. nov.

Desulfurispirillum indicum (in'di.cum L. neut. adj. indicum, of India, Indian). Gram negative highly motile spiral-shaped bacterium approximately 2 x 7 µm in length. Strictly anaerobic. Strain S5^T is a non-fermenting bacterium. Respires selenate to elemental Se coupled to utilization of short chain fatty acids. Is also capable of selenite, nitrate and arsenate respiration. Mesophilic with a maximum temperature for growth at 37 °C and an optimum at 28 °C. Tolerates a NaCl range from 0.1 to 0.75 M. The predominant cellular

fatty acids are $C_{12:1\ 3\text{-}OH}$, $C_{16:0}$, $C_{16:1\ \omega7c}$, $C_{17:0\ iso3OH}$, and $C_{18:1\ \omega7c}$. Groups within the family *Chrysiogenetes*. The genomic DNA G + C content of the type strain is 56 mol%.

The type strain $S5^T$ (= $DSM2283^T$ = ATCC $BAA\text{-}1389^T$) was isolated for its ability to respire selenate to elemental Se from an estuarine canal in Chepauk in Chennai, located in Southern India.

Acknowledgements

We are grateful to Valentin Starovoytov for his help with the electron microscopy and to J.P. Euzeby for advice in nomenclature. This study was supported in part by the New Jersey Water Resources Research Institute.

Figure 3.1 Phylogenetic tree showing Strain S5^T with closely related strains. The tree was constructed with aligned 16S rRNA gene sequences using the Neighbor-joining method with global gap removal algorithm. The evolutionary distances were computed using the Maximum Composite Likelihood method (1251 positions in analysis). Bootstrap values above 50 are indicated. The 16S rRNA gene sequence of *Thermotoga maritima* was used as outgroup.

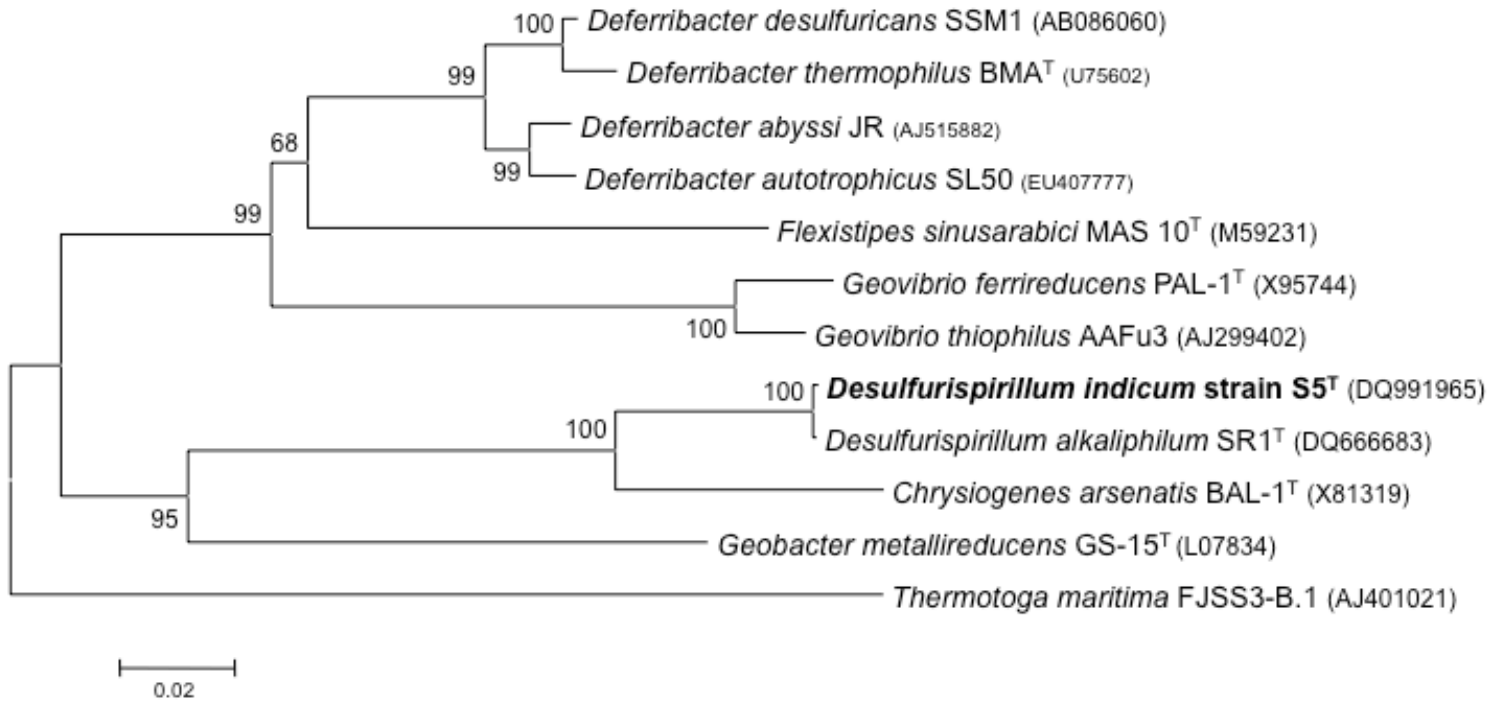


Figure 3.2 Cell morphology of strain S5. (a) Transmission electron micrographs of two week old cultures with intracellular deposits of elemental Se granules. (b) Phase contrast microphotograph of cells grown with nitrate and pyruvate and stained with flagella stain.

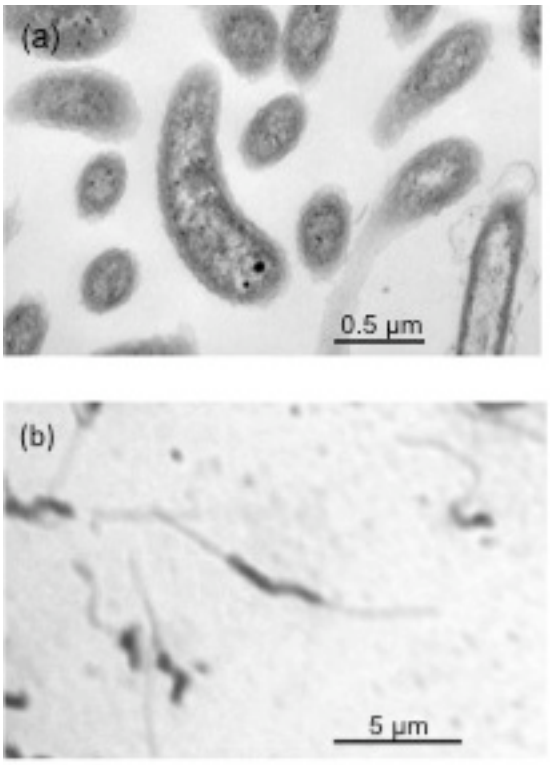


Table 3.1 Differential physiological and metabolic characteristics of strain S5^T and closely related species. Taxa: 1, Strain S5^T (data from this study); 2, *Desulfurispirillum alkaliphilum* SR 1^T (Sorokin et al. 2007); 3, *Chrysiogenes arsenatis* BAL1^T (Macy et al. 1996).

Characteristics	1	2	3
Morphology	Spirillum	Spirillum	Vibrio
Motility	Highly motile	Highly motile	Motile
Cell length (µm)	2-7	2-5	1-2
Cell diameter	0.1-0.15	0.15-0.2	0.5-0.75
DNA G+C content (mol%)	56.1	44.8 ± 0.5[5]	49
Fermentation	-	+	-
Growth temperature (°C) Optimum	28	30	25 to 30
pH Range for Growth (Optimum)	6.8 – 7.6 (7.4)	8.0 – 10 (9.0)	7.4 - 7.8
NaCl concentration (M Na+)			
Range	0-0.75	0.1-2.5[5]	
Optimum	0.4	0.4	0.02[6]
Electron Donors			
Succinate	-	-	+
Fumarate	-	+	+
HS-	-	+[5]	ND
H_2	-	+	-
Malate	-	-	+
Propionate	-	+	ND
Citrate	-	-	-
Electron acceptors			
Arsenate	+	-	+
Chromate	-	+	-
DMSO	-	-	ND
Nitrite	-	+	+
Selenite	+	-	-
Selenate	+	-	-
Sulfur	-	+	-

For strain S5, electron acceptors were tested with 20 mM pyruvate as electron donor and electron donors were tested with 10 mM nitrate as electron acceptor. +, Positive; -, Negative; ND, not determined. All strains were positive for reduction of nitrate and oxidation of short chain fatty acids acetate, lactate, and pyruvate. All strains were negative for reduction of oxygen, chlorate, Fe^{3+}[5], sulfate[5], fumarate, and $S_2O_3^{2-}/SO_3^{2-}$[5] and oxidation of formate[5].

[5] Data from Sorokin et al. (2007)
[6] Data from Macy et al. (1989)

Table 3.2 Generation times of strain S5^T grown on various electron acceptors grown with 10 mM pyruvate as carbon source and electron donor and various electron acceptors (10 mM).

Electron Acceptor	Generation Time (in Hours)
Nitrate	19.2 ± 2
Arsenate	4.3 ± 0.2
Selenate	28.6 ± 2.6

Table 3.3 Cellular fatty acid composition of strain S5^T (1) compared to selected fatty acids present in *Desulfurispirillum alkaliphilum* SR 1^T (2). Strain S5^T was grown with 10 mM nitrate as electron acceptor and 20 mM pyruvate as carbon source and electron donor (- , not detected).

Fatty Acids	Relative abundance (%)	
	1	2[7]
$C_{12:0}$	1.4 ± 0.8	3.2 ± 0.3
$C_{12:1\ 3OH}$	4.1 ± 1.8	1.7 ± 0.1
$C_{12:0\ 3OH}$	1.4 ± 0.7	0.4 ± 0.1
$C_{14:0}$	-	0.7 ± 0.4
$C_{15:0\ iso\ 3OH}$	0.5 ± 0.7	-
$C_{15:0\ 2OH}$	0.5 ± 0.7	-
$C_{16:1\ \omega9c}$	1.1 ± 0.2	1.3 ± 0.2
$C_{16:1\ \omega7c}$	6.7 ± 0.8	4.8 ± 0.4
$C_{16:1\ \omega5c}$	4.3 ± 0.6	3.5 ± 0.2
$C_{16:0}$	20.6 ± 3.6	14.9 ± 0.9
$C_{17:1\ ISO\ \omega5c}$	-	8.9 ± 0.6
$C_{17:1\ \omega8c}$	0.9 ± 0.4	-
$C_{17:1\ \omega6c}$	2.1 ± 0.1	1.4 ± 0.2
$C_{17:0\ iso\ 3OH}$	7.8 ± 11	-
$C_{18:1\ \omega9c}$	-	0.6 ± 0.1
$C_{18:1\ \omega7c}$	38.5 ± 10.1	39.6 ± 3.3
$C_{18:1\ \omega5c}$	2.9 ± 0.7	2.1 ± 0.3
$C_{18:0}$	3.2 ± 1.5	3.1 ± 0.4
$C_{18:1\ iso\ H}$	1.0 ± 0	-
$C_{18:1\ iso\ H\ \omega8c}$	3.9 ± 1.6	-

[7] *D. alkaliphilum* was grown at 28 °C with 20 mM acetate as electron donor and 10 mM nitrate as electron acceptor.

CHAPTER 4

ENERGY METABOLISM AND MULTIPLE RESPIRATORY PATHWAYS REVEALED BY GENOME SEQUENCING OF *DESULFURISPIRILLUM INDICUM* STRAIN S5

Env Microbiol 2011

Abstract

Desulfurispirillum indicum strain S5, a novel obligate anaerobe belonging to the phylum *Chrysiogenetes*, is a dissimilatory selenate-, selenite-, arsenate-, nitrate- and nitrite-reducing bacterium. The circular genome of this metabolically versatile bacterium is 2.9 Mbp, with a G+C content of 56.1% and 2619 predicted protein-coding genes. Genome analysis uncovered the components of the electron transport chain, providing important insights into the ability of *D. indicum* to adapt to different conditions, by coupling the oxidation of various electron donors to the reduction of a wide range of electron acceptors. Sequences encoding the subunits of dehydrogenases and enzymes with roles in the oxidation of several electron donors, including acetate, pyruvate and lactate were identified. Furthermore, five terminal oxidoreductase complexes were encoded in the *D. indicum* genome. Phylogenetic analyses of their catalytic subunits, operon structure and co-transcription of subunit-coding genes indicate a likely role of three of them as respiratory arsenate reductase (Arr), periplasmic nitrate reductase (Nap) and the membrane-bound nitrate reductase (Nar). This study is the first description and annotation of the genome of a dissimilatory selenate- and arsenate-respiring organism, and *D. indicum* represents the first, sequenced member of its phylum. Our analysis demonstrates the complexity of the microorganism's respiratory system provides the basis for the functional analysis of metalloid oxyanions respiration and expands our knowledge of the deep branching phylum of *Chrysiogenetes*.

Introduction

Microbes control the biogeochemical cycles of many trace elements found in the Earth's crust. The microbial transformation of these elements, including change in their chemical form and valence state, may be the result of detoxification, assimilatory processes or dissimilatory processes (Silver and Phung 1996; Stolz et al. 2002, 2006; Lloyd 2003). As and Se are two intriguing elements involved in microbial metabolism. Although the natural abundance of both elements is low, less than 0.0002% for As and less than 0.0001% for Se, they can accumulate in the environment to much higher concentrations under certain conditions (Stolz and Oremland 1999). Speciation of As and Se, which alters their toxicity and mobility in the environment, is greatly affected by both environmental conditions and the activity of the microbial populations that mediate these transformations (Oremland and Stolz 2003). At the same time, the biochemical transformation of As and Se oxyanions play an important role in supporting the growth of these microbial communities in anaerobic environments (Stolz et al. 2006).

The enzymes that catalyse the oxidation-reduction reactions of many metalloids depend on the transition metal molybdenum (Kisker et al. 1997; Schwarz et al. 2009). Enzymes containing the molybdenum cofactor are found in virtually every organism and their roles are fundamental in the catalysis of key steps in microbial metabolism (Hille et al. 1999; Schwarz et al. 2009). They are divided into three groups, sulfite oxidase, xanthine oxidase and dimethyl sulfoxide (DMSO) reductase, based on sequence similarity, cofactor composition and catalytic function (Kisker et al. 1997; Schwarz et al. 2009). Key members of the DMSO family of reductases are the periplasmic reductase (Nap), respiratory nitrate reductase (Nar), respiratory arsenate reductase (Arr) and selenate reductase (Ser) (Kisker et al. 1997; Malasarn et al. 2008; Lowe et al. 2010). Even though many phylogenetically diverse organisms have been isolated that are capable of dissimilatory arsenate and selenate respiration (e.g. Macy et al. 1993;

Switzer Blum et al. 1998; 2001; Stolz and Oremland, 1999; Huber et al. 2000; Narasingarao and Häggblom 2006, 2007a,b; Rauschenbach et al. 2011), the genes and regulation patterns associated with the respiration of these oxyanions are yet to be fully understood. *Desulfurispirillum indicum* belongs to the *Chrysiogenetes*, a deep branching phylum that currently only includes two other cultured species, *Chrysiogenes arsenatis* (Macy et al. 1996) and *Desulfurispirillum alkaliphilum* (Sorokin et al. 2007). The three microorganisms are all strict anaerobes and metabolically versatile, in that they use a wide variety of alternative terminal electron acceptors and carbon sources. *Desulfurispirillum indicum* is unique in the phylum *Chrysiogenetes*, being the only one capable of respiring both arsenate (10 mM) and selenate (10 mM), in addition to selenite and nitrate, with pyruvate, lactate or acetate serving as electron donors and carbon sources (Narasingarao and Häggblom, 2007a; Rauschenbach et al. 2011).

As a first step towards the characterization of the metabolic pathways for energy conservation in arsenate and selenate-respiring microorganisms, the genome of *D. indicum* was completely sequenced. Our analysis provides an in-depth view of the genes encoding components of the electron transport chain, with a special emphasis on those responsible for the terminal dissimilatory reduction of arsenate and nitrate. Additional molybdoenzymes were identified, revealing a complex energy metabolism. A model is proposed that will serve as the basis for the elucidation of the mechanisms of dissimilatory reductions in this selenate and arsenate reducer.

Material and Methods

Growth of* Desulfurispirillum indicum *and DNA Extraction. *D. indicum* strain S5 was anaerobically grown in mineral salts medium (Fennell et al. 2004) containing 10 mM nitrate as electron acceptor and 20 mM pyruvate as electron donor at 28 ºC, unless

otherwise stated. Genomic DNA was extracted by phenol-chloroform extraction as previously described (Kerkhof and Ward 1993) from a 80 mL culture.

Genome Sequencing and Assembly. The draft genome of *D. indicum* strain S5 was generated at the DOE Joint genome Institute (JGI) using a combination of Illumina (Bennett, 2004) and 454 technologies (Margulies et al. 2005). The following libraries were constructed and sequenced: an Illumina GAii shotgun library; a 454 Titanium standard library; and a paired end 454 library. The 454 Titanium standard and paired end data were assembled together with Newbler, version 2.3. Illumina sequencing data was assembled with VELVET, version 0.7.63 (Zerbino and Birney 2008). The 454 Newbler consensus shreds, the Illumina VELVET consensus shreds and the read pairs in the 454 paired end library were integrated using parallel phrap, version SPS - 4.24 (High Performance Software, LLC). The software Consed (Ewing and Green 1998; Ewing et al. 1998; Gordon et al. 1998) was used to correct potential base errors and the software Polisher (developed at JGI, Alla Lapidus, unpublished) was used to increase consensus quality of the Illumina data. Possible mis-assemblies were corrected using gapResolution (Han, unpublished), Dupfinisher (Han and Chain 2006), or by sequencing cloned bridging PCR fragments with subcloning. Gaps between contigs were closed by editing in Consed or by PCR and bubble PCR (Cheng, unpublished) primer walks. The final assembly is based on 220 Mb of 454 draft data which provides an average 108 x coverage of the genome and 607 Mb of Illumina draft data which provides an average 222 x coverage of the genome. Annotation of the project is available at http://genome.ornl.gov/microbial/sind/14sep10 and http://img.jgi.doe.gov/cgi-bin/pub/main.cgi?section=TaxonDetailandpage=TaxonDetailandtaxon_oid=647000350. The sequence of the genome has been deposited in GenBank with accession number

CP002432 and NCBI (NC_014836). The circular genome (Figure 4.1) was generated using DNAPlotter (Carver et al. 2008).

Comparative and Phylogenetic Analyses. For comparative sequence analyses, BlastP searches were conducted against the *D. indicum* genome database, using sequences of characterized enzymes obtained from GenBank. Dehydrogenases were identified by search of the genome annotation and then comparing putative sequences to GenBank via BlastP searches. Molybdoenzymes were identified on the basis of protein similarity to sequences of the molybdopterin oxidoreductase family (Pfam00384) and biochemically characterized reductases. Accession numbers of molybdenzymes sequences are indicated in Figure 2. The protein sequence of the five *D. indicum* putative molybdoenzymes Selin_0378, Selin_2609, Selin_2495, Selin_0752 and Selin_2443, the protein sequences of characterized reductases, as well as selected putative homologs of the *D. indicum* catalytic subunits retrieved via BlastP searches (Altschul et al. 1997) of non-redundant databases at NCBI, were aligned using ClustalW (Chenna et al. 2003) implemented in MEGA4 (Tamura et al. 2007). The same program was used to conduct phylogenetic and molecular evolutionary analyses of all molybdenum containing enzyme sequences. Evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The evolutionary history was inferred from these distances using the Neighbor-Joining method with bootstrap test (1000 replicates) (Tamura et al. 2007). All positions containing gaps and missing data were eliminated from the dataset. There were a total of 157 positions in the final dataset.

Gene Expression Analysis of Arsenate, Nitrate, and Selenate Reductases via RT-PCR. *D. indicum* strain S5 was pregrown in mineral salts medium amended with 20 mM

pyruvate as electron donor and carbon source and either 10 mM nitrate, 10 mM selenate or 10 mM sodium arsenate as sole electron acceptors. Cultures were subcultured for at least 3 months to ensure that only one electron acceptor was present and utilized at the time of RNA extraction. Total RNA was isolated as previously described (Villafane et al. 2009) using the RNeasy Midi Kit (Qiagen, USA) from 5 mL of a log phase culture (data not shown). DNAse treatment (Ambion, USA) was followed by PCR using primers targeting the 16S rRNA gene to rule out DNA contamination. RT-PCR reactions were set up using 0.5 µg of RNA in a two step RT-PCR approach in 25 µL reactions using the Enhanced Avian RT-PCR Kit, according to the manufacturer's protocol (Sigma Aldrich, USA). Primers for the specific amplification of individual genes of the *arr*, *nar*, *nap* and *ynfEF* operons (Table 4.1) were designed using the Invitrogen OligoPerfect™ Designer tool (http://tools.invitrogen.com/content.cfm?pageid=9716). Two negative controls were included, one omitting the reverse transcriptase and one without template. Amplicons were separated by 1.2% agarose gel electrophoresis and the gel images were acquired using a GelLogic 440 Imaging System (Eastman Kodak, USA). Visible bands were extracted (Qiagen, USA) and sequenced (Genwiz Inc., USA) to validate primer specificity.

Results and Discussion

General Features of the Genome. The genome of *D. indicum* strain S5 consists of a single circular chromosome of 2,928,377 bp with an average G+C content of 56.1% (Figure 4.1; Table 4.1). It is estimated to contain 2619 protein coding genes, of which 2144 were assigned to specific COG functional groups (Figure 4.1, Table 4.1), while the rest of the sequences were homologous to proteins with unknown function (5%) or were assigned a general function (11.6%). Three copies of the rRNA operon, including 16S, 23S and 5S ribosomal RNAs, were found in the *D. indicum* genome and 37 tRNA genes.

Additional genome features are listed in Table 4.1. The analysis of the genome focused on the elucidation of various modes of respiration of *D. indicum*. In accord with the ability of strain S5 to couple the utilization of pyruvate, lactate and acetate to dissimilatory nitrate, arsenate, and selenite reductions, genes encoding the putative components of the electron transport chains for the respiration of these terminal electron acceptors were identified.

Genes Involved in Oxidation of Electron Donors. During dissimilatory nitrate, arsenate or selenate reduction, *D. indicum* utilizes pyruvate, lactate or acetate, as electron donors and carbon sources. In *Escherichia coli*, oxidation of pyruvate occurs through pyruvate : ferredoxin oxidoreductase, an enzyme complex made of 24 subunits of pyruvate dehydrogenase, 24 subunits of dihydrolipoate transacetylase and 12 subunits of dihydrolipoate dehydrogenase (Packman et al. 1984).
Putative homologues of each of these subunits were identified in strain S5. Selin_2129-2128 encode a pyruvate oxidoreductase domain containing a FAD binding site, and an enzyme with a binding site for thiamine pyrophosphate (TPP), consistent with the pyruvate dehydrogenase architecture respectively. Interestingly, these sequences are located near the cluster of genes encoding the enzymes succinate, malate and isocitrate dehydrogenases of the tricarboxylic acid (TCA) cycle. Two additional clusters encoding putative subunits of the pyruvate oxidoreductase were Selin_0679-0678 and Selin_1937-38. A homologue of the lactate dehydrogenase (Selin_0121) is present in the *D. indicum* chromosome, thus the oxidation of lactate is predicted to proceed using this enzyme. Through this pathway, lactate is oxidized to pyruvate, which can be further oxidized as described above. A phosphate acetyltransferase (Selin_1054) and an adjacent acetate kinase (Selin_1055) are encoded in the strain S5 genome, which is in agreement with the ability of *D. indicum* to use acetate as an electron donor,

and indicates that the process is likely to occur through the conversion of acetate to acetyl-CoA and CO2. A glycerol-3-phosphate dehydrogenase (Selin_1822) was also identified, suggesting that the strain might also use glycerol-3-phosphate as an electron donor. The subunits of various NADH : quinone oxidoreductase complexes were also identified (Complex I: Selin_0606-0616; Complex II: Selin_0878-0891). Such oxidoreductase complexeshave the fundamental function to re-oxidize NADH generated during the oxidation of carbon substrates in eukaryotes and prokaryotes.

Homologues of enzymes belonging to the TCA cycle were also detected in the *D. indicum* genome. These include citrate synthase (Selin_1730), aconitate hydratase (Selin_0779), isocitrate dehydrogenase (Selin_2134), 2-oxoglutarate ferredoxin oxidoreductase (Selin_2128-2129), succinyl-CoA synthase (Selin_0303-0304), succinate dehydrogenase (Selin_2130-2131), fumarate hydratase (Selin_1547) and malate dehydrogenase (Selin_2133).

Identification of Molybdenum-Containing Enzymes and Putative Respiratory Reductases. The processes involved in the reduction of terminal electron acceptors in the respiratory metabolism are catalyzed by highly conserved molybdenum containing enzymes (Kisker et al. 1997). We identified in the genome of *D. indicum* strain S5 five proteins belonging to the family of the molypdopterin oxidoreductases, Selin_0378, Selin_0752, Selin_2443, Selin_2495, and Selin_2609, that may be responsible for the reduction of oxyanions and organic compounds used as terminal electron acceptors and are expected to have important roles during anaerobic respiration. The deduced protein sequences of Selin_0378, Selin_0752, and Selin_2443, Selin_2495, Selin_2609 were used to construct a phylogenetic tree in order to gain insights into the evolutionary relationships with other characterized molybdoenzymes (Figure 4.2). Selin_0378 was most closely related to the respiratory arsenate reductase subunit ArrA of *Chrysiogenes*

arsenatis (AAU11839). Both the ArrA of strain S5 and *C. arsenatis* clustered with ArrA subunits of phylogenetically diverse organisms, such as *Shewanella* sp. ANA-3, *Desulfosporosinus* sp. Y5, *Bacillus selenitireducens*, and *Bacillus arseniciselenatis* (Figure 4.2) (Krafft and Macy 1998; Switzer Blum et al. 1998; Afkar et al. 2003; Pérez-Jiménez et al. 2005; Malasarn et al. 2008). Selin_2443 displayed the signature amino acids that characterize the NarG subunit of the respiratory nitrate reductase present in *Escherichia coli* (NP_415742) and *Pseudomonas fluorescens* (AAG34373). Selin_0753 was found to be most closely related to the NapA subunit of the periplasmic nitrate reductase found in *Desulfovibrio desulfuricans* (CAI72603) (Figure 2). Selin_2609 and Selin_2495 were distantly related to the polysulfide reductase found in *Shewanella oneidensis* MR-1 and *Wolinella succinogenes*, and YnfEF, a putative selenate reductase of *E. coli*, respectively (Figure 4.2).

Arsenate Reductase. The respiratory arsenate reductase enzyme (Arr) has been isolated and characterized in *C. arsenatis, B. selenitireducens,* and *Shewanella* sp. Strain ANA-3 (Krafft et al. 1998; Afkar et al. 2003, Malasarn et al. 2008) and identified in many other organisms (see Stolz et al. 2006). It is highly conserved and usually it is expressed as a heterodimer with a larger molybdopterin containing subunit, ArrA, and a smaller, iron-sulfur containing subunit, ArrB (Krafft et al. 1998; Afkar et al. 2003; Malasarn et al. 2008). The predicted protein sequence of Selin_0378 was 79% identical and 87% similar to ArrA found in *C. arsenatis*. Further analysis of the sequence showed that the predicted protein was 846 amino acids long and contained a leader twin arginine (tat) signal, required for protein translocation. Based on sequence similarity and clustering with known ArrA subunits (Figure 4.2), we designated Selin_0378 as ArrA.

To determine whether a homolog of ArrB is also found downstream of Selin_0378 (ArrA), we analyzed its gene neighborhood. We indeed found a sequence,

Selin_0379, immediately downstream of Selin_0378 that was 53% similar and 40% identical to ArrB of *C. arsenatis* (Figure 4.3). Similar to other ArrB sequence, the 261 amino acid long protein of Selin_0378 lacked a tat signal sequence in the N-terminus. Thus we designed this sequence ArrB. Both subunits, ArrA and ArrB, had N-proximal cysteine rich iron-sulphur cluster binding motifs. The motif CX_2CX_3C is present in ArrA and it is predicted to anchor a [4Fe-4S] cluster, while the motif present in ArrB ($CX_2CX_2CX_3C$) typically binds [Fe-S] centers. Based on the protein signatures and tat signal sequences, it is suggested that ArrA is secreted into the periplasm while ArrB remains bound to the membrane. By analogy with other Arr reductases (Macy et al. 2000), the *D. indicum* ArrA and ArrB are proposed to form a reductase complex in which ArrA binds and reduces arsenate to arsenite and ArrB is responsible for conducting the electrons from the respiratory chain (Figure 4.4).

An arsenical resistance operon, designated *arsRBC*, was also found upstream of Selin_0378 (Figure 4.3). Each gene encoded a protein predicted to be 140, 368, and 145 amino acids long, respectively. The *ars* operons are highly conserved among bacteria and have been shown to confer arsenical resistance to microorganisms carrying this locus on either plasmids or chromosomes (Carlin et al. 1995; Silver and Phung 1996; Li and Krumholz 2007). The *arsRBC* operon encodes an arsenate reductase (ArsC), a membrane protein forming an ion channel (ArsB) and a trans-acting repressor (ArsR). Arsenite binds to ArsR causing a conformational change of the transcriptional regulator that results in dissociation of the repressor from the promoter and transcription of the *ars* operon. The arsenate reductase ArsC would reduce arsenate to arsenite, which in turn is removed from the cell via the arsenite specific efflux pump ArsB (Figure 4.4) (Rosen 1999; Li and Krumholz 2007). Arsenate is probably taken up by *D. indicum* cells via the inorganic phosphate transport systems Pst and/or Pit, similar to what has been observed in other microorganisms (Páez-Espino et al. 2009). In fact, putative

components of both systems were identified in the genome, and they were encoded by Selin_1029 (Pit) and Selin_2587-2590 (Pst).

Nitrate and Nitrite Reductases. The catalytic subunit NarG of the respiratory nitrate reductase has been shown to form a heterotrimeric structure with two other subunits, NarH and NarI (Blasco et al. 2001). The gene product of Selin_2443 was found to be homologous to the large subunit NarG of the respiratory nitrate reductase Nar of *E. coli* (64% similar; 48% identical). It was also 61% similar (47% identical) to NarG of *P. fluorescens.* Furthermore, conserved amino acids Thr^{236}, Gln^{235} and Thr^{236} were present within the *D. indicum* sequence, bearing the hallmark signature of NarG (Martinez-Espinosa et al. 2007). Visual inspection of the region surrounding *narG* revealed that the complete *narGHIJ* operon was indeed present in strain S5 (Figure 4.3). The size of their gene products, predicted to be 1207, 493, 185, and 227 amino acids long, respectively, is in good agreement with the size of the subunits of the *E. coli* Nar complex (Gonzáles et al. 2006). None of the subunits encoded by the *D. indicum* operon contained a tat signal sequence, suggesting their cytoplasmic location (Figure 4.4). In *E. coli*, NarI has been shown to anchor the other subunits to the cytoplasmic membrane. Its heme groups receive electrons from the quinone pool. Electrons are then transferred to the iron sulfur clusters present in NarH and finally to the catalytic subunit NarG (Blasco et al. 2001; Martinez-Espinosa et al. 2007). Thus, the presence of a NarI homolog in *D. indicum* suggests that NarGH is oriented toward the cytoplasmic side of the membrane (Martinez-Espinosa et al. 2007). This observation is also consistent with NarI predicted cytoplasmic location, based on the lack of a tat signal sequence. Even though not part of the final membrane complex, NarJ plays an integral role in the maturation of the final NarGHI membrane complex (Dubourdieu and DeMoss 1992; Gonzáles et al. 2006). Transporters were identified upstream of *narG* (*narL*) and immediately downstream of

narI (*narK*) and are predicted to be involved in the transport of nitrate into the cell. NarK, initially thought to be a nitrate importer, has been demonstrated to be involved in nitrite export (Rowe et al. 1994).

A second type of nitrate reductase, Nap (periplasmic nitrate reductase), is common in Gram negative bacteria. In contrast with Nar, Nap is not anchored to the membrane but is found in the soluble periplasmic fraction (Richardson et al. 2001). Nap function is controversial. It has been reported to contribute to energy conservation (Sears *et al.* 2000) and also to be involved in the disposal of excess reducing equivalent (Richardson 2000). In *D. indicum* we have identified sequence Selin_0752 as a putative NapA. The protein encoded by Selin_0752 is 793 amino acids long and had highest similarity to NapA of *D. desulfuricans* (CAI72603, 65% similarity, 47% identity). *D. indicum* NapA contains a highly conserved cysteine rich iron-sulfur cluster motif CX_2CX_3C, at the N-terminal region, as well as a tat signal sequence, similar to the NapA of *D. desulfuricans* (Dias et al. 1999). Analysis of the gene neighborhood of napA revealed that it was located within a gene cluster comprised of five open reading frames (Figure 3). Nap operons have been identified in many prokaryotes, however, in contrast to nar operons, they display greater heterogeneity and their gene organization is highly dependent on the organism (Richardson et al. 2001; Marietou et al. 2005; Correira et al. 2006). The organization of the putative *nap* locus of *D. indicum* strongly resembles the *nap napMADGH* operon structure of *D. desulfuricans.* Accordingly, the first gene in the *D. indicum* operon encodes NapM, a c-type cytochrome which has presently only been identified in *D. desulfuricans* (Marietou et al. 2005). The second gene, *napA*, encodes for the catalytic subunit, responsible for the reduction of nitrate to nitrite. Located immediately downstream of *napA* is the gene encoding NapD. The role of NapD is poorly understood but suggested to be required for maturation and export of NapA. *The genes napG* and *napH* are predicted to encode for a membrane-bound menaquinol

complex, similar to the NapGH complexes found in organisms such as *E. coli* (Brondijk et al. 2004), *W. succinogenes* (Kern and Simon 2009), and *Shewanella* sp. (Simpson et al. 2010). Most *nap* operons also encode NapB and/or NapC, involved in the mediation of electrons to the catalytic subunit NapA. Surprisingly, strain S5 does not encode for either of these enzymes, which leads us to speculate that NapA could receive electrons via two alternate pathways, one via NapG and the other through NapM, as proposed for *D. sulfuricans* (Marietou et al. 2005).

D. indicum strain S5 reduces nitrate to ammonium (Rauschenbach et al. 2011a) and is also capable of respiring low levels of nitrite (up to 5 mM) to ammonium (data not shown). We identified in the genome a *nrf* operon, consisting of two genes *nrfA* and *nrfH* (Selin_2182-2181), that are probably responsible for dissimilatory nitrite reduction. NrfA, a cytochrome c reductase, catalyzes the reduction of nitrite to ammonium without the release of any intermediate products (Simon 2002). The *D. indicum* homolog of NrfA is most closely related to NrfA found in *Desulfovibrio vulgaris* (YP_002435906.1, 60% identical and 73% similar). Similar to other characterized NrfA subunits (Simon, 2002; Kern and Simon 2009), it also contains five haem groups, four CXXCH and one CXXCK motif responsible for haem attachment. NrfH is a member is the NapC/NirT family and forms a stable membrane complex with NrfA (Simon 2002). The *D. indicum* gene encoding a NrfH homolog was identified immediately downstream on *nrfA*. It contains four putative haem attachment sites, CXXCH, and 18 hydrophobic amino acids residues in the N-terminus, suggesting a membrane location for the subunit .

Selenate Respiration*.* In addition to arsenate and nitrate, strain S5 can also utilize selenate as a terminal electron acceptor to gain energy. To date, few studies have identified selenate reductases and only one respiratory selenate reductase has been purified and characterized (Schröder et al. 1997). Using the information available, we

also searched the genome for a putative selenate reductase. No protein sequence homology was found between any of the five molybdoenzymes in strain S5 and SerA, the respiratory selenate reductase of *Thauera selenatis* (Schröder et al. 1997) (Figure 4.2). *E. coli* is capable of SeVI reduction, although it has not been shown to utilize this process for energy conservation. Based on mutagenesis data, its *ynfEFGH* operon has been shown to be responsible for selenate reduction (Guymer et al. 2009). In *D. indicum* we identified the protein sequence Selin_2609, which was 41% similar and 25% identical to YnfE, and 42% similar and 26% identical to YnfF. Even though Selin_2609 had similarity with YnfE and YnfE, no other genes of the *ynfEFGH* were detected upstream and downstream. Further analysis is needed to positively identify the selenate reductase in strain S5.

Expression of Arsenate and Nitrate Reductase Genes. After the identification of putative Arr, Nar, and Nap and their respective subunits, we wanted to verify whether the genes belonging to the *arr*, *nar*, and *nap* clusters were co-transcribed and expressed under arsenate or nitrate reducing conditions, respectively. Primers were designed to specifically detect the genes encoding the catalytic subunits (*arrA*, *narG* and *napA*), as well as each of the other subunits (Table 4.2). Genes that cluster into operons encode the proteins necessary to perform a coordinated function and thus are transcribed into mRNA in a single transcript. Using RT-PCR with primers that amplify across adjacent genes we demonstrated that all the contiguous genes identified as putative *arr*, *nar*, and *nap* subunits were co-transcribed, as indicated by the bands narGH, narHI, narIJ (Figure 4.3b-I), arrAB (Figure 4.3B-II), and napAD, napDG, napGH (Figure 4.3B-III), and thus they constitute functional operons.

Interestingly, the *arrA* transcript was detected both in the presence (Figure 4.3) and in the absence (not shown) of arsenate. However, it is possible that the *arr* operon

may be upregulated following exposure to arsenate as it was previously shown in *Shewanella* sp. strain ANA-3 (Saltikov et al. 2005), and real time RT-PCR analyses of their transcripts will be required to verify this hypothesis.

Conclusions

The genome of *D. indicum* strain S5 provides insights into the energy metabolism of an arsenophilic and selenophilic organism. Its analysis revealed the presence of multiple respiratory pathways, characterized by a modular architecture, with components that can be arranged in different combinations to allow the utilization of a vast array of carbon and energy sources, coupled to the reduction of organic and metallic terminal electron acceptors. Five molybdoenzymes (Selin_0378, Selin_0752, Selin_2443, Selin_2495, and Selin_2609) were identified, to only three of which we were able to assign putative functions as components of the Arr, Nar, and Nap reductases (Figure 4.4) through comparative sequence and genome context analyses. (Figure 4.2, Figure 4.3). Two of the *D. indicum* molybdoenzymes were more distantly related to known molybdoenzymes and they were also encoded within cluster of genes organized as typical terminal reductase operons. Although we have not yet been able to assign the function of Selin_2609 and Selin_2495, they are likely to have a role in the reduction of additional terminal electron acceptors to be tested experimentally. The identified reductases and their predicted localizations on either the cytoplasmic or the periplasmic side of the cell membrane is represented in Figure 4.4. We also show the components of the arsenic detoxification system, which may have a role in the reduction of excess arsenate and efflux of arsenite.

Our current analyses did not clearly identify selenate and selenite reductases. This is due to the lack of reference sequences, given that only one respiratory selenate reductase (Schroeder et al. 1998) and no selenite reductase have been characterized

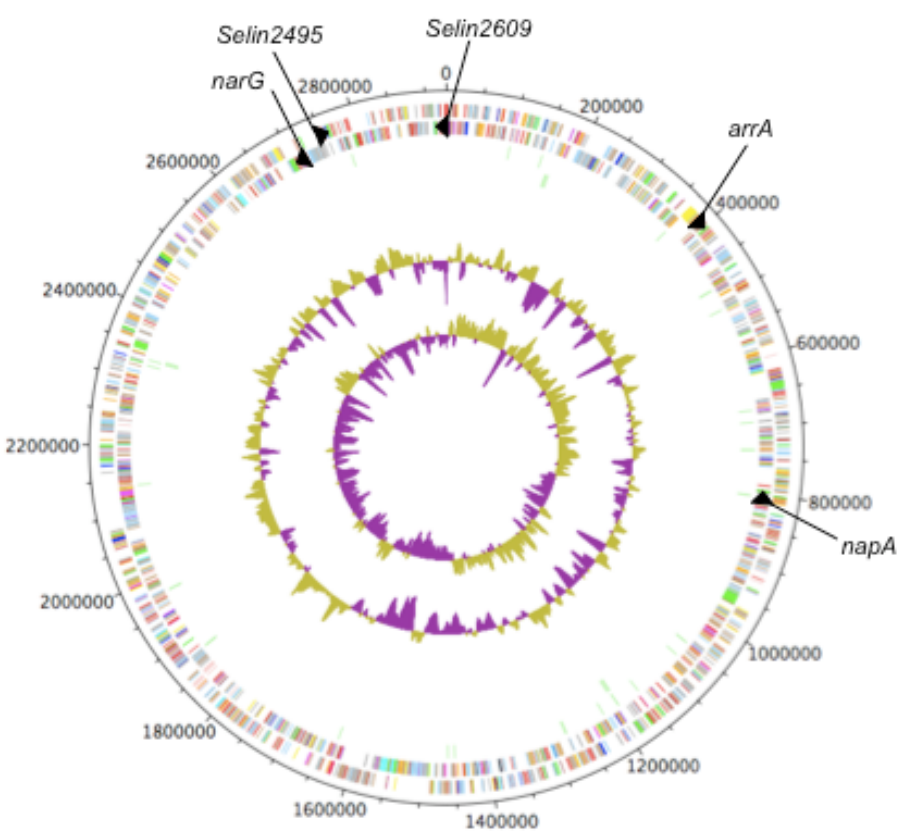
thus far. We propose that strain S5 may harbor novel, unknown selenate and selenite reductases which may likely be encoded in either Selin_2609 or Selin_2495; alternatively, selenate and selenite reduction may be carried out via Nap or Nrf respectively, as suggested for other selenate/selenite respiring bacteria (DeMoll-Decker and Macy 1993; Oremland et al. 1999; Sabaty et al. 2001).

Further work is needed to experimentally identify all the substrates and inducers of the molybdenum containing enzymes present in *D. indicum*. Also, transcription level profiling will be needed to characterize the regulation of all genes involved in the respiratory systems. The molecular information expands our knowledge of the deep branching phylum of *Chrysiogenetes*, will help enhance the investigation of the biochemistry of arsenate, selenate, selenite and nitrate reduction and will provide a better understanding in the mechanisms involved in the mobilization of trace metals. The information from comparative genomics studies of *D. indicum* strain S5 and upcoming genomes of dissimilatory oxyanions reducers will advance our understanding of the evolutionary relationships between the enzymes of the DMSO reductase family and help us identify respiratory systems that are functionally equivalent.

Acknowledgements

The work was funded in part by the National Science Foundation (EAR 0843295). The work conducted by the U.S. Department of Energy Joint Genome Institute was supported by the Office of Science of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231.

Figure 4.1 Circular representation of whole genome of . *indicum* strain S5. From the outer ring inwards, forward and reverse strands, tRNA and rRNA content, % GC plot (G + C distribution) and GC skew (difference in base composition between leading and lagging strand). Positions of the putative genes encoding the catalytic subunits are indicated. Colors assignment and description can be found in the table.



Information Storage and Processing	
Translation, Ribosomal Structure and Biogenesis	
Transcription; Chromatin Structure and Dynamics	
DNA Replication, Recombination and Repair	
Cellular Processes	
Signal Transduction Mechanisms; Cell Motility and Secretion; Defense Mechanisms	
Cell Division and Chromosome Partitioning	
Cell Envelope Biogenesis, Outer Membrane	
Postranslational Modification, Protein Turnover, Chaperones	
Metabolism	
Inorganic Ion Transport and Metabolism; Secondary Metabolites Biosynthesis, Transport and Catabolism	
Energy Production and Conversion	
Carbohydrate Transport and Metabolism	
Amino Acid Transport and Metabolism	
Coenzyme Metabolism; Nucleotide Metabolism	
Lipid Metabolism	
Uncharacterized Proteins	
Putative Uncharacterized Proteins	

Figure 4.2 Neighbor-joining tree of putative molybdoenzymes identified in strain S5. Putative molybdoenzymes Selin_0378, Selin_ 0752, Selin_2443, Selin_2495, and Selin_2605 are compared to sequences of known molybdoenzymes. Bootstrap values greater than 50 are shown. Arr, respiratory arsenate reductase; Psr, polysulfide reductase; Nar, nitrate reductase; DMS DH, dimethyl sulfide dehydrogenase; Ser, selenate reductase; Chr, chlorate reductase; DMSO, dimethyl sulfoxide reductase; Bis, biotin sulfoxide reductase; TMNO, trimethylamine N-oxide reductase; YnfE, putative selenate reductase; Nap, respiratory nitrate reductase.

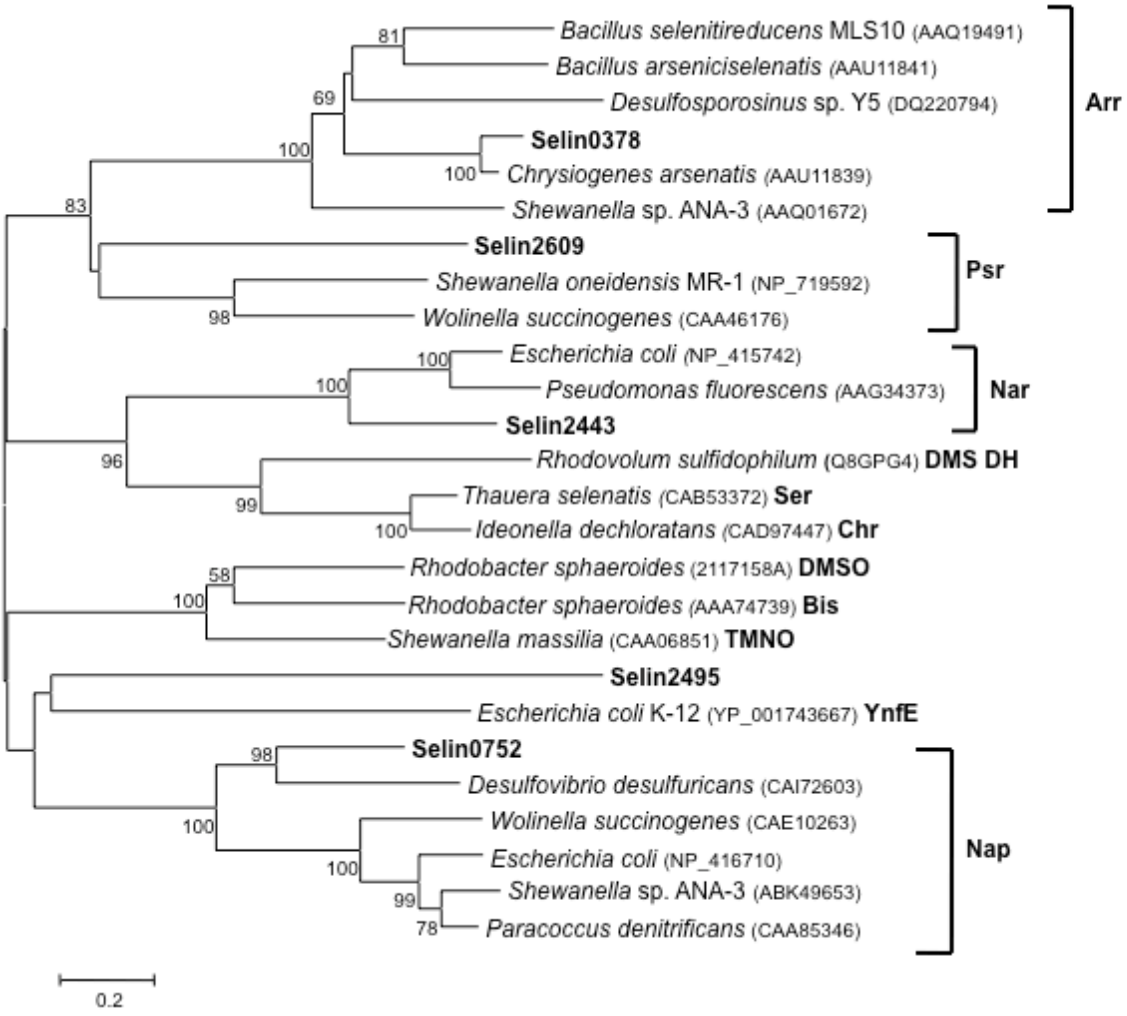
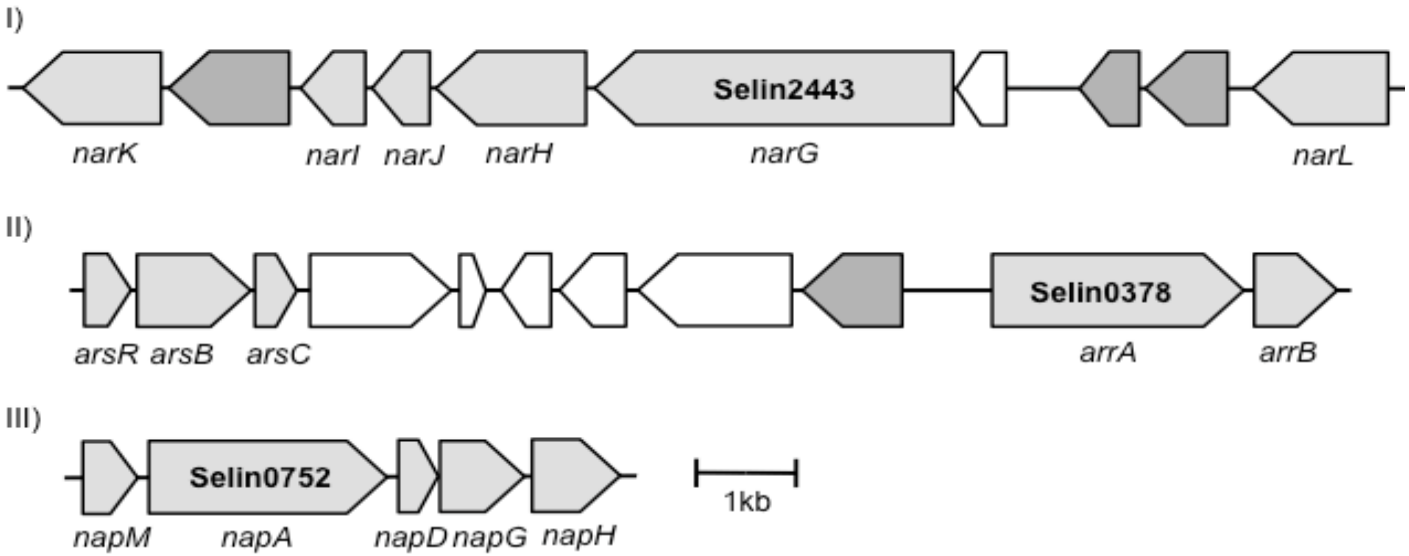


Figure 4.3 Operons encoding putative nitrate and arsenate reductases identified in the genome of strain S5. A) Organization of I) Nar operon II) Arr and Ars operon and III) Nap operon and surrounding gene environment. Genes are drawn to scale. B) Expression of putative operons under I) and III) nitrate reducing conditions (nar and nap) II) arsenate reducing conditions (arr). The number to the left of each gel picture indicate the size of the band in that position.

a)



b)

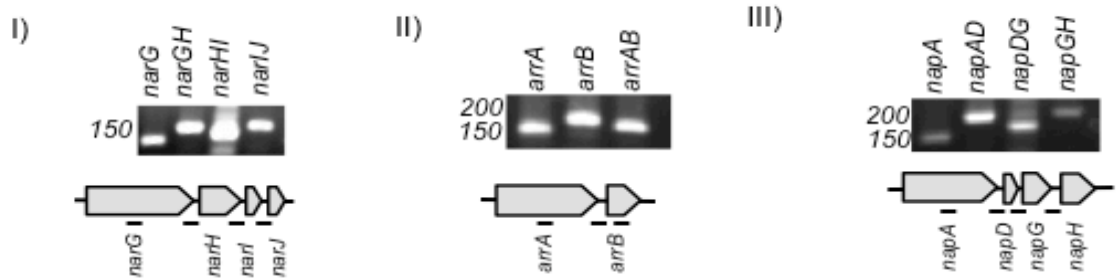


Figure 4.4 Whole cell model of metabolism of *D. indicum*. Proteins belonging to each reductase are indicated in one color. (Circles – putative subunits of reductases; Rectangles – putative transporters; Stars – regulators.)

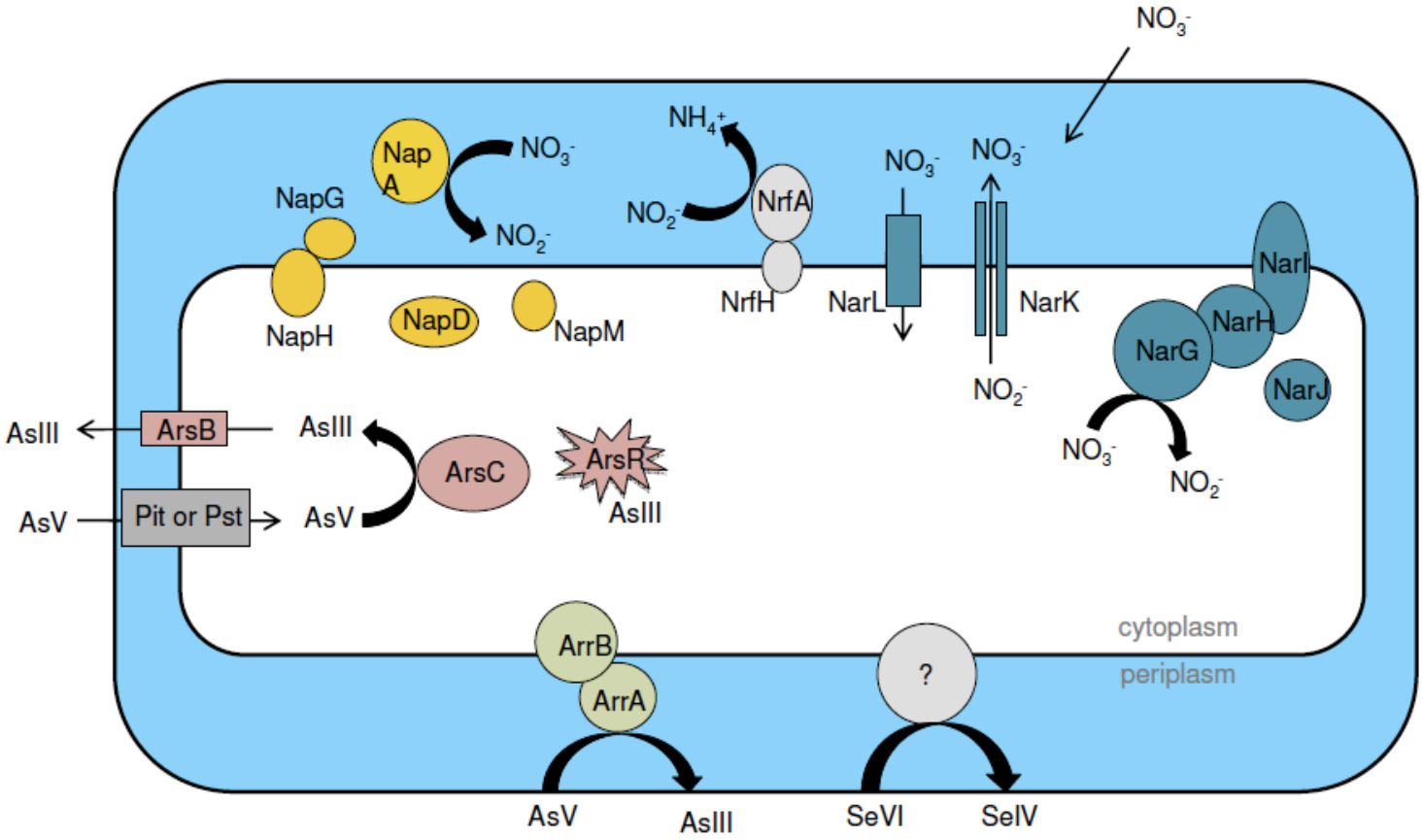


Table 4.1 Primers used to amplify putative reductases in transcription analysis of Arr, Nar, and Nap.

Primer Name	Sequence	Product Length	Target
RTArrA 4F	TCGTATCAAGACTCCCATGAAG	145	Selin_0378
RTArrA 4R	ACTTGTGGGTTTCGTTGTTTTT		
RTArrB 4F	TACAAAAAGGACAATGGCATCA	165	Selin_0379
RTArrB 4R	CCTTGTATTTCGCCTGTACCTC		
RTArrAB 1F	GCCAACTTCAATGAAATCATGC	158	Selin_0378-Sin0379
RTArrAB 1R	GCGCACTTCAGCAGATCTATAA		
RTNarG 4F	GAGATGCAGGCCACTGACTAC	132	Selin_2443
RTNarG 4R	CACGCAGATAGGGGTATTTGAC		
RTNarGH 3F	TTAACTACTGGGGTCCCACCG	150	Selin_2443-Selin_2442
RTNarGH 3R	AGACCTGTGCCCGGATATTCAT		
RTNarHI 1F	GGATAGCGCATGAGGACACT	144	Selin_2442-Selin_2441
RTNarHI 1R	CCATTGAGGCCCATACTGAT		
RTNarIJ 4F	TGTAACCACGCTTTGTACTGCT	153	Selin_2441-Selin_2440
RTNarIJ 4R	GAGTGCGAAGAGTGGGAGTAAC		
RTNapA 5F	CTTCCTGGCCTACAAGTCCAT	149	Selin_0752
RTNapA 5R	TACCATGCTTTTCGTGGAAATA		
RTNapAD 1F	AGCTGAACCCGAGTACAAAATC	185	Selin_0752-Selin_0751
RTNapAD 1R	CTGATAATGCATGCGGAAAGTA		
RTNapDG 1F	CCTGCACGAAGAGATCAGTAAA	169	Selin_0751-Selin_0750
RTNapDG 1R	ATCCTTAAAGGGGTTTTCGTTC		
RTNapGH 1F	AAACAGGAGCTCTCACCTCAAG	191	Selin_0750-Selin_0749
RTNapGH 1R	TTCAGAAAGGGGATCAAAAAGA		

Table 4.2 General features of the *Desulfurispirillum indicum* strain S5 genome.

General Characteristics	Chromosome
Size (base pairs)	2,928,377
G+C Percentage	56.1
No. of Protein Coding Genes	2619
Proteins Assigned to COG Functional Groups	2591
Information Storage and Processing	443
Cellular Processes	932
Metabolism	1154
Poorly Characterized	427
rRNA Genes	9
RNA Genes	37
Transposases	13

CHAPTER 5

THE RESPIRATORY ARSENATE REDUCTASE IN *DESULFURISPIRILLUM INDICUM* STRAIN S5 IS AN INDUCIBLE ENZYME

Abstract

The arsenate respiratory enzyme Arr is found in phylogenetically diverse microorganisms. The genome analysis of the arsenate respiring bacterium *Desulfurispirillum indicum* strain S5 revealed the presence of an operon encoding a putative Arr enzyme. To positively verify that this operon to encodes for the arsenate respiratory enzyme, gene expression was profiled and the results showed 100 times the expression of this gene during arsenate respiration over expression during nitrate respiration. We also determined whether Arr was an inducible enzyme. After growth of strain S5 with nitrate, protein synthesis was inhibited with chloramphenicol and the utilization of arsenate was determined. We found that arsenate was no longer reduced, as compared to cultures without chloramphenicol, indicating the Arr enzyme was an inducible enzyme. This data shows that *D. indicum* strain S5 can regulate its arsenate reductase and respond to arsenate present in its environment.

Introduction

Exposure to elevated levels of As in the ground water is one of the most important environmental, global health risks, threatening the lives of tens of millions of people. Arsenic is considered a human carcinogen, affecting various organs such as skin, liver or the bladder (Das et al. 1996). It is also associated with skin lesions, circulatory disorders, gastrointestinal disease, diabetes, and death. Most non-occupational exposures to inorganic arsenic are related to the ingestion of contaminated drinking water. In order to prevent high concentrations of arsenic in groundwater, the WHO guidelines for As in drinking water are 10 µg/L (WHO, 2004).

As is the twentieth most abundant element in the Earth's crust, is an ubiquitous metalloid found in aquatic and terrestrial environments, where it is mainly associated with metals such as lead, copper and gold (Garelick 2008; Smedley and Kinniburgh 2002). Anthropogenic sources that contribute to accumulation of As include mining activities, waste processing, and the use of pesticides (Nordstrom 2002). Arsenic has four oxidation states of which As(V) and As(III) are the most prevalent in nature. As(V) is mainly associated with aerobic environments and is readily adsorbed to the surface of several common minerals that reduces its hydrologic ability, thus making it a less toxic. Arsenite (As(III)) is prevalent in anaerobic conditions. It adsorbs less strongly and to fewer minerals and thus is more mobile and more toxic (Oremland and Stolz 2003).

Even though As is highly toxic, it is readily metabolized by some microbes which heavily influence the biogeochemical cycle of this element (Oremland and Stolz 2003, Paez-Espino et al. 2009, Rowland et al. 2009). Most microorganisms not only display As resistance, some utilize As as alternate terminal electron acceptor in the process of dissimilatory reduction (Macy et al. 1996, Stolz and Oremland, 1999, Silver and Phung 2005). As respiration is directed by the arsenate respiratory reductase Arr, consisting of

the molybdenum containing catalytic subunit ArrA and the membrane boung, heme-containing subunit ArrB (Stolz et al. 2006).

To date, arsenate reductases (Arr) have only been purified from *Chrysiogenes arsenatis, Bacillus selenitireduces*, and *Shewanella* sp. strain ANA-3 (Schroeder et al. 1997; Krafft et al. 1998; Affkar et al. 2003; Malasarn et al. 2008). The sequences of all three enzymes are highly conserved and consisted of two main subunits. Arr is only functional when expressed as a heterodimer, with a larger molybdopterin containing subunit ArrA and a smaller, iron-sulfur containing subunit, ArrB. The characteristic features of respiratory arsenate reductases are summarized in Table 1.5. A putative arsenate reductase Arr was also identified in the genome of *D. indicum* strain S5 (Rauschenbach et al. 2007b; Chapter 4). The protein sequence for the putative subunit ArrA (Selin_0378) was 79% identical and 87% similar to Arr found in *C. arsenatis.* Downstream, a putative ArrB was identified and found to be 53% similar and 40% identical to ArrB of *C. arsenatis*.

Studying the respiratory arsenate reductase in *Shewanella* sp. strain ANA-3 (Saltikov et al. 2003) had advantages, as it is a facultative anaerobe, allowing for easier molecular laboratory manipulation and characterization of the expression patterns of *arr*. The two genes encoding the Arr sunits, ArrA and ArrB, were cloned into *E. coli* and shown to be required for As respiration (Saltikov and Newman 2003). In addition, studies of *arrAB* regulation revealed the upregulation of these genes during anaerobic respiration even in the presence of low concentrations of As (Saltikov et al. 2005).

Based on the knowledge about the three characterized arsenate reductases and the studies performed with *Shewanella* sp. strain ANA-3, we also wanted to understand the posttranscripional activity of the putative Arr identified in *D. indicum* strain S5.

Materials and Methods

Growth of D. indicum and DNA Extraction. *D. indicum* strain S5 was anaerobically grown in mineral salts medium (Fennell et al. 2004) containing 8 mM nitrate or arsenate as electron acceptor and 10 mM pyruvate as electron donor at 28 ºC, unless otherwise stated. Growth of strain S5 was analyzed by measuring the optical density at 600 nm (As(V) and nitrate); loss of e^-acceptors (As(V) and nitrate) via ion chromatography (Rauschenbach et al. 2011a; Chapter 2)**.** Genomic DNA was extracted by phenol-chloroform extraction as previously described (Kerkhof and Ward, 1993) from a 5 mL culture. The DNA was used to check the specificity of primers (Table 5.1), developed for the narG, arrA, Selin_2495, and Selin_2609 genes (Rauschenbach et al. 2011b; Chapter 3).

Gene Expression Analysis of Molybdoenzymes via qRT-PCR. *D. indicum* strain S5 was pregrown in mineral salts medium amended with 10 mM pyruvate as electron donor and carbon source and either 10 mM nitrate, 10 mM selenate or 10 mM arsenate as sole electron acceptors. Cultures were subcultured at least 10 times to ensure that only one electron acceptor was present and utilized at the time of RNA extraction. Total RNA was isolated from 7 mL of a log phase culture using TRIZOL as described by Invitrogen with the following amendments. Cells were pelleted, resuspended in 1 mL RNA Protect (Quiagen) and incubated for 15 minutes at room temperature. They were then again pelleted by centrifugation; and the supernatant was discarded. After centrifugation, cells were washed twice with Tris-NaCl buffer (20 mM Tris, 15 mM NaCl in H_2O; pH 8). 500 µL of buffer were used in the first and 250 µL in second wash. The cells were resuspended and flash frozen with dry ice and ethanol. The cells were thawed and 3 µL of lysozyme (5 mg/mL lysozyme in TE buffer) were added. RNA was extracted as described in the protocol provided by Invitrogen (USA).

After RNA extraction, DNAse treatment (Invitrogen, USA) was followed by PCR using primers targeting the 16S rRNA gene of strain S5 to rule out DNA contamination. Quantitative Reverse Transcription-PCR (qRT-PCR) reactions were set up using 30 ng of RNA in a one step qRT-PCR approach in 15 µL reactions using the iScript One-Step qRT-PCR with SYBR Green Kit, according to the manufacturer's protocol (BIORAD, USA). Primers used in the analysis are summarized in Table 5.1. Two negative controls were included, one omitting the reverse transcriptase and one without template.

Inhibition Studies of Arsenate Respiration. Strain S5 was anaerobically grown in minimal salts medium with 10 mM pyruvate as carbon source and electron donor and either 5 mM arsenate or nitrate as electron acceptor. The minimal inhibitory concentration (MIC) of chloramphenicol was determined by adding a range of 5 to 50 µg/mL of the antibiotic to the medium. Growth was followed by measuring the optical density of each culture at 600 nm and by analysis of reduction of nitrate and arsenate. Growth of strain S5 was inhibited by chloramphenicol at a concentration of 30 µg/mL. Cultures were then grown with 10 mM pyruvate as electron acceptors and 8 mM nitrate or arsenate. Once cultures were depleted of nitrate, 30 µL chloramphenicol and 8 mM of either nitrate or arsenate were added. Controls without chloramphenicol were also spiked with the same concentration of electron acceptors. The concentrations of nitrate, selenate, and arsenate were measured via ion chromatography (Chapter 2).

Results and Discussion

The characterization of the properties of arsenate respiration and expression of Arr are important to elucidate the contribution of the enzyme to the bio-geochemical cycle of As. About 8 mM arsenate was respired within 24 hours of growth (Figure 5.1). Cell increase was lower than on other tested electron acceptors (Figure 5.2), yet

proceeded at a faster rate as compared to growth with either nitrate or selenite as electron acceptors.

We also studied the levels of gene expression of the previously identified putative Arr of strain S5. During exponential growth of strain S5, the putative arrA was expressed almost 100-fold as compared to levels of arrA expressed under nitrate respiring conditions (Figure 5.3). None of the other genes encoding for molybdoenzymes monitored in strain S5 showed any significant increase. The expression level of *arr* in strain S5 is similar to levels found in *Shewanella* sp. strain ANA-3 (Saltikov et al. 2005). ArrA was upregulated at the beginning of the exponential phase and reached maximal expression before the end of the exponential phase (Saltikov et al. 2005; Malasarn et al. 2008). In addition, Arr activity persisted beyond the time point at which Arr transcripts were detected (Malasarn et al. 2008). The expression of *arr* in strain S5 is consistent with the proposed function of Arr as a respiratory arsenate reductase. Levels of *arr* expression were significantly high during the exponential growth of the organism.

The antibiotic chloramphenicol is known to inhibit protein synthesis and has been used to determine whether genes are inducible or whether they are constitutively expressed (Oremland 1999). We also employed this approach to find out whether Arr is induced only in the presence of arsenate. The MIC of chloramphenicol in strain S5 was 30 µg/mL (Figure 5.4a). Cells pregrown on nitrate were either spiked with both chloramphenicol and arsenate or just arsenate as comparison. While nitrate reduction proceeded, none of the arsenate was reduced when protein synthesis was inhibited with chloramphenicol (Figure 5.4b, c). These results suggest that Arr is an inducible enzyme that is only expressed when strain S5 is exposed to arsenate. Similarly, in *Shewanella* sp. strain ANA-3, *arr* was only expressed in the presence of arsenate (Saltikov et al. 2005). Even low concentrations of arsenate, such as 100nM, induced the expression of

this enzyme. In contrast, *arr* was repressed under nitrate respiring conditions (Saltikov et al. 2005).

Conclusions

In summary, our results verify the identity of the putatively identified *arr* operon as the respiratory arsenate reductase in *D. indicum* strain S5. *Arr* was the only terminal reductase gene highly expressed during As respiration. Also, we concluded that Arr was an inducible enzyme that is only expressed in the presence of arsenate. These results are in accordance with characterizations of other Arr systems (Saltikov et al. 2005; Malasarn et al. 2008) suggesting that Arr systems not only bear sequence homology, but that they are also functionally homologous.

Arsenic oxyanion speciation in nature is greatly influenced by the microbial communities in various environments. Further comparative and functional studies of different arr operons, enzymes, and Arr families will also help elucidate the mechanisms by which these enzymes function, possibly resulting in the development of specific inhibitors for microbial reduction of As oxyanions.

Figure 5.1 Growth of strain S5 with arsenate as electron acceptor and pyruvate as carbon source and electron donor (◆Loss of As(V); ▲Cell density; ■ Pyruvate Only, No electron acceptor).

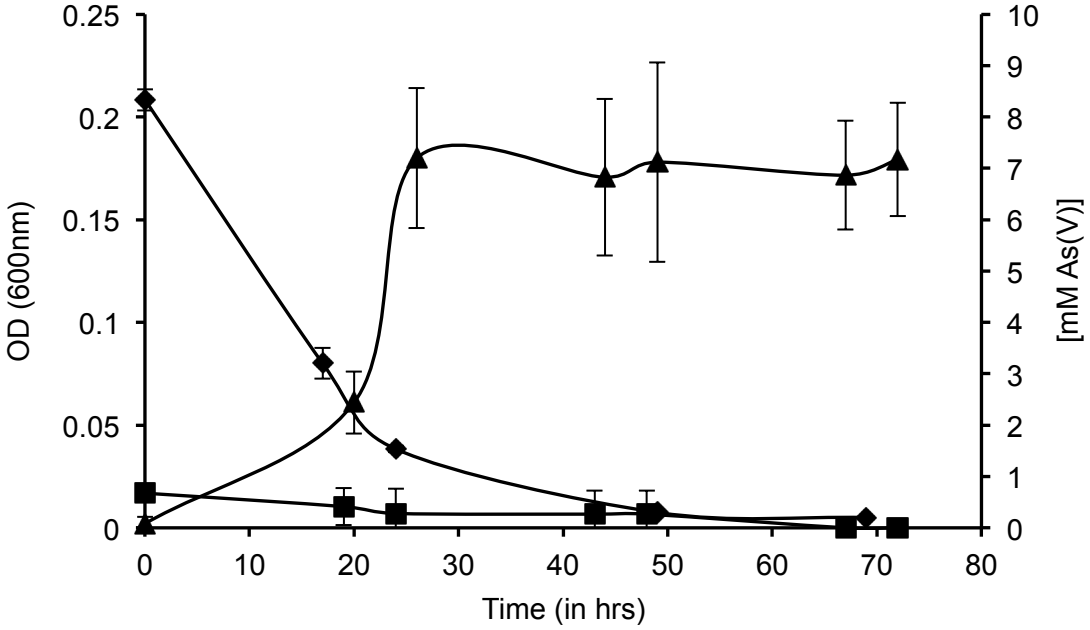


Figure 5.2 Growth of strain S5^T with 10 mM pyruvate as electron donor and carbon source and various electron acceptors (10 mM) (▲ Nitrate; ■ Se(VI); ● As(V); ◇ No Electron Acceptor).

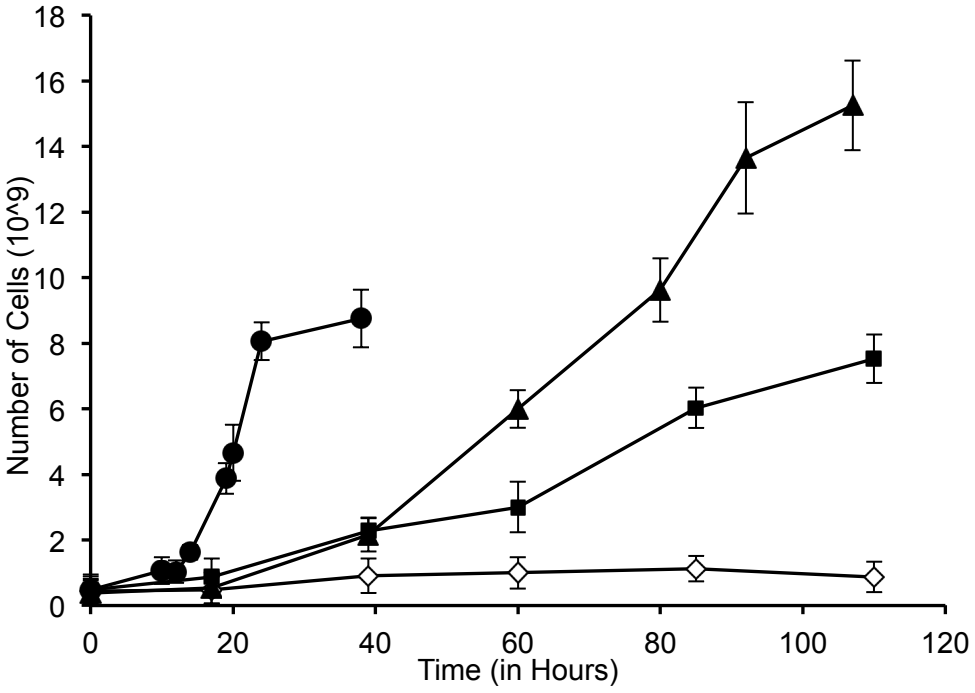


Figure 5.3 Fold expression of molybdoenzymes in *D. indicum* strain S5 as compared to gene expression levels under nitrate respiring conditions.

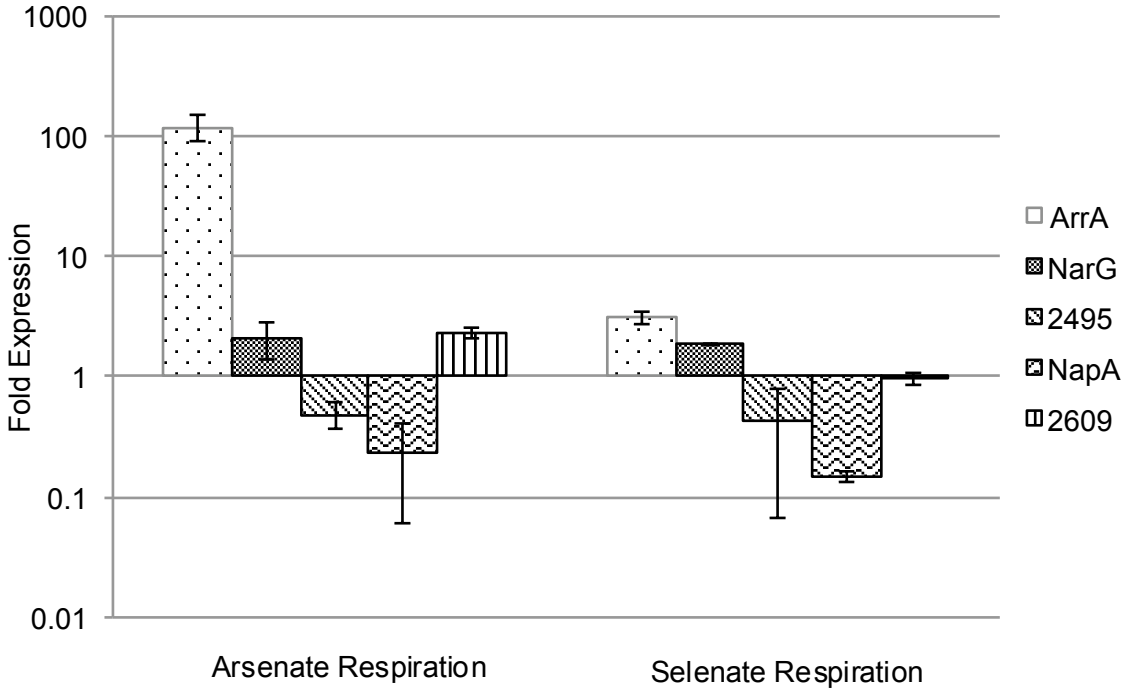
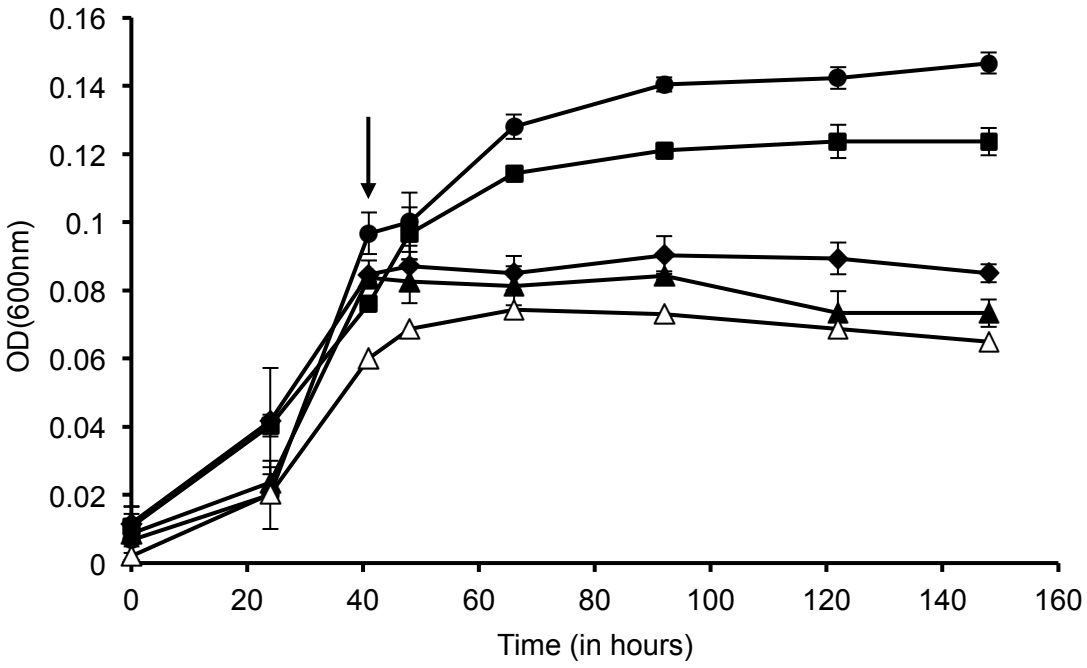
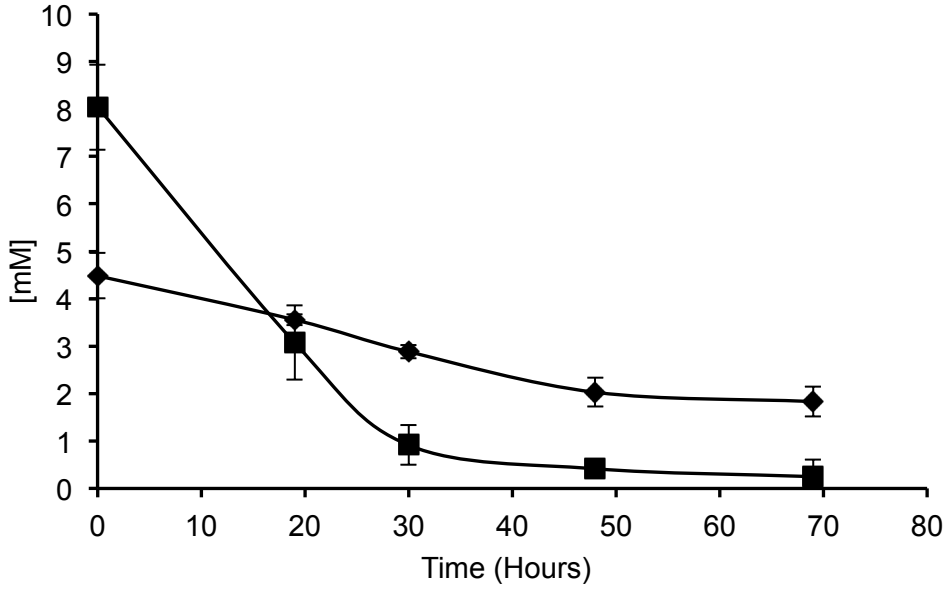


Figure 5.4 Inhibition of arsenate reduction with chloramphenicol a) Minimal inhibitory concentration of chloramphenicol of strain S5 (Chloramphenicol concentrations: ■ 5 µg /mL; ◆ 10 µg/mL; ▲ 20 µg/mL; △ 50 µg/mL; • 0 µg/mL) b) Nitrate and arsenate respiration of strain S5 (◆Nitrate; ■ As(V)) c) Inhibition of arsenate reduction during nitrate respiration (◆Nitrate; ■ As(V))

a)



b)


10
9
8
7
6
5
4
3
2
1
0
[mM]
0
10
20
30
40
50
60
70
80
Time (Hours)

c)

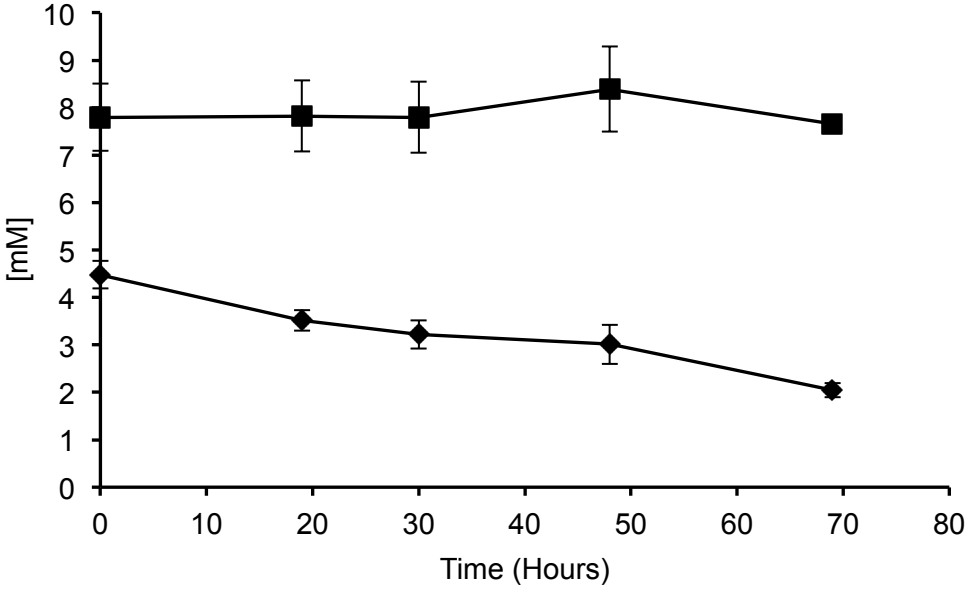

10
9
8
7
6
5
4
3
2
1
0
[mM]
0
10
20
30
40
50
60
70
80
Time (Hours)

Table 5.1 Table of Primers used for qRT-PCR analysis.

Primer Name	Sequence	Product Length	Target
RTArrA 4F	TCGTATCAAGACTCCCATGAAG	145	Selin_0378
RTArrA 4R	ACTTGTGGGTTTCGTTGTTTTT		
RTNarG 4F	GAGATGCAGGCCACTGACTAC	132	Selin_2443
RTNarG 4R	CACGCAGATAGGGGTATTTGAC		
RTNapA 5F	CTTCCTGGCCTACAAGTCCAT	149	Selin_0752
RTNapA 5R	TACCATGCTTTTCGTGGAAATA		
RT2495F	GCTACAGCGAAACCCTCAGTT	169	Selin_ 2495
RT2495R	GTACAGCCATTACACACCGAAG		
RT1482F	TACAAGCAGAACGTGGTTGACT	192	Selin_ 2609
RT1482R	GCTCAATACCGTTTACCCCATA		

CHAPTER 6

TELLURIUM RESPIRING BACTERIAL COMMUNITY ISOLATED FROM SLUDGE

Abstract

The rare element tellurium (Te) is found in the same group as selenium in the periodic table and has similar chemical characteristics. The biological function of the element is unknown, the natural Te-cycle has not been investigated and the role of microbes in this process is poorly understood. In order to learn more about microorganisms that may be capable of dissimilatory Te reduction, activated sludge sediments were incubated with lactate, pyruvate, and acetate as carbon sources and either tellurate or tellurite as electron acceptors. The enrichment cultures progressively turned black, evidence for the reduction of the tellurium (Te) oxyanions and precipitation of elemental Te. Denaturing gel gradient electrophoresis and clone libraries showed the presence of two different microorganisms (Figure 6.1). This study contributes to the increasing variety of environments from which dissimilatory Te-reducing microbes have now been isolated.

Tellurium (Te) is a toxic metalloid that can be found native or combined with other metals, such as lead, gold, silver, copper and antimony (Rathgeber et al. 2002). It is relatively rare, composing only about $2x10^{-7}$ percent of the lithosphere (Rathgeber et al. 2002). The major source of tellurium is the anode sludge produced during the refinement of blister copper (Bagnall 1966; Rezanka and Siegler 2008). It is currently used in various alloys, glasses, and semiconductors (Chasteen and Bentley 2003; Taylor 1999). Te improves machinability of copper and lead without decreasing conductivity. When added to lead, it improves strength, hardness, and resistance to vibration and fatigue (Bagnall 1966; Taylor 1999). Te is also added to cast iron for chill control, to steel for toughness, and used as a reagent in producing the black finish on silverware. It has been employed as the major photosensitive material in infrared detectors and in semiconductors and solar cells (Taylor 1999). Demand for Te in these industrial applications is increasing since native tellurium is rare and mostly found as part of a metal compound (George 2006).

Tellurium has four chemical oxidation states: Te(VI+) (tellurate), Te (IV+) (tellurite), Te(0) (elemental tellurium), and Te(II-) (telluride) (Bagnall 1966, Cooper 1971; Taylor 1999). Te(IV) is more toxic to most gram-negative bacteria than Se(IV) (Zannoni et al. 2008). In fact, in the 1930s, Alexander Fleming first reported on the antimicrobial properties of tellurium, as it was routinely used a selective antimicrobial agent in growth media (Fleming 1932). Te compounds also found applications as therapeutic agents for the treatment for leprosy, tuberculosis, dermatitis, and eye infections (Taylor 1999).

The mechanisms of resistance to Te-oxyanions involve their physical removal from the cell's immediate aqueous environment (Csotonyi et al. 2006). This can be achieved by reductive precipitation of insoluble, elemental Te or the formation of dimethyl telluride (Baesman et al. 2007; Summers and Jacoby 1977). The precipitated Te(0) has a black color and will localize on the inside or outside of the cells (Tucker et al.

1961; Rathgeber et al. 2002; Baesman et al. 2007; Taylor 1999). Recently, research has focused more on the microbial reductive capability of Te oxyanions (George 2006). Few organisms have been described that can reduce Te oxyanions (Switzer Blum et al. 1998; Stolz et al. 1999; Baesman et al. 2007; Baesman et al. 2009; Csotonyi et al. 2006). This study describes a dissimilatory tellurate and tellurite reducing bacterial community that was enriched from activated sludge.

Genomic DNA was extracted by phenol-chloroform extraction (Kerkhof and Ward 1999) with the following changes: 5 mL of either tellurate or tellurite respiring cultures were pelleted and approximately 600 ng/μL of archaeal DNA from *Sulfolobus solfataricus* strain P2 was added to bind the tellurite minerals and increase DNA yield. Microbial community composition was determined by DGGE (Figure 6.1) (Muyzer et al. 1989). An 8% acrylamide/bis gel with a 40 to 80 % gradient was utilized, which was exposed to 55 Volts for 17.5 hours. The gel was stained with ethidium bromide. The DGGE analysis revealed the presence of two distinct bands, evidence that two different organisms were present in the culture. The bands were excised and sequenced by Genewiz, Inc. (South Plainfield, NJ, USA). In addition, clone libraries were constructed using pGEM-T Easy Vector System I (Promega, WI, USA) and cloned into Library Efficiency DH5α Cells (Invitrogen, CA, USA) following the directions of the manufacturer. Eight plasmid clones with 16S rRNA gene insert were extracted with Qiagen QIAprep Spin Miniprep Kit (Qiagen, USA). The clones were sequenced by Genewiz, Inc. (South Plainfield, NJ, USA).

Sequence data collected for the clone libraries were compared to related microorganisms by BLAST search (Altschul et al. 1997) and downloaded from GenBank. Phylogenetic and molecular evolutionary analyses of all sequences were conducted

using MEGA5 (Tamura et al. 2011), which utilized ClustalW (Chenna et al. 2003) to align the sequences. Neighbor-joining phylogenetic trees were constructed, which were computed using the Maximum Composite Likelihood method with 1000 bootstrap replications (Tamura et al. 2004). The selected clones were most closely related to *Clostridium* spp. (Figure 6.2).

To demonstrate respiration of tellurate and tellurite, acetate utilization was measured using HPLC analysis (Shimadzu; C_{18} column, 250 mm by 4.6mm, 5μm particle size; Phenomenex Sphereclone, as described in Narasingarao and Häggblom (2007a). No acetate was utilized in the absence of an electron acceptor, indicating that the culture was able to respire tellurate or tellurite. Acetate is a non-fermentable substrate and no other fermentable substrates, such as yeast extract or peptone had been added to the culture (Figure 6.3). About 5 mM acetate was reduced in the presence of either tellurate and tellurite (Figure 6.3).

In order to test for optimum pH, the enrichment cultures were grown with 10 mM acetate as electron donor and carbon source and 250 μM tellurate or tellurite as electron acceptor in anaerobic growth medium. The pH of the medium was adjusted with HCl or NaOH. The optimum pH for the culture was found to be between 6.8 and 7.2. Growth at different temperatures (4 °C and 37 °C) was determined by growing cells in anaerobic medium (pH 7) at the above decribed conditions. The cultures grew at a temperature range between 25 °C and 37 °C, with an optimum at 28 °C.

This study is only one of a few studies that have demonstrated the capability of bacterial cultures to respire tellurate and tellurite. *Bacillus selenitireducens,*

Sulforospirillum barnesii, and *Bacillus beveridgei* and other strains isolated from hydrothermal vents can also utilize Te oxyanions as electron acceptors in respiration (Baesman et al. 2007; Baesman et al. 2009). The process of Te-oxyanions reduction is still poorly understood thus the isolation and characterization of Te-respiring microorganisms is important to further the knowledge about this chemical and elucidate its biogeochemical cycle in the environment.

Figure 6.1 DGGE gel picture of tellurate and tellurite respiring community.

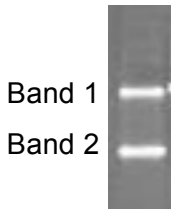


Figure 6.2 Phylogenetic tree comparing six clones from tellurate respiring bacterial community with closely related species. The tree was constructed by aligning 16S rRNA gene sequences using Neighbor-joining algorithm. The evolutionary distances were computed using Maximum Composite Likelihood method (498 positions in analysis). Bootstrap values above 50 are indicated.

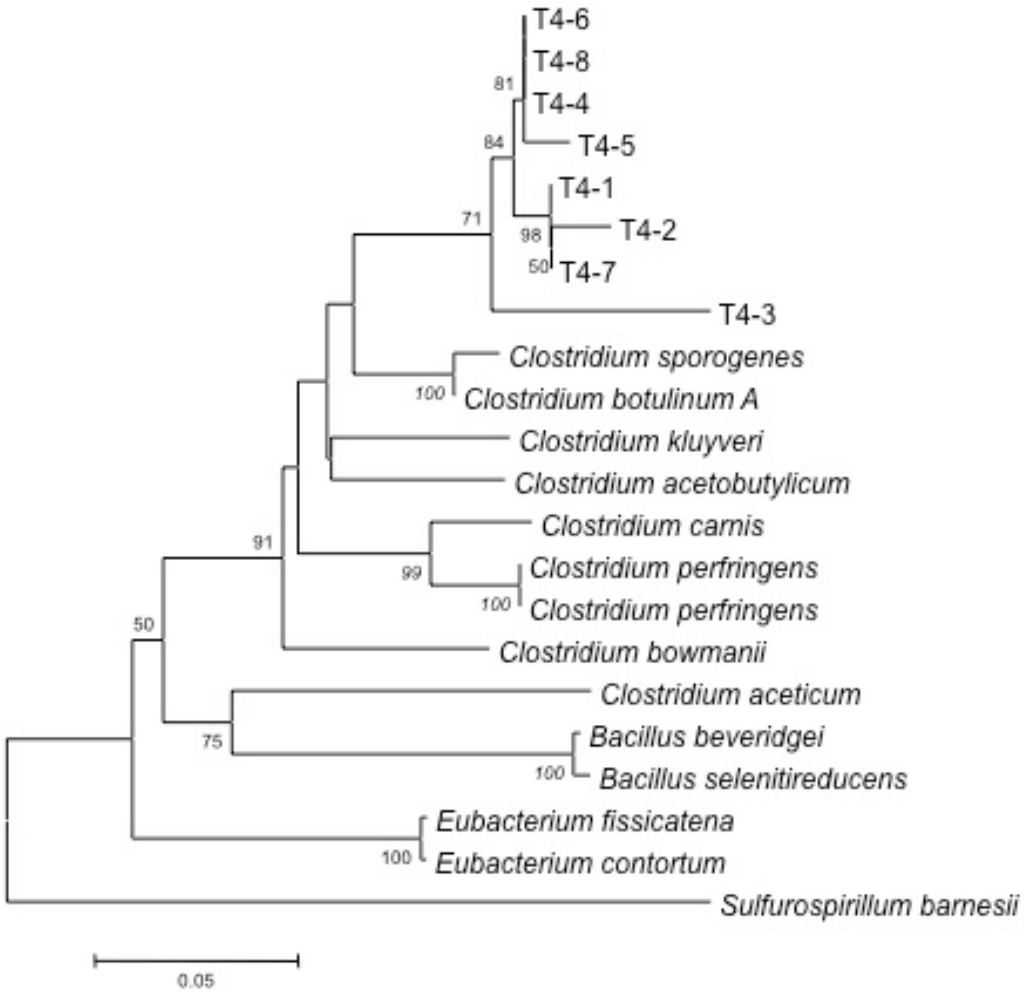
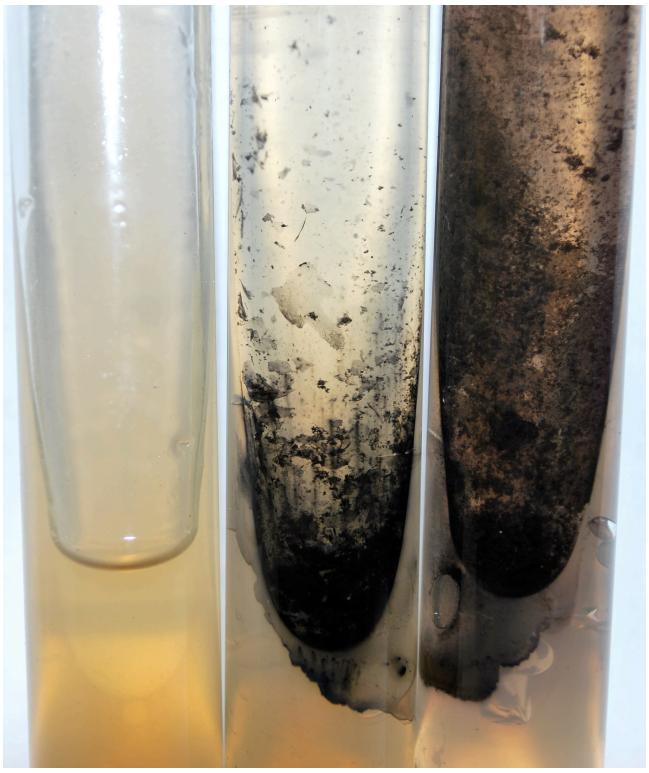


Figure 6.3 Tellurium respiring cultures. Visible black precipitate indicating the presence of elemental tellurium. a) From left to right: uninoculated slant, tellurite-respiring culture, tellurate-respiring culture b) Bacterial colonies growing in 250 µM tellurate and 10 mM acetate.

a)



b)

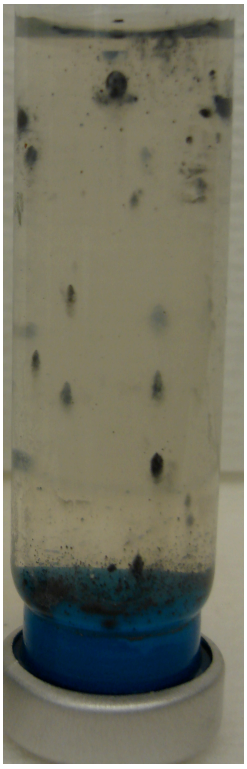
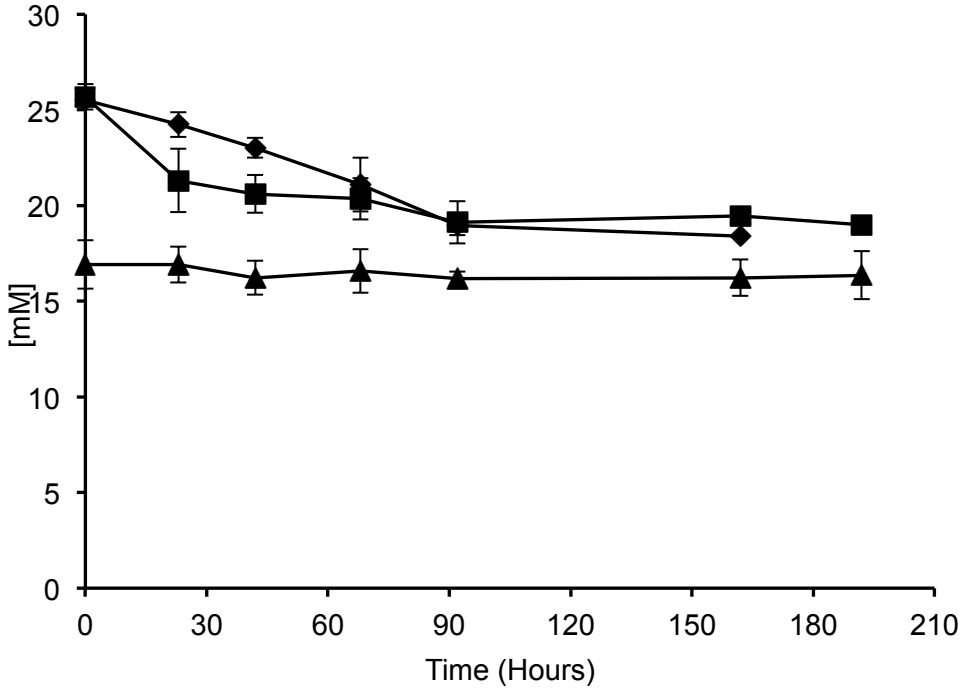


Figure 6.4 Acetate oxidation in the presence of tellurate and tellurite as electron acceptors. (◆ Te(IV); ■ Te(VI); ▲Acetate Only).



CHAPTER 7

CONCLUSIONS

Speciation of As and Se, which affects their toxicity and mobility in the environment, is greatly affected by activity of the microbial populations (Oremland and Stolz 2003). Over the past years, intensive efforts have been made to isolate and characterize new microorganims that are capable of generating energy from oxidation/reduction reactions of Se and As oxyanions. These efforts were supported by the identification of environments greatly influenced and altered by the presence of Se and As, development of selective media, and testing Se and As oxyanions as electron acceptors while characterizing new isolates. As a result, the list of microorganisms capable of Se and As redox reaction has continued to grow (Table 1.4).

In this study, we were able to isolate two novel, arsenate and selenate-respiring bacteria, "*Selenovibrio woodruffii*" strain S4^T (Chapter 2) and *Desulfurispirillum indicum* strain S5^T (Chapter 3; Rauschenbach et al. 2011a). Both were strictly anaerobic, non-fermenting, Gram-negative bacteria. They differed in their capabilities to utilize various electron acceptors. Strain S4^T only utilized acetate as carbon source in the presence of arsenate and selenate (Chapter 2), while the metabolically diverse strain S5^T was able to utilize lactate, acetate, and pyruvate as carbon sources and selenate, selenite, nitrate, nitrite, and arsenate as electron acceptors (Chapter 3, Rauschenbach et al. 2011a). Surprisingly, nitrate, a common electron acceptor in respiration, was not utilized by strain S4^T. Some researchers had previously argued that the nitrate reductase systems might also be responsible for selenate reduction (Oremland et al. 1999). The lack of nitrate reduction in strain S4^T may hint to the presence of a novel, nitrate independent selenate reductase.

The isolated strains belonged to different phyla within the bacterial domain (Figure 7.1). "*Selenovibrio woodruffii*" strain S4^T belongs to the family Deferribacteres and shares only 92% homology with its closes relative, *Denitrovibrio acetiphilus* (Chapter 2). *Desulfurispirillum indicum* strain S5^T, is only the third described member of the family Chrysiogenetes (Macy et al 1996). Strain S5^T shares 93% 16S rRNA gene homology with *Chrysiogenes arsenatis* and 98% 6S rRNA gene homology with *Desulfurispirillum alkaliphilum*, its closest known relatives (Macy et al. 1996; Narasingarao and Häggblom 2007, Sorokin et al. 2007).

Se is only the 69th most abundant in the Earth's crust, yet microorganisms can readily utilize Se oxyanions for cellular respiration (Table 1.3). The question remains as to how these microorganisms were able to evolve systems that can reduce Se oxyanions. Perhaps, selenate reduction developed as a mean to survive in the absence of other more abundant electron acceptors. Other possibilities could be that selenate fits into the active sites of multiple other molybdenzymes or that genes encoding selenate reductases may have been acquired through gene transfer. As can be highly toxic to cells and As respiratory systems may have evolved to help detox cells containing high amounts of As. Microorganisms, e.g., *D. indicum* (Chapter 4) and *Shewanella* sp. ANA-3 (Saltikov et al. 2005) have a separate detoxification systems Ars to help facilitate As detoxification.

Archaea and bacteria can readily oxidize, reduce, methylate, and demethylate As and Se. In addition to unraveling the phylogenetic diversity of these organisms, studies of the genes and enzymes involved in reduction and respiration of As and Se have contributed to the elucidation of the biogeochemical cycles of these elements in nature (Figure 7.2) and helped develop models to predict microbial activity, which mechanisms are involved, and under what conditions these mechanisms will function. We utilized *D. indicum* as a model organism to characterize the gene and enzyme systems that govern

anaerobic respiration.

The annotation of the genome sequence of *D. indicum* enabled us to identify and characterize the reductases and molybdoenzymes involved in selenate, arsenate, and nitrate respiration (Chapter 4). Genome analysis uncovered five sequences with signatures of molybdoenzyme oxidoreductases (Selin_0378, Selin_0752, Selin_2443, Selin_2495, and Selin_ 2609). These protein sequences were aligned with sequences of characterized reductases and compared in phylogenetic analysis. Based on sequence homology, were able to deduce the putative functions of three of the five molybdoenzyme sequences, Selin_0378, Selin_ 0752, and Selin_2443. Selin_0378 clustered with the respiratory arsenate reductase Arr; Selin_ 0752 with the periplasmic nitrate reductase subunit NapA; and Selin_2443 with the membrane-bound nitrate reductase NarG. The two others were more distantly related to known molybdoenzymes and their role in the metabolism of strain $S5^T$ remains unknown (Figure 4.1).

Further genome analysis showed evidence for the presence of orfs encoding additional subunits of each enzyme and their gene organization resembled typical *arr, nar*, and *nap* operons (Figure 4.3). RT-PCR analysis across contiguous genes demonstrated that these genes indeed constitute functional operons. The results of this genomic analysis provided new insights into metabolic pathways for energy production in contaminated environments, and how these pathways are regulated depending on the availability of oxyanions.

The identification of an As detoxification system (Ars), as well as respiratory nitrate (Nar, Nap) and As reductases (Arr) in strain $S5^T$ to previously studied, phylogenetically diverse organisms (Chapter 4) indicates possible gene transfer. The presence of multiple mechanisms for As reduction in strain S5 and other organisms (Salktikov et al. 2005) is an indication of convergent evolution, while the high similarity of the respiratory reductase Arr between strain S5 and other phylogenetically diverse organisms is

suggestive for horizontal gene transfer.

The gene(s) and enzyme(s) responsible for Se reduction in Strain S5^T are still unknown and remain to be studied. Our assumption was that the Se reductase would be among the five molydoenzymes found in the genome of Strain S5^T but we were unsuccessful in identifying it (Chapter 4). Selin_2495 had low similarity with a recently described selenate reductase Srd in *B. selentarsenatis* (Kuroda et al. 2011). However, expression studies of this gene showed that is was not upregulated, just as any of the putative molybdoenzymes, during selenate respiration. These results suggest that selenate reduction may be a secondary capability of some of the identified molydoenzymes present in Strain S5^T (Figure 4.1) or that the process is carried out by a yet to be identified, novel cytochrome of the organism. Nitrate seems to be an effective inhibitor of selenate respiration, suggesting that in the absence of nitrate, selenate reduction could be mediated via the nitrate reductase (Appendix C).

RNA expression studies of the molybdoenzymes identified in strain S5^T clearly showed that Selin_0378 was the respiratory As reductase (Chapter 5). *Arr* was the only gene highly expressed under arsenate respiring conditions (Figure 5.3). Inhibition of protein synthesis with chloramphenicol also showed that the gene was inducible (Figure 5.2). These results are in accordance with other Arr systems (Saltikov et al. 2005; Malasarn et al. 2008). The identification of the genetic and biochemical factors that influence the expression dynamics of *arr* operons may help develop tools to specifically sense different forms of inorganic arsenic in the environment. Further comparative and functional studies of different *arr* operons, Arr enzymes, and Arrfamilies will also help elucidate the mechanisms by which these enzymes function, possibly resulting in the development of specific inhibitors for microbial reduction of As oxyanions in environments with high concentrations of As.

Te is element Group 16 of the periodic table sharing similarities with selenium, sulfur and oxygen. Around the 1930s, German scientist Wilhelm Blitz coined the term “chalcogen,” from chalcos old Greek for “ore,” to collectively describe this group of elements. In comparison to the growing number of phylogenetic diverse Se and As-respiring microorganisms, there have only been four Te-reducing microorganisms characterized (Csotonyi et al. 2006; Baesman et al, 2007, 2009). Since not much is known about the Te biogeochemical cycle in nature, we wanted to apply our knowledge about culturing and isolating unknown microorganisms to characterize the microorganisms present in Te-respiring enrichments and contribute to elucidation of Te-cycling and speciation in nature. A bacterial community capable of respiring tellurate and tellurite in the presence of acetate as carbon source was enriched. Analysis of the 16S rRNA gene sequence from clones obtained from the enrichment culture demonstrated that they were closely related to *Clostridium* spp. (Chapter 6).

Similar to Se(0) crystals that closely associate with bacterial cells (Figure 2.2, Figure 3.2), Te-nanocrystals have been found to accumulate extracelluary in Te-respiring bacteria, *Bacillus selenitireducens* and *Sulfurospirillum barnesii* (Baesman et al. 2007). The presence of these nano-sized Te(0) particles in the cultures could have potential practical industrial applications. Our enrichment cultures should also evaluated for the presence of Te(0) nanocrystals. Microbially excreted Te(0) crystals could find application in solar panels and other nano-optics and replace the harsh reagents and high temperatures employed for their chemical synthesis

This thesis has provided new insights into the microbial metabolism of Se and As, as well as Te. The phylogenetic diversity and ubiquity of the processes involving these elements show that they have been important selective factors in microbial evolution. Available genome data with continue to help researchers explore the metabolic diversity of these organisms, confirming common themes among known reductases and discover

new pathways and regulatory elements. It is well established that annotation data does not necessary give the right clue of a sequence's function, thus their function needs to be verified by physiological, molecular, and biochemical experiments.

The global geochemical cycles of selenium and arsenic have been greatly influenced by irresponsible anthropogenic activities (Figure 7.2). The most important challenges ahead are to translate the knowledge derived from the many studies carried out on these elements into science-based programs to intervene and stop further adverse effects on humans, animals, and the wider ecosystem.

Figure 7.1 Phylogenetic tree comparing selenate and arsenate respiring microorganisms. The tree was constructed with aligned 16S rRNA gene sequences using the Neighbor-joining method. The evolutionary distances were computed using Maximum Composite Likelihood method with 1334 positions in the final analysis. Bootstrap values above 50 are indicated.

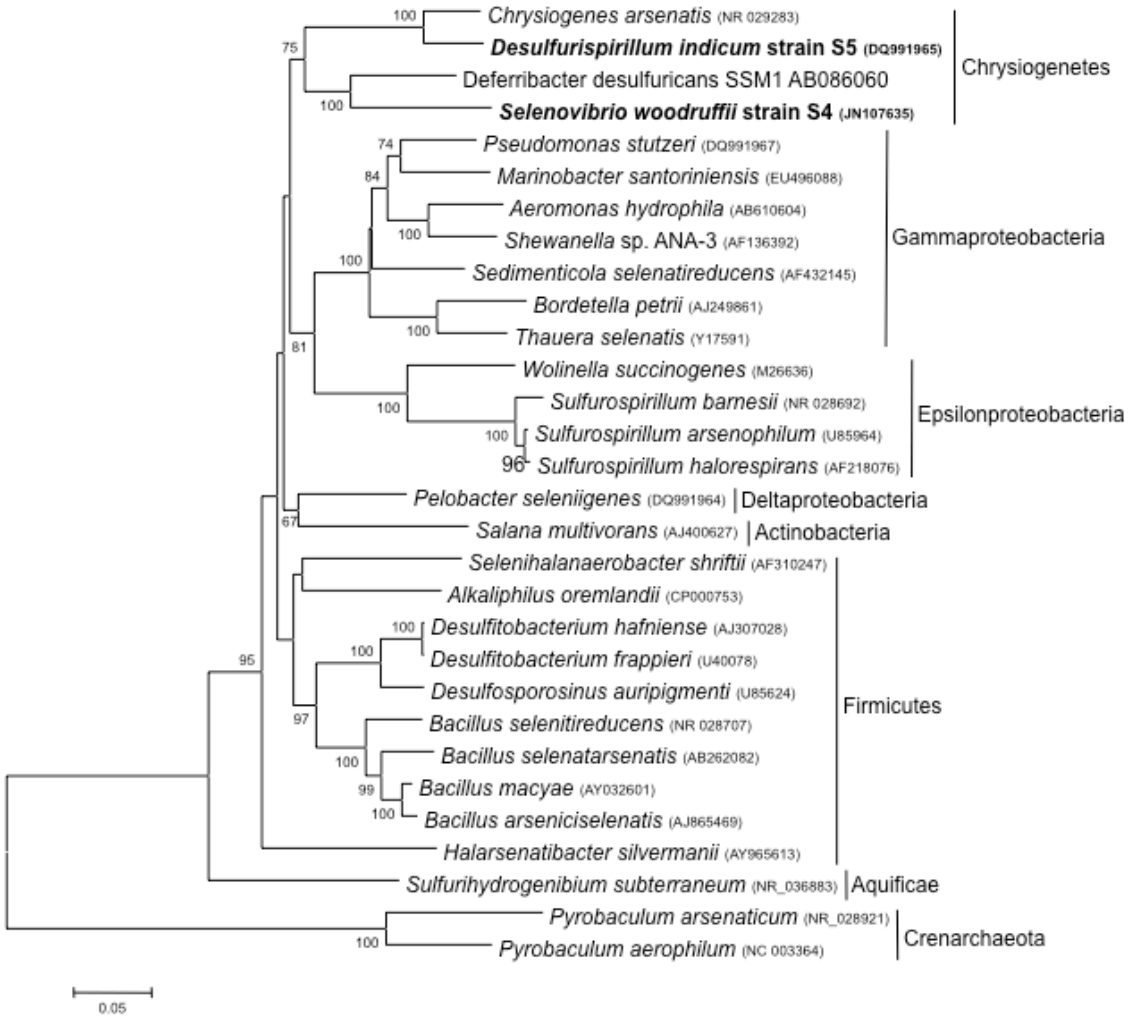
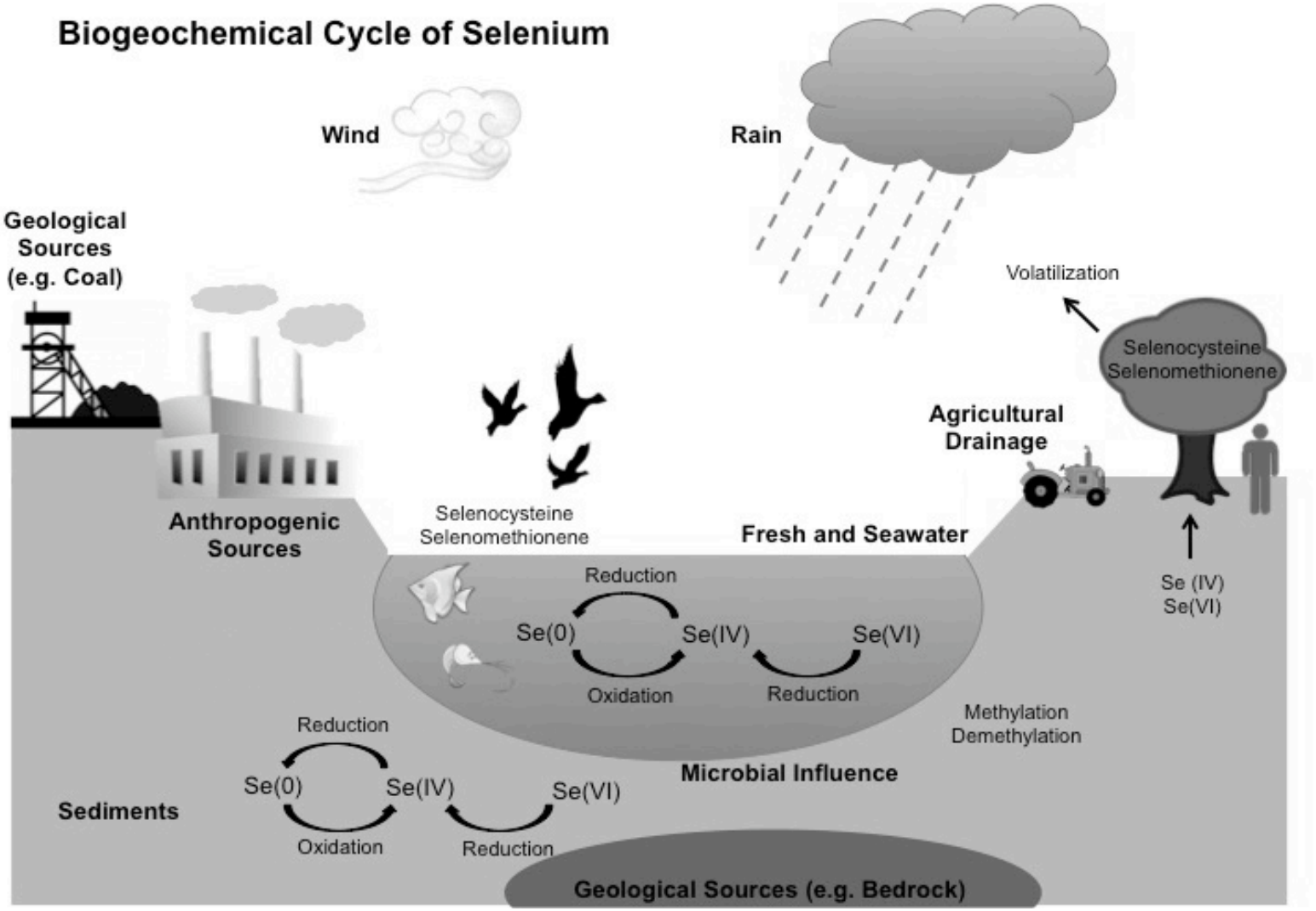
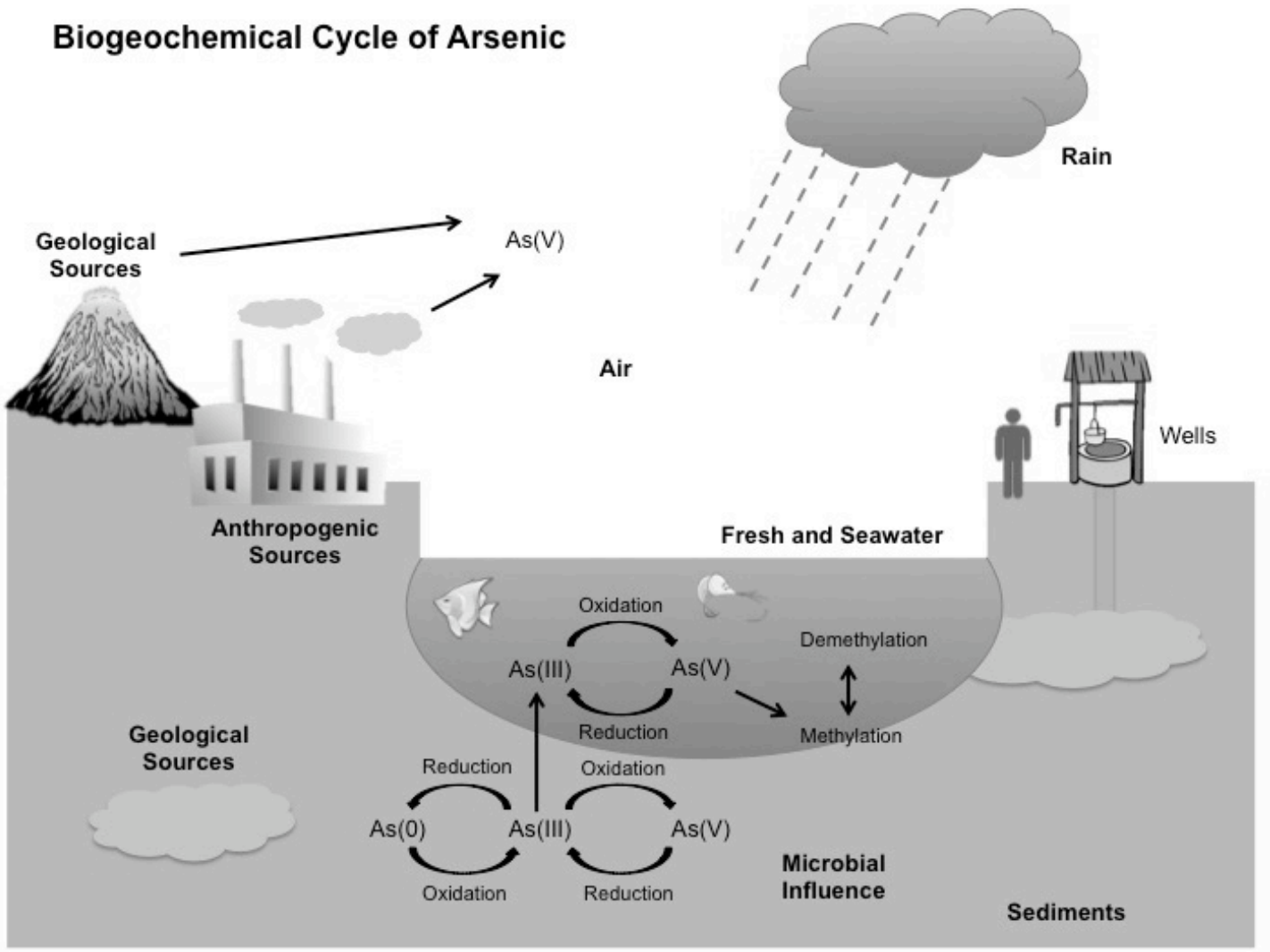


Figure 7.2 Biogeochemical Cycles of a) Selenium and b) Arsenic.

a)



b)


Biogeochemical Cycle of Arsenic
Rain
Geological Sources
As(V)
Air
Wells
Anthropogenic Sources
Fresh and Seawater
Oxidation
Demethylation
As(III)
As(V)
Reduction
Methylation
Geological Sources
Reduction
Oxidation
As(0)
As(III)
As(V)
Oxidation
Reduction
Microbial Influence
Sediments

Appendix A. Partial 16S rRNA Gene Sequence of Strain S4

1474 nucleotide bases, GenBank Accession Number JN107635

ATGCAAGTCAGGGGTTTATCCTTTCGGGGATGGGCAACTGGCGCACGGGTGAGTA
ACGCGTGAGAAATCTGCCTCAGAGATTGGGACAACAACCCGAAAGGGTTGCTAATA
CTAAATAAGCTCACAATTCGCATGTTTTGTGAGAAAAGGTCGGGGTAACCTGACGC
TTTGAGATGATCTCGCGTGTCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGC
TACGATGACTAGCCGGCCTGAGAGGGTGGCCGGCCACATAGGGACTGAGACACG
GCCCTAACTCCTACGGGAGGCAGCAGTGGGGAATTTTGCGCAATGATCGAAAGATT
GACGCAGCGACGCCGCGTGGACGATGAAGGTTTTCGGATCGTAAAGTCCTTTCAG
CAAGGAAGAATGGATATGTAAGTAACTGTGCATATATTGACGGTACTTGCAGAAGC
AGCCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGGGCAAGCGTT
GTTCGGAGTCACTGGGCGTAAAGCGCATGTAGGTGGATGTATAAGTCAGGAGTCA
AAGGCGTCGGCTCAACCGGCGTACGGCTTTTGAAACTATATGTCTAGAGTGTCGGA
GAGGAATATGGAATTCCCGGTGTAGCGGTGAAATGCGTAGATATCGGGAGGAACA
CCAGTAGCGAAGGCGATATTCTGGCCGACAACTGACACTGAGATGCGAGAGCATG
GGTAGCAAACAGGATTAGATACCCTGGTAGTCCATGCTGTAAACGATGGACGCTAG
GTGTTGGGGTTTTAAAGCCTCAGTGCCGAAGCAAACGCGTTAAGCGTCCCGCCTG
GGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGC
GGTGGAGCACGTGGTTTATTCGATGCTAACCGAAGAACCTTACCTAGGCTTGACAT
CCTAAGGATATTTTAGAGATAAGATAGTGCCGTTCTTTGAACGGAACTTAGAGACAG
GTGCTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTTGGGTTAAGTCCCGCAA
CGAGCGCAACCCCTATTTTTAGTTGCCATCAGGTTAAGCTGGGCACTCTAAAGAGA
CTGCCGTCGATAAGGCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTT
ATGTCTAGGGCTACACACGTGCTACAATGGTTTATACAGAGGGCAGCTAACTCGTG
AGAGTATGCGAATCCCTTAAAGTAATCCTCAGTTCGGATTGTAGTCTGCAACTCGAC
TACATGAAGTTGGAATCGCTAGTAATCGCAGGTCAGCAAAACTGCGGTGAATACGT
TCCCGGGCCTTGTACACACCGCCCGTCACACCACGGGAGTCGACCGTACCTGAAG
CCGGTGGCCTAACCGTAAGGAGGGAGCCGTCTATGGTATAGTTGGTAACTGGGGT
GAAGTCGTAACAAGGTAGCCGTACCGGAAGG

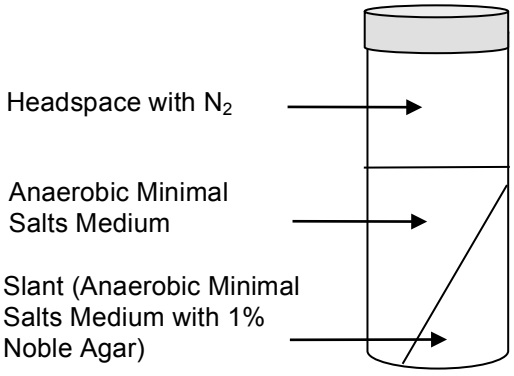
Appendix B. Storage and Revival of Strain S4 and S5

Strain S4^T:

Short-Term Storage: 4 °C, in minimal salts medium with 10mM acetate and 10 mM selenate.

For Long-Term Storage: Actively growing cells should be preserved 40% glycerol and stored at -80 °C. In order to revive frozen cells, inoculate 1mL of the frozen cells in a combination of 10 mL fresh minimal salts medium with 10 mM Acetate as carbon source and 10 mM selenate as electron acceptor and 10 mL agar slant (1% noble agar, Difco; in minimal salts medium with vitamins, trace salts, Na_2S x $9H_2O$, resazurin, 10 mM acetate, and 10 mM selenate.)

Figure B.1 Diagram of culture conditions to revive frozen stocks of strain S4^T.



Strain S5^T:

Short-Term Storage: 28 °C, in minimal salts medium with 10mM pyruvate and 10 mM selenate or nitrate. Cultures should be respiked with donor/acceptor at least 2 days before it is reinoculated into fresh medium (pH 7.3 to 7.4)

Long-Term Storage: Strain S5^T is extremely difficult to revive from frozen cultures (preserved in 40% glycerol at -80 °C) and will take a long time to grow.

Appendix C. Selenate Respiration Inhibited by Nitrate

Materials and Methods

Growth Conditions of Strain S5. Strain S5 was grown under standard conditions as described in Rauschenbach et al. (2011a). 10 mM pyruvate were added as carbon source and electron donor and either 10 mM nitrate, 10 mM selenate or 10 mM arsenate, or a combination of the electron acceptors was added to cultures.

Analsis of Respiratory Metabolism in the Presence of Various Electron Donors. The reduction of Se(VI), As(V), and nitrate was analyzed using ion chromatography (IC) (Dionex Model ICS 1000; Dionex, Sunnyvale, CA) with an AS9-HC column (Dionex) and an eluent of 11.4 mM $NaHCO_3$, with a flow rate of 1.2 mL/min. Standards were prepared in the minimal medium used to cultivate strain *D. indicum*.

.

Inhibition of selenate reduction. Strain S5 was grown in minimal salts medium under four different conditions. (1) Cells were grown with 10 mM Se(VI) and then transferred (1:10) to fresh medium with 10 mM Se(VI) and 10 mM nitrate as electron acceptors. (2) Strain S5 was pregrown on 10 mM nitrate Cells were then transferred into medium with 10 mM nitrate and 10 mM Se(VI). (3) Cells were pregrown on 10 mM Se(VI) before transfer into fresh medium with 10 mM Se(VI) and 10 mM As(V) (Figure C.2). (4) Cells were grown on 10 mM Se(VI) and 10 mM nitrate was added to actively Se(VI) respiring cultures after 140 hours.

Figure C.1 Utilization of Se(VI) and nitrate by strain S5 with Se(VI) or nitrate as electron acceptors and 10 mM pyruvate as carbon source and electron donor.

(■Nitrate; ◆ Selenate)

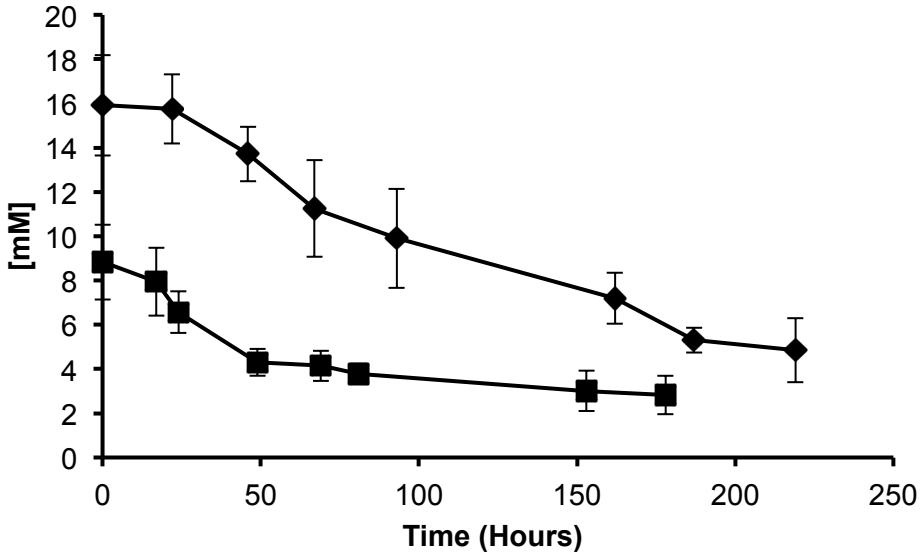
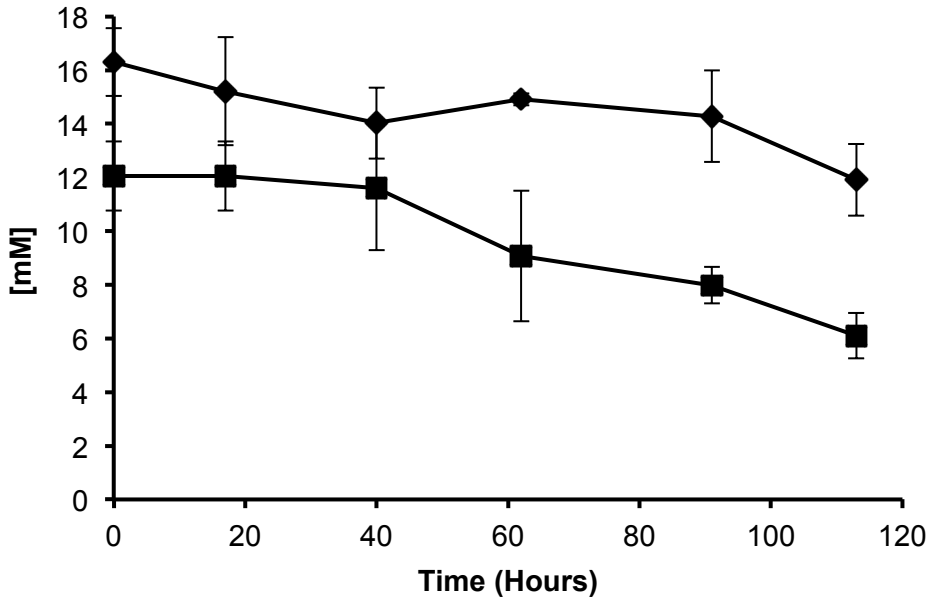
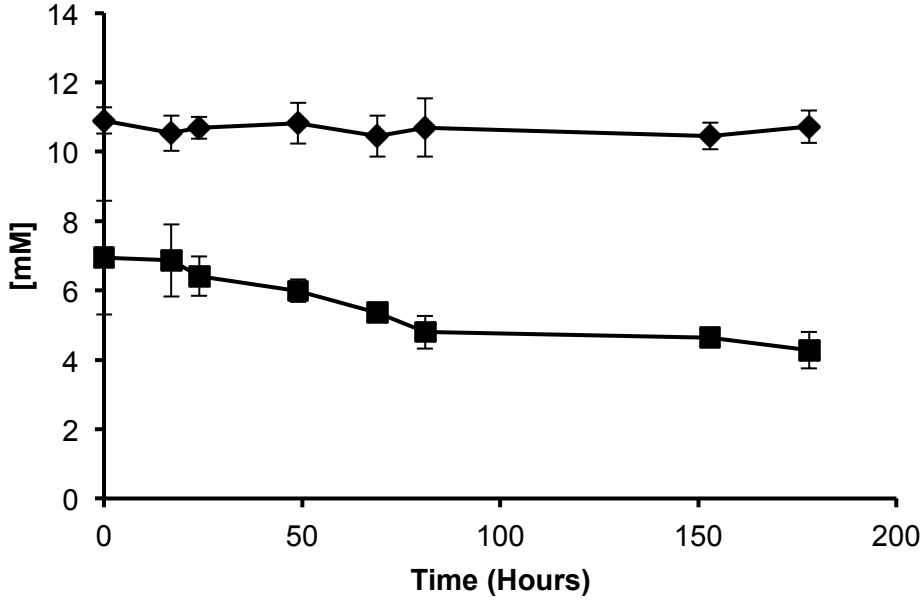


Figure C.2 Inhibition of selenate reduction. a) Strain S5 pregrown on Se(VI), transferred (1:10) to fresh medium with Se(VI) and nitrate as electron acceptors. b) Strain S5 was pregrown on nitrate, then transferred into medium with nitrate and Se(VI). c) Cells were pregrown on Se(VI) before transfer into fresh medium with Se(VI) and As(V) (■Nitrate; ◆ Selenate; ▲Arsenate).

a)



b)



c)

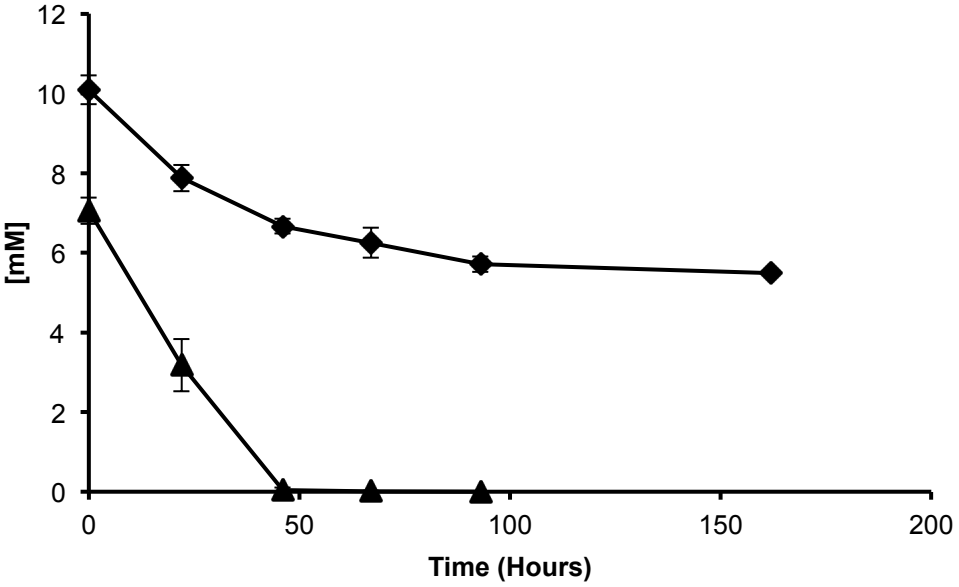
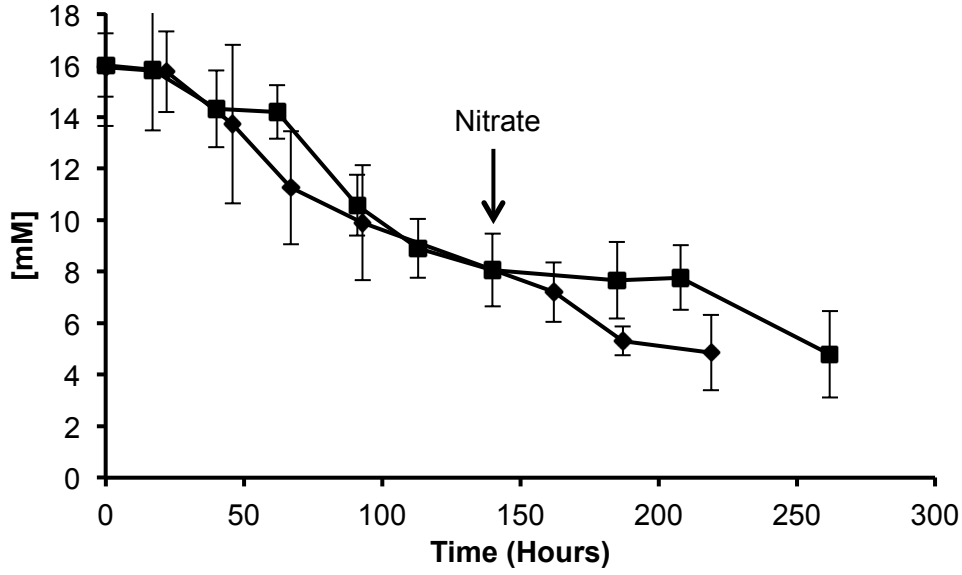


Figure C.3 Nitrate temporarily inhibits selenate respiration (■ Se(VI) with nitrate spiked after 140 hours; ◆ Se(VI) only).



Appendix D. Studies of the Putative Selenate Reductase in *Enterobacter cloacae*

1. Primers used to sequence YnfEF and surrounding genes

ynfC
10F – GATCAAAATCAAATTCATTTGA
Ynf7R – TGTGCCAACGATACCTTTAAAA

ynfD
Ynf7F – GAAACGGTTAACCTGGACTACC
Ynf7R – TGTGCCAACGATACCTTTAAAA

ynfEF
PO1 1F – AATCTGGCTTTACGCTGAACAT
PO1 1R – CACCTGATGATTGCCGTAGATA

PO2 2F – CTGAAAAACGTGGTCGAAAAAT
PO2 2R – TACGCTTTGTAGTGCCCATTAG

PO3 1F – GGGATCACCTACTACCTCGAAC
PO3 1R – CTGCGTTTTTACCGGGTTTT

PO4 2F – CATCTGGAACTATGCCGGTAA
PO4 2R – CATACTGGGTTGTCATCGGTTA

ynfEF/ynfG

Ynf5F – TATGGGCCAGGGAGCCTGGCAT
Ynf5R – ATGTGGCAGTAGCGGCAGCCGA

ynfG/ynfH

8F – TTATCCTCCTGCCAGTCGCCGC
8R – GAACCGGCAGCAATCTCGTT

ynfH/dmsD

9F – AACGAGATTGCCGCCGGTTC
9R - AACAGCACAGATTCACGATCCA

2. Sequence of operon ynfCDEFGH (Bold = sequences between genes)

ynfC
…gccagaagggttatcgctacatatttcttcacatcatttaccgatacagagagattattcccaatcat

attagcaaatacagttgtttacacgaaccctgctaaaattaatctacacacagagaaaaggagctacag

ynfD
atgaaacggttaccctggattaccgccctgctgttaatgagtgcctctaccgccgcgctggcggcaccggattcctgcgaac
gtgtaaaaagcgacattcagcagaaaatcatcaataacggcgtgccggaatctggctttacgctgaacatcgtacccaac
gatcaggccgatcggccggatgcgcaggttgttgggcattgtgccaatgataccttcaaaattttgtacactcgcacaggca
gcggggctgctgctggcgcgcaggagagtccgcaaggcgaaccgcagtgatttttaaccactttccttgcatcaatccccct
tgaattgatgaggattaatatttaatacctccaaataagtaacactcaccccatcattagcccggttgtaatcacgggcgaaa
ataatagtcaattaaaatgatggggtgagtc

ynfEF
atgtccgatgtcgaacatcacggtggaatcagccgccgaacactcgttaagtccaccgcgataggttctctggcgcttgccg
ccggtggtatctcgttgccgtttggattgaaaagcgccgctgctgcagtgcaaaacgccgtacagcctgctgaagataaagt
ggtctggggagcctgctcggtcaactgcggcagccgctgcgcgctgcgccttcatgtccgtgacgatgaagtttactgggtg
gaaacggataataccggggaggatatctacggcaatcatcaggtgcgcgcctgtcttcgcggacgctccatccgccgtcg
cattaatcaccctgaccgccttaactacccgatgaagcgcgtgggcaaacgcggtgaaggtaaatttgagcgcatcacct
gggatgaagcgctggataccctgacccgcagcctgaaaaacgtggtcgaaaaatacggcaacgaagcggtgtacatta
actactcctccggcattgtcggcggcaatatcacccgctcttcccttacgcctcgctggtggcgcgcctgatgaactgttacg
gcggtttcctgagccactatggcacctacagtaccgcgcaaatttcctgcgcgatgccgtacacctacggcagcaacgatg
gcaacagcacctccgacattgaaaacactaaactggtggtgatgttcggcaataatccggcggaaacgcgtatgagcgg
cggcgggatcacctactacctcgaacaggcgcgtgaacgctcaaatgcgcggatgatcgtcatcGacccgcgttatacc
gacaccgctgccggacgtgaagatgaatggatcccgattcgcccgggtaccgatgccgcgctggtagccggacttgcatg
ggtattaatcaacgaaaatctggttgaccagccgttcctcgataaatactgcgttggctatgacgaaaaaacgctgccgga
aggcgcgcctgctaatgggcactacaaagcgtatatcctcggccagggcgacgataatacgccaaaaaccccggagtg
ggcctcacgcatcaccggtattcctgcggatcgcatcgtcaagctggcgcgcgagattggctcggcaaaacccgcctatat
ctgccagggctggggaccgcagcgccaggccaacggtgaactgacgtcgcgcgccatcgccatgctgccgatcttaacc
ggcaacgtgggtatcaacggcggtaacagcggcgcgcgcgaatcgacctataccatcaccatcgaacggatgccgctg
ccggaaaacccggtaaaaacncanatctcctgctttanctggacggatgccatcnnccgcggcccggaaatgaccgcc
ctgcgcgacngcntgcgtggcnaggataanctggatgtgccgatcanatttcatctggaactatgccggtaacaccatcat
caatcaacnctccnacatcaacaaaacccacgatattctgcaggacgaaagcaagtgcgaaaccatcgtggtgatcga
caactttatgacctcttcggcgaaatatgctgacatcctgctgccggacctcatgaccgtcgagcaggaagacatcatcccg
aacgattatgcgggcaacatgggctatctgattttcctgcagccggtcaccgcgccgaagtttgagcgtaagcccatctact
ggatcatgagcgaagtggcgaaacgtctcgggccggatatccatcagaaattcaccgaaggccggacacagtcgcagt
ggctgcagtatttgtacgccaaaatgctggcgaaagatccgcagcttccgtcctatgacgaactgaaaaaaatgggcattt
ataagcgcaaagatcccaacggacacttcgtggcgtataaaaaattccgcgatgatccggatgcccatccgctcaaaacc
ccgtcaggcaagattgaaatctactccagcaagctggcagagattgccgccacctgggagctggaaaaagatgaaacc
atcagcccgctgccggtgtacgcctcaacgtttgacggctgggatgccccagagcgtaaaacatacccgctgcagctgttt
ggcttccacttcaaggcgcgcacccactccagctacggcaacgtggatgtgcttaaagccgcctgtcgtcaggaggtatgg
ctcaatccggttgatgcggcacaacgcggtattcaaaacggcgatatggtgcgcgtgtttaacgaccgcggcgaagtgcg
cattgccgcgaaagtcaccccgcgcatcatgcccggcgtcagcgcgatgggccaggggcctggcacgacgccaacat
gaacggcgatcggatcgaccacggctcatgatcaacacgttgaccacccatcgcccgtcaccgctggcgaaaggcaac
ccgcagcacaccaatctggtgcagatcgagaagcgtaagagtaccg

ynfG
atgacacccagtatggatttttttattgactccagccgttgcaccgggtgcaaaacctgcgagctggcctgcaaggattacaa
agacctgaccccggacgtcagcttccgccggatctacgaatacgcgggcggcgactggcaggaggataacggcgtctg
gcatcagaatgtctttgcctattacctgtcgattgcctgtaaccactgtgaaga

ccctgcctgcaccaaagtgtgcccgagcggggcgatgcacaagcgtgacgacgggtttgtggtggtgaatgag
gatgtctgcatcggctgccgctactgccatatggcctgtccgtacggtgcgccgcagtacaacgccgccaaagg
ccacatgaccaagtgcgacggctgccatgaccgcgtcgcagagggcaaaaagccgatttgcgtggagtcctgt
ccgctgcgcgcgctggacttcggcccgattgacgagctgcgtaaaaaacacggccagcttgcggccgtc

ynfG
gcgccgctgccgtccgcgcacttcacgaagccaagcattgtgattaaacctaacgccaacagccgcccgacgggagat
acctccggctatctggcaaacccgaaggaggtgtgag

ynfH
atgggaagtggatggcatgaatggccgctggtgatcttcaccgtttttgggcagtgcgtggccggggcgttgctcgtcagcg
gtctggcatggatgagggagagtgacgaggcggtcaaagcccgcatcgtgcgcagcatgttctttttatggctggtcatggg
cgttggctttatcgcatcggtgatgcacctgggctccccgctcagggcgttaac

3. *ynfEF* clones

3.1 Primers used to prepare *ynfEF* clones

PCR1
F – CGAATTCTACAGTTGTTTACACGAACC
R – GCTGTAGAGACTCACCCCATCATTTT

PCR2
F – GCTCTAGAACGGGGTCTGACGCTCAG
R – GCACTAGTTACAATTTCAGGTGGCACTT

PCR3
F – GCTCTAGAGGAGTAACCGATGACAACCC
R – GCTCTAGAGGCCGTGTTTTTTACGCA

3.2 Clones generated

pGEM1 (pGEM-T Easy with PCR1)
pGEM2 (pGEM-T Easy with PCR1 and PCR2)
pGEM3 (pGEM-T Easy with PCR1, PCR2, and PCR3)

3.3 Strains, Plasmids and Restriction Enzymes Used

Escherichia coli DH5α, *Enterobacter cloacae* SLD1a-1

pET30 (for kan resistance gene)
pGEM-T-Easy
pJQ200SK
XbaI, SpeI, EcoRI (for screening), NotI (for cutting out pGEM3, cutting pJQ200SK and making final construct)

APPENDIX E. *ESCHERICHIA COLI* SELENATE REDUCTION MUTANTS

0 white, no reduction
\+ reduced activity
++ normal activity

Table E.1 *E. coli* mutants deficient of selenate reduction capability.

Plate #	Well	Name of Gene	Function	Day 1	Day 2	Day 3	Day 4	Day 7
1	A4	fnr	FNR represses genes involved in aerobic respiration and activates genes required for anaerobic respiration.	0	0	0	0	0
3	H2	pykF	Pyruvate kinase I, fructose 1,6-bisphosphate-activated	0	0	0	0	0
5	F8	moaA	Molybdopterin biosynthesis protein A	0	0	0	0	0
19	D11	IscA	Iron Sulfur Cluster Protein	0	0	0	0	0
23	F9	ynbA	Putative diacylglycerol cholinephosphotransferase	0	0	0	0	0
29	F11	hscB	HscB is a co-chaperone that stimulates HscA (Hsc66) ATPase activity. Does not exhibit its own chaperone activity. Required for wild-type stimulation of HscA ATPase activity by the substrate, IscU, and for wild-type interaction between HscA and IscU. This system is involved in iron-sulfur cluster assembly.	0	0	0	0	0

Plate #	Well	Name of Gene	Function	Day 1	Day 2	Day 3	Day 4	Day 7
31	H1	mog	Predicted molybdochelatase	0	0	0	0	0
31	E2	gor	Glutathione reductase	0	+	+	+	+
33	D12	yhbP	Conserved protein	0	++	++	++	++
39	B9 - Dead	priA	The role of PriA in the cell appears to be the restart of stalled replication forks	Dead	Dead	Dead	Dead	Dead
41	E8	aroC	Chorismate synthase (amino acid biosynthesis)	0	0	0	0	0
43	A4	moeB	molybdopterin biosynthesis	0	0	0	0	0
	B4	moeA	molybdopterin biosynthesis	0	0	+	+	+
	C6	menE	MenE, subunit of o-succinyl-CoA ligase	0	0	0	0	0
	D6	menC	o-succinylbenzoate synthase	0	+	+	++	++
	F3	moaC	Molybdopterin biosynthesis protein C	0	0	+	+	+
	G3	moaD	Molybdopterin biosynthesis protein D	0	0	+	+	+
	H3	moaE	Molybdopterin biosynthesis protein E	0	0	+	+	+
	H8	menA	1,4-dihydroxy-2-naphthoate octaprenyltransferase catalyzes the transfer of an octaprenyl side chain to DHNA, the reaction in menaquinone biosynthesis where the pathway becomes associated with the membrane	0	0	0	0	0

Plate #	Well	Name of Gene	Function	Day 1	Day 2	Day 3	Day 4	Day 7
45	E6	atpC	ATP synthase subunit	0	0	+	+	++
	G11	hscA	Member of Hsp70 protein family	0	+	+	+	+
49	H4	Pfs	biotin synthase activity	0	++	++	++	++
	A4	Dgk4		0	++	++	++	++
55	B1	modB	Subunit of molybdate ABC transporter	0	0	0	0	0
	C1	modC	Subunit of molybdate ABC transporter	0	0	0	0	0
57	G5	selD	Selenocysteine biosynthesis	0	++	++	++	++
	G8	ptsI	PTS enzyme 1	0	++	++	++	++
61	B6	tatC	TatC is a subunit of the TatABCE (twin-arginine translocation) complex for the export of folded proteins across the cytoplasmic membrane.	0	0	0	0	0
65	F1	ygjK	glycoside hydrolase	0	++	++	++	++
	F7	no orf		0	++	++	++	++
67	C5	No orf		0	+	+	+	+
69	B3	menD	gene product catalyzes the first committed step in the biosynthesis of menaquinone	0	+	++	++	++
	E2	no orf		Dead	Dead	Dead	Dead	Dead
71	B2	dapF	Diaminopimelate epimerase	0	++	++	++	++
	F1	tatB	Subunit of TatABCE protein complex	0	0	0	0	0

77	G8	yehP	Function unknown (motility, chemotaxis)	0	++	++	++	++
Plate #	**Well**	**Name of Gene**	**Function**	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 7**
79	B10	ubiH	*ubiH* encodes a hydroxylase in the ubiquinone biosynthesis pathway. No direct biochemical evidence for the enzymatic activity of UbiH exists to date.	*0*	++	++	++	++
	C9	modA	Molybdate ABC transporter	*0*	*0*	0	0	0
89	B12	hflD	Predicted lysogenization regulator	0	++	++	++	++
	A11	cydD	Part of the *cydDC* operon encodes two ATP-binding cassette (ABC) transporter proteins that are most closely similar to ABC transporters involved in export. studies have shown that both of these proteins are essential for functional cytochrome *bd* and cytochrome *c*	0	++	++	++	++
	H6	dmsD	essential for the biogenesis of DMSO reductase	0	0	0	0	0

Table E.2 Reduced Reduction/Hyperfunction Mutants **(**+ Reduced Function; ++ Normal Function**;** +++ Hyperfunction)

Plate Number	Well Number	Gene	Function	Day 1	Day 2	Day 3	Day 4	Day 7
25	G8	ubiF	Enzyme involved in the ubiquinone-8 biosynthesis pathway	++	+++	+++	+++	+++
	H8	ybgJ	Putative carboxylase	++	+++	+++	+++	+++
43	E6	ubiX	Ubiquinone-8 biosynthesis pathway	+	++	++	++	++
	D8	ubiE	Part of menaquinone-8 biosynthesis	+	++	++	++	++
49	A9	IdnK	Gluconokinase	+++	++	++	++	++
	C7	cmk	cytidylate kinase	+	++	++	++	++
51	C5	atoD	Acetoacyl CoA Transferase	+++	+++	+++	+++	+++
	G1	ynfE	Oxidoreductase subunit paralog of DmsA	+	++	++	++	++
55	G7	ydjN	Transporter	+	++	++	++	++
57	F8	ptsH		+	++	++	++	++
61	C7	rpsF	30S ribosomal subunit protein S6	+	++	++	++	++
	G5	selB	specific translation factor subunit of selenocysteine	+	++	++	++	++

Plate Number	Well Number	Gene	Function	Day 1	Day 2	Day 3	Day 4	Day 7
61	H5	selA	Synthase transcription	+	++	++	++	++
	H7	nusB	antitermination protein NusB	+	++	++	++	++
63	E9	tnaA	L-cysteine desulfhydrase / tryptophanase	+++	+++	+++	+++	+++
	A6	rfaE	ADP-heptose formation	+++	+++	+++	++	++
67	D3	tnaC	Leader peptide is required for regulation by attenuation of the *tnaCAB* operon.	+++	++	++	++	++
	C5	No Orf		+	++	++	++	++
77	H5	yebV	Predicted protein	+	++	++	++	++
83	B10	rfaC	ADP-heptose	+++	++	++	++	++
89	E3	cysB	Protein product controls the expression of the	+	++	++	++	++
	E5	ubiG	cysteine regulon *ubiG* deletion mutants are respiration defective and thus unable to grow on	+	+	+	+	+
	A7	ydfW	succinate or glycerol	+	++	++	++	++
	A10	rplA	No info 50S ribosomal subunit protein L1	+	++	++	++	++

References

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 Microbiol Lett* 226:107-12

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

Ahmann, D., Krumholz, L., Hemond, H., Lovley, D., and Morel. F.M. (1997) Microbial mobilization of arsenic from sediments of the aberjona watershed. *Environ Sci Tech* 31:2923-2930.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402.

An, D., Li, D., Liang, Y., and Jing, Z. (2007) Unventilated indoor coal-fired stoves in Guizhou Province, China: reduction of arsenic exposure through behavior changes resulting from mitigation and health education in populations with arsenicosis. *Environ Health Perspec* 115:659-662.

Andren, A.W. and Klein, D.H. (1975) Selenium in Coal-Fired Steam Plant Emissions. *Environ Sci Technol* 9:856-858.

Anawar, H. M., Akai, J., Yoshioka, T., Konohira, E. , Lee, J. Y., Fukuhara, H.E., Alam, M.T.K. and Garcia-Sanchez, A. (2006) Mobilization of arsenic in groundwater of Bangladesh: evidence from an incubation study. *Environ Geochem Health* 28:553-565.

Anderson, L.A., McNairn, E., Leubke, T., Pau, R.N., and Boxer, D.H. (2000) Mod-Dependent Molybdate Regulation of the Molybdenum Cofactor Operon *moa* in *Escherichia coli*. *J Bacteriol* 182:7035-7043.

Astolfi, E., Maccagno, A., Garcia, J.C., Garcia Fernandez, J.C., Vaccaro, R., and Stimola, R. (1981) Relation between arsenic in drinking water and skin cancer. *Biol Trace Elem Res* 3:133-143.

Arnér, E.S. and Holmgren, A. (2000) Physiological functions of thioredoxin and thioredoxin reductases. *Eur J Biochem* 267:6102-6109.

Baesman, S.M., T.D. Bullen, J. Dewald, D. Zhang, S. Curran, F.S. Islam, T.J. Beveridge, and R.S. Oremland (2007). Formation of tellurium nanocrystals during anaerobic growth of bacteria that use Te oxyanions as respiratory electron acceptors. *Appl Environ Microbiol* 73:2135-2143.

Baesman, S.M., Stolz, J.F., Kulp, T.R., and Oremland, R.S. (2009) Enrichment and isolation of *Bacillus beveridgei* sp. nov., a facultative anaerobic haloalkaliphile from Mono Lake, California, that respires oxyanions of tellurium, selenium, and arsenic. *Extremophiles* 13:695-705.

Bagnell, K.W. 1966. The *Chemistry of Selenium, Tellurium, and Polonium*. Elsevier Publishing Co., London.

Barceloux, D.G. (1999) Selenium. *Clinical Toxicol* 37:145-172.

Bebién, M., Chauvin, J. P., Adriano, J. M., Grosse, S. and Vermeglio, A. (2001) Effect of selenite on growth and protein synthesis in the phototrophic bacterium *Rhodobacter sphaeroides*. *Appl Environ Microbiol* 67:4440-4447.

Bebién, M., Kirsch, J., Me!jean, V. and Verméglio, A. (2002) Involvement of a putative molybdenum enzyme in the reduction of selenate by *Escherichia coli*. *Microbiol* 148:3865–3872.

Bennett S. (2004) Solexa Ltd. *Pharmacogenomics* 5:433-8.

Bentley, R. and Chasteen, T.G. (2002) Microbial methylation of metalloids: arsenic, antimony, and bismuth. *Microbiol Mol Biol Rev* 66:250-271.

Berks, B.C. (1996) A common export pathway for proteins binding complex redox cofactors. *Mol Microbio* 22:393-404.

Blasco, F., Guigliarelli, B., Magalon, A., Giordano, G., and Rothery, R.A. (2001) The coordination and function of the redox centers of the membrane-bound nitrate reductases. *Cell Mol Life Sci* 58:179-193.

Bouchard, B., Beaudet, R., Villmur, R., McSween, G., Lepine, F. and J.-G. Bisaillon, J.-G. (1996) Isolation and characterization of *Desulfitobacterium frappieri* sp. nov., an anaerobic bacterium which reductively dechlorinates pentachlorophenol to 3-chlorophenol. *Int J Sys Evol Microbiol* 46:1010-1015.

Brondijk, T.H.C., Nilavongse, A., Filenko, N., Richardson, D.J., and Cole, J.A. (2004) NapGH components of the periplasmic nitrate reductase of *Escherichia coli* K-12: location, topology and physiological roles in quinol oxidation and redox balancing. *Biochem J* 379:47-55.

Bundschuh, J., Farias, B., Martin, R., Storniolo, A., Bhattacharya, P., Cortes, J., Bonorino, G., Albouy, R. (2004) Groundwater arsenic in the Chaco-Pampean Plain, Argentina: case study from Robles county, Santiago del Estero Province. *Appl Geochem* 19:231-243.

Bun-ya, M., Shikata, K., Nakade, S., Yompakdee, C., Harashima, S., and Oshima, Y. (1996) Two new genes, PHO86 and PHO87, involved in inorganic phosphate uptake in *Saccharomyces cerevisiae*. *Curr Genet* 29:344–351.

Caccavo, F., Coates, J.D., Rossello-Mora, R.A., Ludwig, W., Schleifer, K.H., Lovley, D.R., and McInerney, M.J. (1996). *Geovibrio ferrireducens*, a phylogenetically distinct dissimilatory Fe(III)-reducing bacterium. *Arch Microbiol* 165, 370-376.

Camacho, L.M., Gutiealrrez, M., Alarcon-Herrera, M.T., Villalba, M., Deng, S. (2011) Occurrence and treatment of arsenic in groundwater and soil in northern Mexico and southwestern USA. Chemosphere. In Print.

Carlin, A., Shi, W., Dey, S., and Rosen, B.P. (1995) The *ars* operon of *Escherichia coli* confers arsenical and antimonial resistance. *J Bacteriol* 177: 981-986.

Carver, T., Thomson, N., Bleasby, A., Berriman, M., Parkhill, J. (2008) DNAPlotter: circular and linear interactive genome visualization. *Bioinformatics* 25:119-120.

Challenger, F. (1945) Biological methylation. *Chem Rev 36:315-361.*

Chasteen, T.G. and Bentley, R. (2003) Biomethylation of selenium and tellurium: microorganisms and plants. *Chem Rev* 103:1-25.

Chen, X., Yang, G., Chen, J., Chen, X., Wen, Z., and Ge, K. (1980) Studies on the relations of selenium and Keshan disease. *Biol Trace Elem Res* 2:91-107.

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T.J., Higgins, D.G. and Thompson, J. (2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic Acid Res* 31:3497-3500.

Conde, J.E. and Alaejos, M.S. (1997) Se concentrations in natural and environmental waters. *Chem Rev* 97:1979–2004.

Cooper, W. (1971)*Tellurium*. Van Nostrand Reinhold Company, New York.

Corcueram, G.P., Bastidas, A., and Dubourdieu, M. (1993), Molybdenum uptake in *Escherichia coli* K12. *J Gen Microbiol* 139:1869-1875.

Cristóbal, S., de Gier, J.W., Nielsen, H., and von Heijne, G. (1999) Competition between Sec- and Tat-dependent protein translocation in *Escherichia coli*. *EMBO J* 18:2982–2990.

Csotonyi, J. T., E. Stackebrandt, and Yurkov,Y. (2006) Anaerobic Respiration on tellurate and other metalloids in bacteria from hydrothermal vent fields in the Eastern Pacific Ocean. *Appl Environ Microbiol* 72:4950-4956.

Cullen, W. and Reimer, K.J. (1989) Arsenic speciation in the environment. *Chem Rev* 89:713-764.

Das, D., Samanta, G., Mandal, B.K., Chowdhury, T.R., Chanda, C.R., Chowdhury, P.P., Basu, G.K., and Chakraborti, D. (1996) Arsenic in ground water in six districts of West Bengal, India. *Environ Geochem Health* 18:5-15.

DeMoll-Decker, H. and Macy, J.M. (1993) The periplasmic nitrite reductase of *Thauera selenatis* may catalyze the reduction of selenite to elemental Se. *Arch Microbiol* 160:241-247.

Dias, J.M., Than, M.E., Humm, A., Huber, R., Bourenkov, G.P., Bartunik, H.D. *et al.* (1999) Crystal structure of the first dissimilatory nitrate reductase at 1.9 Å solved by MAD methods. *Structure* 7:65-79.

Doran, J.W. and Alexander, M. M. (1977) Microbial transformations of selenium. Appl *Environ Microbiol* 33: 31-37.

Dowdle P.R. and Oremland RS (1998) Microbial oxidiation of elemental selenium in soil slurries and bacterial cultures. *Environ Sci Technol* 32:3749–3755.

Dridge, E.J., Watts, C.A., Jepson, , B.J.N., Line, K., Santini, J.M., Richardson, D.J., and Butler, C.S. (2007) Investigation of the redox centres of periplasmic selenate reductase from *Thauera selenatis* by EPR spectroscopy. *Biochem J* 408:19-28.

Dubourdieu, M. and DeMoss, J.A. (1992) The *narJ* gene product is required for biogenesis of respiratory nitrate reductase in *Escherichia coli*. *J Bacteriol* 174:867-872.

Dungan, R.S., Yates, S.R. and Frankenberger, W.T. (2003) Transformations of selenate and selenite by *Stenotrophomonas maltophilia* isolated from a seleniferous agricultural drainage pond sediment. Environ Microbiol 5:287–295.

Ewing, B. and Green, P. (1998) Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res* 8:186-194.

Ewing, B., L. Hillier, M.C. Wendl, and Green, P. (1998) Base-calling of automated sequencer traces using phred. I. Accuracy assessment. *Genome Res* 8:175-185.

Fennell, D., Rhee, S.K., Ahn, Y.B., Häggblom, M.M. and Kerkhof, L.J. (2004) Detection and characterization of a dehalogenating microorganism by terminal restriction fragment length polymorphism fingerprinting of 16S rRNA in a sulfidogenic, 2-bromophenol-utilizing enrichment. *Appl Environ Microbiol* 70:1169-1175.

Ferguson, J.F. and Gavis, J. (1972) Review of the arsenic cycle in natural waters. *Water Res* 6:1259-1274.

Finkelman, R.B.(1998) Trace elements in coal environmental and health significance. *Biol Trace Elem Res* 67:197-204.

Finkelman, R., Belkin, H.E., and Zheng, B. (1999) Health impacts of domestic coal use in China. *PNAS* 96:3427-3431.

Fishbein, L. (1983) Environmental selenium and its significance. *Fund Appl Tox* 3:411-419.

Fisher, E., Dawson, A.M., Polshyna, G., Lisak, J., Crable, B., Perera, E., Ranganthan, M., Thangavelu, M., Basu, P., and Stolz, J. (2008) Transformation of inorganic and organic arsenic by *Alkaliphilus oremlandii* sp. nov. strain OhILAs. *Ann NY Acad Sci* 1125:230-241.

Fleming, A. 1932. On the specific antibacterial properties of penicillin and potassium tellurite. *J Path Bacteriol* 35: 831-842.

Garelick, H., Jones, H., Dybowask, A., and Valsami-Jones, E. (2008) Arsenic pollution sources. *Res Environ Contam Toxicol* 197:17-60.

George, M.W. 2007. Selenium and Tellurium. *2006 Minerals Yearbook, U.S. Geological Society*. Pgs. 65.1-65.8.

Gihring, T.M. and Banfield, J.F. (2004) Arsenite oxidation and arsenate respiration by a new Thermus isolate. *FEMS Microbiol Lett* 204:335-340.

González, P.J., Correia, C., Moura, I., Brondino, C.D., and Moura J.J.G. (2006) Bacterial nitrate reductases: Molecular and biological aspects of nitrate reduction. *J Inorg Biochem* 100:1015-1023.

Gordon, D., C. Abajian, and Green, P. (1998) Consed: a graphical tool for sequence finishing. *Genome Res* 8:195-202.

Green, J., Crack, J.C., Thomson, A.J., and LeBrun, N.E. (2009) Bacterial sensors of oxygen. *Cur Opinion Microbiol* 12:145-151.

Gross, R., Simon, J., and Kröger, A. (1999) The role of the twin-arginine motif in the signal peptide encoded by the hydA gene of the hydrogenase from *Wolinella succinogenes*. *Arch Microbiol* 172:227-232.

Grunden, A.M., Self, W.T., Villain, M., Blalock, J.E. and Shanmugam, K.T. (1999) An analysis of the binding of repressor protein ModE to *modABCD* (molybdate transport) operator/promoter DNA of *Escherichia coli. J Bacteriol* 247:24308-24315.

Guymer, D., Maillard, J., and Sargent, F. (2009) A genetic analysis of in vivo selenate reduction by *Salmonella enterica* serovar Typhimurium LT2 and *Escherichia coli*. *Arch Microbiol* 191:519-528.

Han, C. and Chain, P. (2006) Finishing repeat regions automatically with Dupfinisher. *In Proceeding of the 2006 International Conference on Bioinformatics and Computational Biology.* Arabnia, H.R. and Valafar, H. (eds) CSREA Press, pp. 141-146.

Handley, K.M., Héry, M., and Lloyd, J.R. (2009) *Marinobacter santoriniensis* sp. nov., an arsenate-respiring and arsenite-oxidizing bacterium isolated from hydrothermal sediment. *Int J Sys Evol Microbiol* 59:886-892.

Hedges, R. W., and Baumberg, S. (1973) Resitance to arsenic compounds conferred by a plasmid admissible between strains of *Escherichia coli*. *J Bacteriol* 115:459-460.

Heinz, G.H., D.J. Hoffman, and L.G. Gold. 1988. Toxicity of organic and inorganic selenium to mallard ducklings. *Arch Environ Contam Toxicol* 17:561-568.

Herbel, M.J., Switzer Blum, J., Hoeft, S.E., Cohen, S.M., Arnold, L.L., Lisak, J., Stolz, J.S., and Oremland, R.S. (2002) Dissimilatory arsenate reductase activity and arsenate-respiring bacteria in bovine rumen fluid, hamster feces, and the termite hindgut. *FEMS Microbiol Ecol* 41:59-67.

Hille, R., Rétey, J., Bartlewski-Hof, U., Reichenbecher, W., and Schink, B. (1999) Mechanistic aspects of molybdenum-containing enzymes. *FEMS Microbiol Rev* 22:489-501.

Hoffman, D.J. and Heinz, G.H. (1988) Embryonic and teratogenic effects of selenium in the diet of mallards. *J Toxicol Environ Health* 24:477-490.

Holben, D.H. and Smith, A.M. (1999) The diverse role of selenium within selenoproteins: a review. *J Am Diet Assoc* 99:836-843.

Huber, R., Sacher, M., Vollmann, A., Huber, H., Rose, D. (2000) Respiration of arsenate and selenate by hyperthermophilic archaea. *Sys Appl Microbiol* 23:305-314.

Izard, J.W. and Kendall, D.A. (1994) MicroReview: Signal peptides: exquisitely designed transport promoters. *Mol Microbiol* 13:765-773.

Janssen, P.H., Liesack, W., and Schink, B. (2002). *Geovibrio thiophilus* sp. nov., a novel sulfurreducing bacterium belonging to the phylum *Deferribacteres. Int J Sys Env Microbiol* 52:1341-1347.

Ji, G. and Silver, S. (1992) Regulation and expression of the arsenic resistance operon from *Staphylococcus aureus* plasmid p1258. *J Bacteriol* 174:3684-3694.

Joliffe, D.M. (1993) A history of the use of arsenicals in man. *J Royal Soc Med* 86:287-289.

Karttunen A.J., Linnolahti M., and Pakkanen T.A. (2007) Icosahedral and ring-shaped allotropes of arsenic. *Eur J Chemphyschem* 8:2373-8.

Kerkhof, L. and Ward, B.B. (1993) Comparison of nucleic acid hybridization and fluorometry for measurement of the relationship between RNA/DNA ratio and growth rate in a marine bacterium. *Appl Environ Microbiol* 59:1303-1309.

Kern, M. and Simon, J. (2009) Electron transport chains and bioenergetics of respiratory nitrogen metabolism in *Wolinella succinogenes* and other Epsilonproteobacteria. *Biochim Biophys Act* 1787:646-656.

Kessi, J. and Hanselmann, K.W. (2004). Similarities between the abiotic reduction of selenite with glutathione and the dissimilatory reaction mediated by *Rhodospirillum rubrum* and *Escherichia coli. J Biol Chem* 279:50663-50669.

Kisker, C., Schindelin, H., and Rees, D. (1997) Molybdenum-cofactor-containing enzymes: structure and Mechanism. *Annu Rev Biochem* 66:233-267.

Knight, V. and Blakemore, R. (1998) Reduction of diverse electron acceptors by *Aeromonas hydrophila*. *Arch Microbiol* 169:239-248.

Kodoka, H., Armfield, A.Y., Lombard, G.L., and Dowell, V.R. (1982). Practical procedure for demonstrating bacterial flagella. *J Clinical Microbiol* 16:948-952.

Krafft T., and Macy J.M. (1998) Purification and characterization of the respiratory arsenate reductase of *Chrysiogenes arsenatis*. *Eur J Biochem* 255:647-53.

Kuroda, M., Yamashita, M., Miwa, E., Imao, K., Fujimoto, N., Ono, H., Nagano, K., Sei, K. and Ike, M. (2011) Molecular cloning and characterization of the *srdBCA* operon, encoding the respiratory selenate reductase complex, from the selenate-reducing bacterium *Bacillus selenatarsenatis* SF-1. *J Bac* 193:2141–2148.

Laverman, A.M., Blum, J.S., Schaeffer, J.K, Phillips, E.J.P., Loveley, D.R., and Oremland, R.S. (1995) Growth of strain SES-3 with arsenate and other diverse electron acceptors. *Appl Environ Microbiol* 61:3556-3561.

Lehr, C.R., Polishchuk, E., Radoja, U., and Cullen, W.R. (2003) Demethylation of methylarsenic species by *Mycobacterium neoaurum. Appl Organimetal Chem* 17:831–834.

Lemly, D. (1997) Ecosystem Recovery following selenium contamination in a freshwater reservoir. *Ecotoxicol Environ Safety* 36:275-281.

Lemly, D. (1999) Selenium impacts on fish: an insidious time bomb. *Human Ecol Risk Assess* 5:1139-1151.

Lemly A.D. (2002) Symptoms and implications of selenium toxicity in fish: the Belews Lake case example. *Aqua Toxicol* 57:39-49.

Lemly, A.D. (2004) Aquatic selenium pollution is a global environmental safety issue. *Ecotoxicol Environ Safety* 59:44- 56.

Levine, V. (1925) The reducing properties of microorganisms with special reference to selenium compounds. *J Bac* 10:217-263.

Li, X. and Krumholz, L.R. (2007) Arsenate resistance in *Desulfovibrio desulfuricans* G20 by an *arsRBCC* operon and an *arsC* gene. *J Bac* 189:3705-3711.

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.

Lipman, J.G. and Waksman, S.A. (1923) The oxidation of selenium by a new group of autotrophic microorganisms. *Science* 57:60.

Lloyd, J.R. (2003) Microbial reduction of metals and radionucleotides. *FEMS Microbiol Rev* 27:411-425.

Losi, M.E. and Frankenberger, W.T. (1997) Reduction of selenium oxyanions by *Enterobacter cloacae* SLD1a-1: isolation and growth of the bacterium and its expulsion of selenium particles. *Appl Environ Microbiol* 63:3079-3084.

Lowe, E.C., Bydder, S., Hartshorne, R.S., Tape, H.L.U., Dridge, E.J., Debieux, C.M., *et al.* (2010) Quinal-cytochrome c oxidoreductase and cytochrome c4 mediate electron transfer during selenate respiration in *Thauera selenatis*. *J Biol Chem* 285:18433-18442.

Luijten, M., de Weert, J., Smidt, H., Boschker, H., de Vos, W., Schraa, G., and Stams, A. (2003) Description of *Sulfurospirillum halorespirans* sp. nov., an anaerobic, tetrachloroethene-respiring bacterium, and transfer of *Dehalospirillum multivorans* to the genus *Sulfurospirillum* as *Sulfurospirillum multivorans* comb. nov. *Int J Sys Evol Microbiol* 53:787–793

Luoma, S.N. and Presser, T.S. (2009) Emerging opportunities in management of selenium contamination. *Environ Sci Tech* 43:8483-8487.

Macy, J.M., Michel, T.A., and Kirsch, D.G. (1989). Selenate reduction by a *Pseudomonas species*: a new mode of anaerobic respiration. *FEMS Microbiol Letters* 61:195-198.

Macy, J. M., S. Rech, G. Auling, M. Dorsch, E. Stackebrandt, and Sly, L. (1993) *Thauera selenatis* gen. nov. sp. nov., a member of the beta-subclass of Proteobacteria with a novel type of anaerobic respiration. *Int J Syst Bacteriol* 43:135–142.

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 Sys Evol Microbiol* 47:1153-1157.

Macy, J.M., Santini, J.M., Pauling, B.V., O'Neill, A.H., Sly, L.I. (2000) Two new arsenate/sulfate-reducing bacteria: mechanisms of arsenate reduction. *Arch Microbiol* 173:49-57.

Maher, M.J. and Macy, J.M. (2002) Crystallization and preliminary X-ray analysis of the selenate reductase from *Thauera selenatis*. *Acta Cryst* D58:706-708.

Maher, M. J., Santini, J., Pickering, I. J., Prince, R. C., Macy, J. M. and George, G. N. (2004) X-ray absorption spectroscopy of selenate reductase. *Inorg Chem* 43:402-404.

Malasarn, D., Keeffe, J.R., and Newman, D.K. (2008) Characterization of the arsenate respiratory reductase from *Shewanella* sp. strain ANA-3. *J Bacteriol* 190:135-42.

Margulies M., Egholm, M., Altman,W.E., Attiya, S., Bader, J.S., Bemben, L.A*. et al.* (2005) Genome sequencing in microfabricated high-density picolitre reactors. *Nature* 437:326-327.

Marietou, A., Richardson, D.J., Cole, J.A., and Mohan, S. (2005) Nitrate reduction by *Desulfovibrio desulfuricans*: a periplasmic nitrate reductase system that lacks NapB, but includes a unique tetraheme c-type cytochrome, NapM. *FEMS Microbiol Lett* 24:8217-8225.

Martinez Espinosa, R.M., Dridge, E.J., Bonete, M.J., Butt, J.N., Butler, C.S., Sargent, F., and Richardson, D.J. (2007) Look on the positive side! The orientation, identification and bioenergetics of 'Archeal' membrane-bound nitrate reductases. *FEMS Microbiol Lett* 276:129-139.

Masscheleyn, P.H. and Patrick, W.H. (1983) Biogeochemical processes affecting selenium cycling in wetlands. *Env Toxicol Chem* 12:2235-2243.

Maupin-Furlow, J.A., Rosentel, J.K., and Lee, J.H. (1995) Genetic analysis of the *modABCD* (molybdate transport) operon of *Escherichia coli. J Bacteriol* 177:4851-4856.

McBride, B.C. and Wolfez, R. S. (1971) Biosynthesis of dimethylarsine by methanobacterium. *Biochem* 10:4312-4317.

Mendel, R.R. (2007) Biology of the molybdenum cofactor. *J Exp Botany* 58:2289–2296.

Mesbah, M., Premachandran, U. and Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* 39:159–167.

Michalke, K., Wickenheiser, E.B., Mehring, M., Hirner, A.V., and Hensel, R. (2000) Production of volatile derivatives of metal(loid)s by microflora involved in anaerobic digestion of sewage sludge. *Appl Environ Microbiol* 66:2791–2796.

Mondal, P., Majumder, C.B., Mohanty, B. (2006) Laboratory based approaches for arsenic remediation from contaminated water: recent developments. *J Hazard Mater* B137:464-479.

Murphy, E.A. and Aucott, M. (1998) An assessment of the amounts of arsenical pesticides used historically in a geographical area. *Science Total Environ* 218:89-101.

Muscatello, J.R., Bennett, P.M., Himbeault, K.T., Belknap, A.M., and Janz, D.M. (2006) Larval deformities associated with selenium accumulation in northern pike (*Esox lucius*) exposed to metal mining effluent. *Environ Sci* 40:6506-6512.

Mustachich, D. and Powis, G. (2000) Thioredoxin reductase. *Biochem J* 346: 1-8.

Muyzer, G., de Waal, E.C. and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 59:695-700.

Myneni, S.C.B, Tokunaga, T.K. and Brown, G.E. (1997). Abiotic Se redox transformations in the presence of Fe(II,III) oxides. *Science* 278:1106-1109.

Myhr, S. and Torsvik, T. (2000). *Denitrovibrio acetiphilus*, a novel genus and species of dissimilatory nitrate-reducing bacterium isolated from an oil reservoir model column. *Int J Syst Bacteriol* 50:1611-1619.

Naftz DL, Rice JA (1989) Geochemical processes controlling selenium in ground water after mining, Powder River Basin, Wyoming, USA. *Appl Geochem* 4:565-575.

Narasingarao, P. and Häggblom, M.M. (2006) *Sedimenticola selenatireducens*, gen. nov., sp. nov., an anaerobic selenate-respiring bacterium isolated from estuarine sediment. *Sys Appl Microbiol* 29:382-388.

Narasingarao, P. and Häggblom, M.M. (2007a) Anaerobic selenate-respiring bacteria are diverse and ubiquitous in aquatic sediments. *Appl Environ Microbiol* 73:3519-3527.

Narasingarao, P. and Häggblom, M.M. (2007b) *Pelobacter seleniigenes* sp. nov., a selenate-respiring bacterium. *Int J Syst Evol Microbiol* 57:1937-1942.

Natale, P., Brüser, T., Driessen, A.J.M. (2008) Sec- and Tat-mediated protein secretion across the bacterial cytoplasmic membrane – distinct translocases and mechanisms. *Biochim Biophys Act* 1778:1735-1756.

National Institute of Health (1989) Recommended dietary allowances / Subcommittee on the Tenth Edition of the RDAs, Food and Nutrition Board, Commission on Life Sciences, National Research Council. Washington, D.C., National Academy Press.

Newman, D.K., Kennedy, E.K., Coates, J.D., Ahmann, D., Ellis, D.J., Lovley, D.R., and Morel, F.M.M. (1997) Dissimilatory arsenate and sulfate reduction in *Desulfotomaculum auripigmentum* sp. nov. *Arch Microbiol* 168:380-388.

Niggemeyer, A., Spring, S., Stackebrandt, E., and Rosenzweig, R.F. (2001) Isolation and characterization of a novel As(V)-reducing bacterium: implications for arsenic mobilization and the genus *Desulfitobacterium. Appl Environ Microbiol* 67:5568-5580.

Nordstrom, D.K. (2002) Worldwide occurrences of arsenic in ground water. *Science* 296:2143.

Novick, R. P., and Roth, C. (1968) Plasmid-linked resistance to inorganic salts in *Staphylococcus aureus*. *J Bacteriol* 96:1335-1342.

Novick, R. P., Edean, Schweisnger, M.D., Gruss, A.D., Swanson, E.C., and Pattee. P.A. (1979) Genetic translocation in *Staphylococcus aureus*. *Proc Natl Acad Sci* 76:400-404.

Novick, R. P., Murphy, E., Gryczan, T. J., Baron, E., and Edeman, L. (1979) Penicillinase planmids of *Staphylococcus aureus*: restriction-deletion maps. *Plasmid* 2:109-129.

Ohlendorf, H.M., Hoffman, D.J. Saiki, M.K., and Aldrich, T.W. (1986) Embryonic mortality and abnormalities of aquatic birds: Apparent impacts of selenium from irrigation drainwater. *Sci Total Environ* 52:49-63

Ohlendorf, H.M., Hothem, R.L., Bunck, C.M., and Marois, K.C. (1990) Bioaccumulation of selenium in birds at Kesterson Reservoir, California. Arch *Environ Contam Toxicol* 19:495-507.

Oremland, R.S., Hollibaugh, J.T., Maest, A.S., Presser, T.S., Miller, L.G. and Culbertson, C.W. (1989). Selenate reduction to elemental Se by anaerobic bacteria in sediments and culture: biogeochemical significance of a novel, sulfate-independent respiration. *Appl Env Microbiol* 55:2333-2343.

Oremland, R. S., J. Switzer Blum, C. W. Culbertson, P. T. Visscher, L. G. Miller, P. Dowdle, and F. E. Strohmaier (1994) Isolation, growth, and metabolism of an obligately anaerobic, selenate-respiring bacterium, strain SES-3. *Appl Environ Microbiol* 60:3011-3019.

Oremland, R., Switzer Blum, J.S., Burns Bindi, A., Dowdle, P.R., Hrebel, M., and Stolz, J.F. (1999) Simultaneous reduction of nitrate and selenate by cell suspensions of Se-respiring bacteria. *App Env Microbiol* 65:4385-4392.

Oremland, R. and Stolz, J. (2003) The ecology of arsenic. *Science* 300:939-944.

Osborne, F. H. and Ehrlich, H. L. (1976) Oxidation of arsenite by a soil isolate of *Alcaligenes*. *J Appl Bacteriol* 41:295–305.

O'Toole D. and Raisbeck M.F. (1995) Pathology of experimentally induced chronic selenosis (alkali disease) in yearling cattle. *J Vet Diagn Invest* 7:364-373.

Packman, L.C., Hale, G., and Perham, R.N. (1984) Repeating functional domains in the pyruvate dehydrogenase enzyme complex of *Escherichia coli*. *EMBO J* 3:1315-1319.

Páez-Espino, D., Tamames, J., de Lorenzo, V., and Cánovas, D. (2009) Microbial responses to environmental arsenic. *Biometals* 22:117-130.

Pokhrel, D., Bhandari, B.S., and Viraraghavan, T. (2009) Arsenic contamination of groundwater in the Terai region of Nepal: an overview of health concerns and treatment options. *Env Int* 35:157-161.

Presser, T.S. and Ohlendorf, H.M. (1987) Biogeochecmical cycling of selenium in the San Joaquin Valley of California, USA. *Environ Manage* 11:805-821.

Presser, T.S., Sylvester, M.A., and Low, W.H. (1994) Bioaccumulation of selenium from natural geologic sources in Western states and its potential consequences. *Environ Manage* 18:423-436.

Ranjard, L., Prigent-Combaret, C., Favre-Bonte, S., Monnez, C., Nazaret, S., and Cournoyer, B. (2004) Characterization of a novel selenium methyltransferase fromfreshwater bacteria showing strong similarities with the calicheamicin methyltransferase. *Biochim Biophys Act* 1679:80-85.

Rathgeber, C., N. Yurkova, E. Stackebrandt, P. Schumann, E. Humphrey, J.T. Beatty, and V. Yurkov. (2006) Metalloid reducing bacteria isolated from deep ocean hydrothermal vents of the Juan de Fuca Ridge, *Pseudoalteromonas telluritireducens* sp. nov. and *Pseudoalteromonas spiralis* sp. nov. *Current Microbiol* 53:449-456.

Rauschenbach, I., Narasingarao, P., and Häggblom, M.M. (2011) *Desulfurispirillum indicum* sp. nov., a selenate and selenite respiring bacterium isolated from an estuarine canal in southern India. *Int J Syst Evol Microbiol* 61:654-658.

Reamer, D. C., and W. H. Zoller. 1980. Selenium biomethyla-tion products from soil and sewage sludge. *Science* 208:500-502.

Rezanka, T. and K. Sigler. 2008. Biologically active compounds of semi-metals. *Phytochem* 69:585-606.

Richardson, D.J. (2000) Bacterial respiration: a flexible process for a changing environment. *Microbiol* 146:551-571.

Richardson, D. J., Berks, B. C., Russell, D. A., Spiro , S., and Taylor, C.J. (2001) Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. *Cell Mol Life Sci* 58:165-178.

Ridley, H., Watts, C.A., Richardson, D.J., and Butler, C.S. (2006) Resolution of distinct membrane-bound enzymes from *Enterobacter cloacae* SLD1a-1 that are responsible for selective reduction of nitrate and selenate oxyanions. *Appl Environ Microbiol* 72:5173-5180.

Rosen, B.P. (1999) Families of arsenic transporters. *Trends in Microbiol* 7: 207-212.

Rosen, B.P. and Zijuan, L. (2009) Transport pathways for arsenic and selenium: A minireview. *Environ Int* 35: 512–515.

Rosenberg, H., Gerdes, R., and Chegwldden, K. (1977) Two systems for the uptake of phosphate in Escherichia coli. *J Bacteriol* 131:506-511.

Rosenstein, R., Peschel, A., Wieland, B., and Götz, F. (1992) Expression and regulation of the Antimonite, Arsenite, and Arsenate resistance operon of *Staphylococcus xylosus* plasmid pSX267. *J Bac* 174:3676-3683.

Roux, M., Sarret, G., Pignot-Paintrand, I., Fontecave, M. and Coves, J. (2001) Mobilization of selenite by *Ralstonia metallidurans* CH34. *Appl Environ Microbiol* 67:769-773.

Rowe, J.J., Ubbink-Kok, T., Molenaar, D., Konings, W.N., and Driessen, A.J. (1994) NarK is a nitrite-extrusion system involved in anaerobic nitrate respiration by *Escherichia coli*. *Mol Microbiol* 12:579-586.

Sabaty, M., Avazeri, C., Pignoli, D., and Vermiglio, A. (2001) Characterization of the reduction of selenate and tellurite by nitrate reductases. *Appl Environ Microbiol* 67:1522-1526.

Sakaguchi, T., Kato, M., Kuriyama, N., Niiyama, H., Hamada, S., Morita, Y. and Tamiya, E. (2009) Conjugal transformation and transposon and chemical mutagenesis of Gram-negative selenate-respiring *Citrobacter* sp. strain JSA. *Curr Microbiol* 59:88-94.

Saltikov, C.W., Cifuentes, A., Venkateswaran, K., and Newman, D.K. (2003) The *ars* detoxification system is advantageous but not required for As(V) respiration by the genetically tractable *Shewanella* species strain ANA-3. *Appl Environ Microbiol* 69:2800-2809.

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

Saltikov, C. W., R. A. Wildman, Jr., and D. K. Newman. 2005. Expression dynamics of arsenic respiration and detoxification in *Shewanella* sp. strain ANA-3. *J Bacteriol* 187:7390-7396.

Santini, C.L., Ize, B., Chanal, A., Müller, M., Giordano, G., and Wu, L.F. (1998) A novel Sec-independent periplasmic protein translocation pathway in *Escherichia coli*. *EMBO J* 17:101-112.

Santini J.M., Sly LI.., Schnagl R.D., Macy J.M. (2000) A new chemolithoautotrophic arsenite-oxidizing bacterium isolated from a gold mine: phylogenetic, physiological, and preliminary biochemical studies. *Appl Environ Microbiol* 66:92–97.

Saratchandra, S.U. and Watkinson, J.H. (1981) Oxidation of elemental selenium to selenite by *Bacillus megaterium*. *Science* 211:600-601.

Sargent F (2007) Constructing the wonders of the bacterial world: biosynthesis of complex enzymes. *Microbiol* 153:633–651.

Silva, P. (2011) US EPA. Final determination of the U.S. Environmental Protection Agency pursuant to § 404(c) of the Clean Water Act concerning the Spruce No. 1 mine, Logan County, West Virginia.

Smith, A.H., Lingas, E.O. and Rahman, M. (2002) Contamination of drinking-water by arsenic in Bangladesh: a public health emergency. *Bul WHO* 78:1093-1103.

Steinberg, N.A. and Oremland, R.S. (1990) Dissimilatory selenate reduction potentials in a diversity of sediment types. *Appl Environ Microbiol* 56:3550-3557.

Stolz, J.F., Ellis, D.J., Switzer Blum, J., Ahmann, D., Lovley, D.R., and Oremland, R.S. (1999) *Sulfurospirillum barnesii* sp. nov. and *Sulfurospirillum arsenophilum* sp. nov., new members of the Sulfurospirillum clade of the ε Proteobacteria. *Int J Sys Evol Microbiol* 49:1177-1180.

Saltikov, C., Wildman, R.A., and Newman, D.K. (2005) Expression dynamics of arsenate respiration and detoxification in *Shewanella* sp. Strain ANA-3. *J Bacteriol* 187:7390-7396.

Sancha, A.M. and O'Ryan, R. (2008) Managing hazardous pollutants in Chile: arsenic In *Rev of Environ Contam Toxicol* 196:123-146.

Santini, C.L., Ize, B., Chanal, A., Muller, M., Giordano, G., and Wu, L.-F. (1998) A novel Sec-independent periplasmic protein translocation pathway in *Escherichia coli*. *EMBO J* 17:101–112.

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. *Appl Environ Microbiol* 66:92-97.

Santini, J., Stolz, J., and Macy, J. (2002) Isolation of a New Arsenate-Respiring Bacterium—Physiological and Phylogenetic Studies. *Geomicrobiol J* 19:41-52.

Santini, J.M., Streimann, I.C.A., and van den Hoven, R.N. (2004) *Bacillus macyae* sp. nov., an arsenate-respiring bacterium isolated from an Australian gold mine. *Int J Sys Evol Microbiol* 54:2241-2244.

Sarathchandra, S.U. and Watkinson, J.H. (1981) Oxidation of elemental selenium to selenite by *Bacillus megaterium*. *Science* 172: 600-601.

Schwarz, G., Mendel, R.R., and Ribbe, M.W. (2009) Molybdenum cofactors, enzymes and pathways. *Nature* 13:839-847.

Schröder, I., Rech, S., Krafft, T., and J.M. Macy (1997) Isolation and characterization of the selenate reductase from *Thauera selenatis. J Biol Chem* 272:23765-23868.

Sears, H.J., Sawers, G., Berks, B.C., Ferguson, S.J., and Richardson, D.J. (2000) Control of periplasmic nitrate reductase gene expression (*napEDABC*) from *Paracoccus pantotrophus* in response to oxygen and carbon substrates. *Microbiol* 146:2877-2985.

Silva, P. (2011) Final determination of the U.S. Environmental Protection Agency pursuant to § 404(c) of the Clean Water Act concerning the Spruce No. 1 Mine, Logan County, West Virginia. *EPA*.

Silver, S., and Phung. L.T. (1996) Bacterial heavy metal resistance: New surprises. *Annu Rev Microbiol* 50:753-789.

Simon, J. (2002) Enzymology and bioenergetics of respiratory nitrite ammonification. *FEMS Microbiol Rev* 26:285-309.

Simpson, P.J.L., Richardson, D.J., and Codd, R. (2010) The periplasmic nitrate reductase in *Shewanella*: the resolution, distribution and functional implications of two NAP isoforms, NapEDABC and NapDAGHB. *Microbiol* 156:302-312.

Sirko, A., Hryniewicz, M., Hulanicka, D. and Bock, A. (1990) Sulfate and thiosulfate transport in *Escherichia coli* K-12: nucleotide sequence and expression of the cysTWAM gene cluster. *J Bacteriol* 172:3351–3357.

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

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

Sorensen, E.M.B., Bauer, T.L., Bell, J.S., and Harlan, C.W. (1982) Selenium accumulation and cytotoxicity in teleost following chronic, environmental exposure. *Bull Environ Contam Toxicol* 29:688-696.

Sorokin, D.Y., Foti, M., Tindall, B.J. and Muyzer, G. (2007). *Desulfurispirillum alkaliphilum*, gen. nov., sp. nov., a novel obligately anaerobic sulfur- and dissimilatory nitrate-reducing bacterium from a full-scale sulfide-removing bioreactor. *Extremophiles* 11:363-370.

Spallholz, J. (1994) On the nature for selenium toxicity and carcinostatic activity. *Free Rad Biol Med* 17:45-64.

Steinberg, N.A. and Oremland, R.S. (1990). Dissimilatory selenate reduction potentials in a diversity of sediment types. *Appl Env Microbiol* 56:3550-3557.

Stewart, V., Lu, Y., and Darwin, A.J. (2002) Periplasmic nitrate reductase (NapABC Enzyme) supports anaerobic respiration by *Escherichia coli* K-12. *J Bacteriol* 184:1314-1323.

Stolz, J.F., Ellis, D.J., Switzer Blum, J., Ahmann, D., Lovley, D.R., and Oremland, R.S. (1999) *Sulfurospirillum barnesii* sp. nov. and *Sulfurospirillum arsenophilum* sp. nov., new members of the *Sulfurospirillum* clade of the ε-Proteobacteria. *Int J Sys Evol Microbiol* 49:1177-1180.

Stolz, J.F. and Oremland, R.S. (1999) The ecology of arsenic and Se. *FEMS Microbiol Rev* 23:615-627.

Stolz, J.F., Basu, P., and Oremland, R. (2002) Microbial transformation of elements: the case of arsenic and Se. *Int Microbiol* 5:201-207.

Stolz, J., Basu, P., Santini, J.M., and Oremland, R.S. (2006) Arsenic and Se in microbial metabolism. *Annu Rev Microbiol* 60:107-130.

Summers, A.O. and Jacoby, G.A. (1977). Plasmid-determined resistance to tellurium compounds. *J Bacteriol* 129:276-281.

Swearingen J.W. Jr, Frankel D.P., Fuentes DE, Saavedra C.P., Vasquez C.C., Chasteen T.G. (2006) Identifcation of biogenic dimethyl selenodisulfide in the headspace gases above genetically modified *Escherichia coli*. *Anal Biochem* 348:115–122.

Switzer Blum, J., Burns Bind, A., Buzzelli, J., Stolz, J.F., Oremland R.S. (1998) *Bacillus arsenicoselenatis* sp nov., and *Bacillus selenitireducens* sp. nov.: two haloalkaliphiles from Mono Lake, California which respire oxyanions of Se and arsenic. *Arch Microbiol* 171:19–30.

Switzer Blum, J., Stolz, J.F., Oren R.S. Oremland (2001) *Selenihalanaerobacter shriftii* gen. nov., sp. nov., a halophilic anaerobe from Dead Sea sediments that respires selenate. *Arch Microbiol* 175:208–219.

Switzer Blum, J., Han, S., Lanoil, B., Saltikov, C., Witte, B., Tabita, R.F., Langley, S., Beveridge, T.J., Jahnke, L., and Oremland, R.S. (2009) Ecophysiology of "*Halarsenatibacter silvermanii*" strain SLAS-1^T, gen. nov., sp. nov., a facultative chemoautotrophic arsenate respirer from salt-saturated Searles Lake, California. *Appl Environ Microbiol* 75:1950-1960.

Takai, K., Kobayashi, H., Nealson, K.H., and Horikoshi, K. (2003) *Sulfurihydrogenibium subterraneum* gen. nov., sp. nov., from a subsurface hot aquifer. *Int J Sys Evol Microbiol* 53:823-827.

Takai, K., Kobayashi, H., Nealson, K.H., and Horikoshi, K. (2003) *Deferribacter desulfuricans* sp. nov., a novel sulfur-, nitrate- and arsenate-reducing thermophile isolated from a deep-sea hydrothermal vent. *Int J Sys Evol Microbiol* 53:839-846.

Tamaki, S. and Frankenberger, W.T. (1992) Environmental biochemistry of arsenic. Rev *Environ Cont Toxicol* 124:79-109.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molec Biol Evol* 24:1596-1599.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011) Mega5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution (submitted).

Taylor, D.E. (1999) Bacterial tellurite resistance. *Trends in Microbiol* 7:111-115.

Tisa, L. S. and Rosen, B. P. (1989) Molecular characterization of an anion pump – The ArsB protein is the membrane anchor for the ArsA protein. *J Biol Chem* 265:190-194.

Tucker, F.L., Walper, J.F., Appleman, M.D., and Donohue J. (1961) Complete reduction of tellurite to pure tellurium metal by microorganisms. *J Bacteriol* 83: 1313-1314.

Turner, R.J., Weiner J.H. and Taylor, D.E. (1998) Selenium metabolism in *Escherichia coli*. *Biometals* 11:223–227.

Turpeinen, R., Pantsar-Kallio, M., Häggblom, M., Kairesalo, T. (1999) Influence of microbes on the mobilization, toxicity and biomethylation of arsenic in soil. *Sci Total Environ* 236:173-180.

Valentine, J.L. (1997) Environmental occurrence of selenium in waters and related health significance. *Biomed Environ Sciences* 10:292-299.

Van Fleet-Stalder, V., Chasteen, T. G., Pickering, I. J., George, G. N. and Prince, R. C. (2000) Fate of selenate and selenite metabolized by *Rhodobacter sphaeroides*. *Appl Environ Microbiol* 66:4849-4853.

Villafane, A.A., Voskoboynik Y., Cuebas M., Ruhl I., and Bini E. (2009) Response to excess copper in the hyperthermophile *Sulfolobus solfataricus* strain 98/2. *Biochem Biophys Res Commun* 17:67-71.

von Wintzingerode, F., Gobel, U.B., Siddiqui, R.A., Rosick, U., Schumann, P., Fruhling, A., Rohde, M., Pukall, R., and Stackebrandt, E. (2001). *Salana multivorans* gen. nov., sp. nov., a novel actinobacterium isolated from an anaerobic bioreactor and capable of selenate reduction. *Int J Syst Evol Microbiol* 51:1653-1661.

Wang, H., Tseng, C-P., and Gunsalus, R.P. (1999) The *napF* and *narG* nitrate reductase operons in *Escherichia coli* are differentially expressed in response to submicromolar concentrations of nitrate but not nitrite. *J Bacteriol* 181:5303–5308.

Watts, C. A., H. Ridley, K. L. Condie, J. T. Leaver, D. J. Richardson, and Butler, C.S. (2003) Selenate reduction by *Enterobacter cloacae* SLD1a-1 is catalyzed by a molybdenum-dependent membrane bound enzyme that is distinct from the nitrate reductase. *FEMS Microbiol Lett* 228:273–279.

Weiner,J.H., Bilous, P.T., Shaw,G.M., Lubitz, S.P., Frost, L., Thomas, G.H., Cole, J.A., and Turner, R.J. (1996) Novel and ubiquitous system for membrane targeting and secretion of cofactor-containing proteins. *Cell* 93:93–101.

Wilber, C. (1980) Toxicology of selenium: a review. *Clinical Toxicol* 17:171-230.

Williams, M. (1997) Mining related Arsenic Hazards: Thailand case study. Summary report. *British Geol Surv Tech Rep* WC/97/49.

Wittwer, A.J., Tsai, L., Ching, W.M., and Stadtman, T.C. (1984) Identification and synthesis of a naturally occurring selenonucleoside in bacterial tRNAs: 5-[(methylamino)methyl]-2-selenouridine. *Biochem* 23:4650-4655.

Wolin, M.J., Wolin, E.A., and Jacobs, N.J. (1961) Cytochrome-producing anaerobic vibrio, *Vibrio succinogenes*, sp. n. *J Bacteriol* 81:911-917.

Wormell, L. (2006) Reregistration eligibility decision for MSMA, DSMA, CAMA, and cacodylic acid. EPA-HQ-OPP-2006-0201.

Wormell, L. (2008) Human health risk assessment and ecological effects assessment for the reregistraion eligibility decision (RED) document of inorganic arsenicals and/or chromium wood preservatives. EPA-HQ-OPP-2003-0250-0081

Wu J, Rosen BP The arsD gene encodes a second trans-acting regulatory protein of the plasmid-encoded arsenical resistance operon. *Mol Microbiol* 8:615-623.

Wysocki R, Chery CC, Wawrzycka D, Van Hulle M, Cornelis R, Thevelein JM, Tamas MJ (2004) The glycerol channel Fps1p mediates the uptake of arsenite and antimonite in *Saccharomyces cerevisiae*. *Mol Microbiol* 40:1391–401.

Xia, Y. and Liu, J. (2004)An overview on chronic arsenism via drinking water in PR China. *Toxicology* 198:25-29.

Xu,C., Zhou, T., Kuroda, M., and Rosen, B.P. (1989) Metalloid resistance mechanisms in prokaryotes. *J Biochem* 123:16-23.

Yang, G., Wang, S., Zhou, R., and Sun, S. (1983) Endemic selenium intoxication of humans in China. *Am J Clin Nut* 37:872-881.

Zannoni, D., Borsetti, F., Harrison, J.J. and R.J. Turner (2008) The bacterial response to the chalcogen metalloids Se and Te. *Adv Microb Physiol* 53:1-72.

Yee, N., Ma, J., Dalia, A., Boonfueng, T. and Kobayashi, D.Y. (2007) Se(VI) reduction and the precipitation of Se(0) by the facultative bacterium *Enterobacter cloacae* SLD1a-1 are regulated by FNR. *Appl Environ Microbiol* 73:1914–1920.

Zawislanski PT, Benson SM, Terberg RT, Borglin SE (2003) Selenium speciation, solubility and mobility in land-disposed dredged sediments. *Environ Sci Technol* 37:2415-2420.

Zehr, J. and Oremland, R.S. (1987) Reduction of selenate to selenide by sulfate-respiring bacteria: experiments with cell suspensions and estuarine sediments. Appl *Environ Microbiol* 53:1365-1369.

Zerbino D.R., and Birney E. (2008) Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res* 18:821-9.