Development of a Biochemical Probe for Arsenate Respiring Bacteria using Bacillus selenitireducens strain MLS10

Mirunalni Thangavelu

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Development of a Biochemical Probe for Arsenate Respiring Bacteria using Bacillus selenitireducens strain MLS10

A Thesis presented to the Department of Biological Sciences
Duquesne University

In partial fulfillment of the requirements for the Degree of Master of Science

By

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ABSTRACT

To date, eighteen phylogenetically diverse prokaryotes have been found to utilize arsenic as the terminal electron acceptor in anaerobic respiration, converting arsenate into the more toxic and mobile arsenite. This process can lead to ground water contamination, making it imperative to detect in situ, active arsenate-respiring prokaryotes.

A biochemical probe that targets the catalytic subunit (ArrA) of the respiratory arsenate reductase (Arr) from Bacillus selenitireducens strain MLS10 was developed. Polyclonal antibodies were raised against a fifteen amino acids long highly conserved sequence at the C-terminus of ArrA. Highly specific antibodies obtained by affinity purification using the synthesized ArrA polypeptide, reacted with ArrA from B. selenitireducens, B. arsenicoselenatis, Clostridium sp. strain OhILAs, and strain SLAS-1, but not Bacillus sp. strain JMM-4, strain MLMS-1, and the epsilon Proteobacteria Sulfurospirillum barnesii, S.deleyianum, or S. arsenophilum. Western blot analysis and activity assays indicated that Arr from B. selenitireducens is up regulated by arsenic.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Arr</td>
<td>Arsenate Reductase</td>
</tr>
<tr>
<td>ArrA</td>
<td>Catalytic Subunit of Arsenate Reductase</td>
</tr>
<tr>
<td>ArrB</td>
<td>Fe-S Cluster of Arsenate Reductase</td>
</tr>
<tr>
<td>DMAs(V)</td>
<td>Dimethyl Arsenate</td>
</tr>
<tr>
<td>DMAA (V)</td>
<td>Dimethyl Arsenic Acid</td>
</tr>
<tr>
<td>DMAA (III)</td>
<td>Dimethyl Arsenous Acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme Linked ImmunoSorbent Assay</td>
</tr>
<tr>
<td>MV</td>
<td>Methyl Viologen</td>
</tr>
<tr>
<td>MAs(III)</td>
<td>Monomethyl Arsenite</td>
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<td>MMAA(V)</td>
<td>Monomethyl Arsenic Acid</td>
</tr>
<tr>
<td>MMAA (III)</td>
<td>Monomethyl Arsenous Acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenyl Methyl Sulfonyl Fluoride</td>
</tr>
<tr>
<td>PTPase</td>
<td>Protein Tyrosine Phosphatase</td>
</tr>
<tr>
<td>RAsR</td>
<td>Respiratory Arsenate Reductase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>TMAO</td>
<td>Trimethyl Arsine Oxide</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloro Acetic Acid</td>
</tr>
</tbody>
</table>
I. INTRODUCTION

A. Background

Arsenic (As) is a naturally occurring element present in rocks, water and air. It is makes up only 0.0002% of the Earth’s crust and is the 20th most abundant element. Arsenic is mainly associated with igneous and sedimentary rocks. It enters aquatic systems by weathering of arsenic containing minerals and enters the atmosphere through volcanic emissions, wind erosion and other such natural phenomena. The anthropogenic release of arsenic, however is an alarming 28,000 metric tons per year, nearly four times the natural emission (Cullen and Reimer, 1989). The background concentration in the earth’s crust is about 2 mg kg\(^{-1}\) but the concentration in ground water is about 0.001 mg L\(^{-1}\) (Welch et al., 2000).

Arsenic is a poison. Its toxicity is due to the similarity, in chemical and biochemical properties, that it shares with nitrogen and phosphorus. Arsenate is an analog of phosphate and can uncouple mitochondrial oxidative phosphorylation of glyceraldehyde 3-phosphate dehydrogenase (Oremland et al., 2001). Arsenite binds with thiol groups and destabilizes proteins. Arsenate toxicity can affect the skin, liver, kidneys, and lungs, prostate and can also cause diabetes and coronary disease. Arsenic poses a threat to wildlife and human health due to its occurrence at increasingly high concentrations in terrestrial and aquatic environments. Industrial activities, such as mining and ore smelting, have further elevated the concentration of arsenic in the environment to significant levels (g/kg) (Newman et al., 1998).

Arsenic concentration of the soil of Northern Chile has been reported to be as high as 1099 mg kg\(^{-1}\). Aquifers have been found to contain 11.25 mg L\(^{-1}\) of arsenic. Geologic
factors have contributed to this increase in arsenic content. There are reports that populations of Northern Chile consume drinking water containing arsenic concentration $> 0.05 \, \text{mg L}^{-1}$ during the period of 1955-1970 resulting in an increased death rate from lung and bladder cancer (Munoz et al., 2002).

Human exposure to arsenic can be from air, food and drinking water. Vegetables and cereals are also a source of dietary arsenic exposure. A study conducted on 16 agricultural products for total and inorganic arsenic content grown in Northern Chile revealed that the total arsenic contents varied in the range between 0.008-0.604 mg kg$^{-1}$ of wet weight whereas inorganic arsenic was found to be between 28 and 114% of the total arsenic. The arsenic concentration was higher in edible roots and leaves, especially spinach, than those found in fruit (Munoz et al., 2002).

The estimated average dietary intake of inorganic arsenic is 0.03-0.04 mg per day (US Food and Drug Administration Total Diet Study) but the Center for Disease Control’s Agency for Toxic Substances and Disease Registry has estimated that 0.025-0.05 mg of inorganic arsenic is ingested per day. These are permissible levels of arsenic uptake from food (Holson et al., 2000). Algal food products have become a source of dietary arsenic exposure in many Western countries because of their nutritional content. The total arsenic and inorganic arsenic in 18 different algal food products in Spain have been reported as high as 2.3-141 mg kg$^{-1}$ and 0.15-88 mg kg$^{-1}$ of dry weight respectively due to bio-accumulation of heavy metals. (Almela et al., 2002).

Arsenic is a public health hazard in many parts of the world, drinking water being the most significant source of arsenic. There have been reports of large numbers of people being subject to arsenic exposure in India, Bangladesh, China and Mangolia...
The high arsenic concentration in drinking water maybe attributed to different facts. Hot spring waters normally contain 1-10 mg L\(^{-1}\) arsenic and, As concentrations up to 50 mg L\(^{-1}\) have been reported in some areas and biological activity are believed to be the cause for the high arsenic values (Gihring and Banfield, 2001). Hence, knowing the biological mechanisms of microbial arsenic transformation is very important (Martin et al., 2001). The microbial oxidation of arsenite with nitrate as a terminal electron acceptor has implications for the mobility of arsenic in aquifers that have a low dissolved organic matter content. Oxidation of arsenite can also be important as a detoxification process or can serve as an energy source for chemolithoautotrophic metabolism. Removal of arsenic from the drinking water is a primary environmental concern. The identification and understanding of the biogeochemistry of arsenic is crucial to eliminate or reduce the high concentrations of arsenic in contaminated water in areas like Bangladesh and Taiwan.

**B. Geochemistry Of Arsenic**

Arsenic, a group V element with atomic number 33, can exist primarily in four oxidation states: arsine (As\(^{-3}\)), elemental arsenic (As\(^0\)), arsenate (As\(^{+5}\)) and arsenite (As\(^{+3}\)). Native arsenic occurs rarely while in aerobic environments arsenate [As(V) as H\(_2\)AsO\(_4^-\) and HAsO\(_4^{2-}\)] predominates and arsenite [As (III) as H\(_3\)AsO\(_3\)] is most prevalent in anoxic environments. As(III) is poorly adsorbed onto iron oxy-hydroxides and is more mobile than As(V) in aquatic environments, greatly increasing arsenic toxicity. The adsorbed arsenic is released with the occurrence of the reductive dissolution of solid phase iron oxyhydroxides. This process can account for the occurrence of soluble As in reduced aquatic environments.
Naturally occurring arsenic sources, include rock types (felsic-volcanic rocks of acidic and intermediate composition), certain minerals (arsenopyrite and cinnabar), and iron oxide. Arsenic has also been found adsorbed in clays such as illite and kaolinite at pH 7.5 and pH 9.5, whereas humic acids adsorb arsenic at pH 5.0 - 7.0. Arsenic adsorption studied with clay pillared with titanium(IV), iron (III) and aluminium (III) showed that amorphous iron hydroxide has the highest adsorption capacity. In goethite, an iron oxyhydroxide, arsenite adsorption is stronger than arsenate thus indicating the adsorption of arsenic onto Fe-pillared clay to be a potential method for arsenic removal in water treatment (Lenoble et al., 2002).

Differential pulse polarography studies on a pond that receives effluent from a gold mine have helped in distinguishing between the various oxidation states and the physical forms of arsenic. Speciation depends on the physicochemical properties of water with two thirds of the total arsenic in the dissolved phase and one-third in the particulate phase (Sproal et al., 2002).

Geochemical conditions, such as oxidation-reduction, associated or competing ions, and high rates of evaporation have significant effects on arsenic concentrations in ground water. In parts of Maine, Michigan, Minnesota, South Dakota, Oklahoma, and Wisconsin arsenic concentrations exceed 10 µg/L, resulting from upflow of geothermal water, desorption from iron-oxide, and dissolution of sulfide minerals (Figure 1) (Welch et al., 1999).

Chemical factors, sediment type, pH and reducing conditions determine the speciation and mobility of arsenic. Reductants like sulfide, and oxidants like Fe(III) and Mn(IV) affect speciation. Arsenate reduced to arsenite in the presence of sulfide is
precipitated as orpiment while Fe(III) and Mn(IV) oxidize arsenite into arsenate (Oremland and Stolz, 2001). In Mono lake California, where the arsenic concentration is 200 µM, arsenate and arsenite are more prevalent whereas the methylated forms are not significant (Oremland and Stolz, 2004). The speciation of arsenic changed from arsenate in the oxic mixolimnion to arsenite in the anoxic monimolimnion. Using radiotracer techniques it was determined that the highest rate of arsenate reduction (~5.5 µl mol l⁻¹ d⁻¹) was at the bottom of the oxycline (18 m), and reduced to 0.5–1.0 µl mol l⁻¹ d⁻¹ at greater depths. Seasonal variation affecting the dissimilatory arsenate reduction is due to mixing of the lake water between March and April, corresponding to ~6 µl mol l⁻¹ d⁻¹, after which it declines to 1.0 µl mol l⁻¹ d⁻¹ from April to May (Oremland et al., 2004).

Figure 1: Data-density-based map showing arsenic concentration (Welch et al., 2000)
Organic forms of arsenic occur when arsenic is taken up by phytoplankton and other marine organisms and then converted to methylated forms. The methylated forms include monomethyl arsenic acid [MMAA(V)], dimethyl arsenic acid [DMAA (V)], monomethyl arsenous acid [MMAA (III)] and dimethyl arsenous acid [DMAA (III)] (Reimer, 1988). A number of methylated forms of arsenic are found in natural water as a result of metabolic activity of the aquatic biota like algae, clams, lobsters and shrimp. Complex organoarsenical compounds like arenosugars and arsenolipids are suggested to be the adaptive mechanisms in marine organisms when nitrate sources are limiting. The major organoarsenic compound isolated from marine organisms is arsenobetaine, which can be degraded into methylarsonic acid and to inorganic arsenic (Mukhopadhyay et al., 2002). The mechanisms for conversion of arenosugars and arsenolipids into arsenobetaine have still not been revealed.

C. Toxicity Of Arsenic

The chemical similarity of arsenate and phosphate enables arsenate to act as an analog to phosphate. Arsenic trioxide has been a most common homicidal agent in very small dosages. Arsenate prevents formation of phosphoglyceroyl phosphate by uncoupling mitochondrial oxidative phosphorylation. Arsenite has increased toxicity because it binds to sulphydryl groups thereby impairing the function of some proteins. It binds to the vicinal thiols in pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase key enzymes in Kreb’s cycle. Arsenite interacts with the glucocorticoid receptor harming signal transduction pathways, suppressing transcription of genes involved in tumor suppression increasing the risk of cancer (Oremland and Stolz, 2003).

Arsenic has been classified as a carcinogen (International Agency for Research on
Cancer, 1987). The chronic exposure to arsenic also causes non-cancer health effects. Studies on the drinking water in northeastern Taiwan showed that high levels of arsenic (>0.1 mg/L) presented the risk of preterm delivery and a greater risk for reduced infant birth weight (Yang et al., 2003).

Methylated and dimethylated arsenicals, with arsenic in the trivalent oxidation state MAs(III) and DMAs(V), are cytotoxic and genotoxic and methylation is a means of activation, or detoxification, depending on the oxidation state of arsenic which determines the reactivity and toxicity. One method by which As causes cellular injury is by inducing oxidative stress affecting signaling molecules and gene transcription (Thomas et al., 2001). Humans methylate arsenic species when amounts below 0.2-0.25 mg/day are ingested. While this provides protection from toxic non-carcinogenic effects, it does not necessarily protect against the carcinogenic effects. One hypothesis suggests that methyl groups used for arsenic detoxification when not routed away from DNA synthesis, cause chromosomal damage and indirectly, cancer.

The human erythrocyte membrane (HEM) is the first target after systemic absorption of arsenic, because of the ability of arsenate to pass across the membrane through the anion exchange protein. It has been found that arsenite can be absorbed more rapidly than arsenate. The sulfhydryl groups of the membrane proteins are the binding sites for arsenate and arsenite, affecting membrane integrity. The tertiary and quaternary structures of the HEM are affected by arsenite and arsenate binding reflected in the lower fluidity, change in the charge and increase in the fragility of the membrane. (Zhang et al., 2000)

Inhibition of DNA ligase, interference of DNA methylation and signal
transduction are the various modes of action for arsenicals. MAs\textsuperscript{(III)} and DMAs\textsuperscript{(V)} damage naked DNA and have been reported to be direct acting forms of arsenic that are genotoxic (Mass \textit{et al}., 2001). MMAs\textsuperscript{(III)} inhibit glutathione reductase and also NADPH-dependent oxido-reductase and thioredoxin reductase. HPLC/ICPMS studies have detected eight species of arsenic in the urine of humans exposed to inorganic arsenic in their drinking water in West Bengal, India with 4-21\% present as DMAs\textsuperscript{(III)} and 44-74\% as DMAs\textsuperscript{(V)} in urine species. (Mandal \textit{et al}., 2001)

Arsenic is not essential for plant metabolism but may be necessary for animal metabolism. The bioavailability and phytotoxicity of As depends on the source and concentration of arsenic species. Application of herbicides increases the concentration of the toxic metals in agricultural soil used for cultivating vegetables such as turnips, radishes, tomatoes, and beans. The levels of As in the tissues of turnip ranged from 24.6-to 116 mg/kg dry weight. This addition of arsenic to the fertile soil through pesticide and herbicide use, poses a health hazard due to the accumulation of As in the edible portions of the plants (Carbonelli-Barrachina \textit{et al}., 1999).

Immobilized metals are less toxic which allows higher plants to use phytochelatins for the detoxification mechanism. Phytochelatins [(\textgamma-glutamate-cysteine)\textsubscript{n}-glycine] are glutathione-derived peptides that bind to metals. The PC synthase has been found to be induced by either arsenate or arsenite. Gel filtration studies and Electro spray Ionization Mass spectroscopy (ESI-MS) have established the enzymatic activation by As leading to a As-PC complex which results in the sequestering of As by the phytochelatins.(Schmoger \textit{et al}., 2000)

Arsenic resistance has been studied in many plant species by high-affinity
phosphate uptake systems. The Chinese brake fern *Pteris vittata* is an arsenic hyperaccumulator. This fern has shown potential for use in arsenic contaminated soils. The As-contaminated soil was amended with phosphate and it was observed that this caused an increase in the uptake of arsenic by the fern (Cao *et al.*, 2003). The enhanced mobility of arsenic is due to the replacement of the arsenic by phosphate at the soil binding sites, transformation of arsenate to soluble arsenite increased the uptake and adsorption of arsenate onto iron bound fractions of the soil. The compost and phosphate amendments in concert with the fern effectively decreased the leaching of arsenic from the soil.

Long-term contamination with arsenic adversely affects a soil’s microbial properties. Fungal growth is the most sensitive. Chronic exposure to DDT and arsenic at cattle dipping sites can result in the lowering of soil pH, a decrease in microbial biomass and rate of metabolic activities, and a steady decline in fungal populations (Edvantoro *et al.*, 2003). Studies on growth and reproduction of the Great Salt Lake brine shrimp, *Artemia franciscama* determined the chronic toxicity of arsenic. 15 mg/L arsenic limits survival and at 56 mg/L reproduction was reduced by 40% after 21 days. (Brix K.V., *et al.*, 2003).

In Bangladesh, arsenic poisoning from drinking water has affected 80% of the population. Recent reports indicate that 60 million people in Bangladesh are ingesting arsenic contaminated water and more than 7,000 people are already suffering from symptoms of arsenic poisoning such as general malaise, decreased appetite and weight loss. In a study of water samples from Bangladesh and West Bengal, India, 59 and 34% respectively contained more than 50 mg/L of arsenic and over 33% of the
population exhibiting arsenicosis, were affected by arsenic neuropathy (Chowdhury et al., 2000). Interestingly, the arsenic species present in the water sample were not methylated arsenic species like MMAA or DMAA, but arsenate and arsenite. Buccal mucus membrane melanosis and keratosis were the most prevalent manifestations of arsenic toxicity.

The concentration of arsenic increases with depth in wells in Bangladesh. The arsenic is released by reductive dissolution of iron oxyhydrxides. Bacterial reduction of Fe(III) to Fe(II) can affect arsenic solubility, as the process should release any bound As(V), making it available for further biological reduction. Such a mechanism has been proposed as the source of arsenic in the wells of Bangladesh (Oremland and Stolz, 2003).

**D. Arsenic Resistance - *Ars C* system**

Microorganisms have evolved various mechanisms by which they evade arsenic toxicity - increasing the uptake of phosphate, oxidizing the arsenite by enzyme Arsenite oxidase or by peroxidation reactions with membrane lipids. Two methods for detoxification have been proposed. The first method is the methylation of the arsenic species with S-adenosyl methionine (SAM) as the methyl donor, but it has been reported that methylation is a means of activation. The second mechanism found in microbes like bacteria, yeast and lower prokaryotes is the ArsC system. Bacteria guard against arsenic using an arsenate resistance pathway (*arsC* system) removing internal pools of arsenate by reducing it to arsenite thereby lowering the intracellular concentrations of this toxic chemical.

The bacterial *ars* operon is comprised of *arsR*, the regulatory gene for the
operon, *arsC* the arsenate reductase, and *arsB*, the transmembrane pump specific for arsenite, and in some bacteria the additional genes *arsA* the gene product of which is an arsenite-stimulated ATPase and *arsD* a regulatory gene for *ars* expression. The arsenate that has entered the cell via the phosphate-transport systems is first reduced to arsenite by the soluble cytoplasmic arsenate reductase, and the arsenite is transported out of the cell via an energy dependent arsenite transmembrane pump. In the Gram negative bacteria that have been studied, the efflux pump is a two component ATPase complex - *arsA* encodes for a soluble ATPase subunit and *arsB* encodes for an integral membrane protein. The arsenite is pumped out to prevent binding with sulfhydryl groups that render it more toxic.

The ArsC families are distributed among Gram-positive, Gram negative bacteria and Eukaryota (yeast). The arsenate reductases from *Escherichia coli* (Gram-negative), *Bacillus subtilis*, *Staphylococcus aureus* (Gram positive), and *Saccharomyces cerevisiae* (Eukaryota) have been well characterized. The interfamilial sequence identity was less than 20%. The enzymes from Gram-positive and Gram-negative share very low sequence homology and distinct catalytic features, but function similarly by catalyzing the same chemical reaction and utilizing cysteine residues at the catalytic site.

The *ars* operon from *E.coli* plasmid R773 was found to offer resistant to both arsenic and antimony and deletion mutants of the *ars* operon were found to be several fold more hypersensitive to arsenate, arsenite and antimony (Carlin *et al.*, 1995). ArsC from *E.coli* plasmid R773 has a distinct catalytic motif (HX₃CX₃R). The ArsA has been purified in *E.coli* as a soluble ATPase (63kDa) in the absence of the transporter
protein ArsB(45kDa) that is activated by arsenite. Four cysteines (Cys-26, Cys-113, Cys-172, and Cys-422) are involved in the allosteric activation of the enzyme. Site directed mutagenesis and X-ray Absorption Spectroscopy have demonstrated that two cysteines (Cys-113 and Cys-172) of the A1 subunit and Cys-422 of the A2 subunit coordinate with arsenite drawing together the two subunits of the ATPase into the correct conformation, thereby activating the enzyme. The energy produced from ATPase activity drives the transmembrane export of the metalloid oxyanion As(III) (Rosen et al., 1999).

The Gram-positive family of arsenate reductases has been shown to be structurally and functionally similar to mammalian low molecular weight tyrosine phosphatases. The arsenate reductase expressed in B. subtilis has been found to share a 64% homology with the S. aureus enzyme, including the three cysteine residues. The enzyme from B. subtilis is structurally similar to low molecular weight protein tyrosine phosphatases (PTPase) sharing just 18% sequence homology.

The cloned staphylococcal ars operon confers resistances to arsenate, arsenite, and antimonite in S. aureus and B. subtilis. A deletion mutation in arsB resulted in decreased resistance to arsenate and total loss of arsenite and antimonite resistance. Partial deletion from the 3' end of the arsC gene resulted in loss of resistance to arsenate but the mutant was still resistant to arsenite and antimonite (Ji and Silver, 1992). Interestingly, the staphylococcal operon did not have a corresponding gene for ArsA that is found in the ars operon of E.coli.

The structure of the purified ArsC from S.aureus has been elucidated by 15N-, 13C-nuclear magnetic resonance (NMR) and X-ray crystallography (Messens et al.,
The ArsC expressed in *S. aureus* plasmid p1258 has a tyrosine phosphatase I (PTPase) fold which includes a P-loop and a sequence motif (CX₅R). The P-loop and the three cysteines (Cys 10, 82 and 89) of ArsC are required for the catalytic activity of the enzyme. The oxidized enzyme is regenerated by means of a reaction involving thioredoxin, thioredoxin reductase and NADPH. Steady-state kinetics of mutants from site directed mutagenesis of ArsC, have unraveled the intermediates of the arsenate reductase mechanism in *S. aureus* p1258. The ArsC is unique that it involves a nucleophilic displacement reaction resulting in an intramolecular disulfide bond cascade that triggers a reversible “conformational switch” to release the reduced substrate. The formation of a disulfide bond between the Cys10-Cys82 triggers the change in structure allowing for the Cys89 to attack Cys82. Site directed mutagenesis also demonstrated that Arg16 is essential for the catalytic activity.

A parallel study has indicated that the arginine in the CX₅R sequence motif is essential for the activity of Acr2p from yeast. In yeast, arsenic resistance involves three genes- *Acr1, Acr2, and Acr3*. Mutants of *Acr1* leads to hypersensitivity to arsenate and arsenite where as deletion of *Acr3* renders the cell incapable of resistance to both oxyanions while Acr2 mutants lack resistance to arsenate alone (Mukhopadhyay and Rosen, 1998). The regeneration of the active Acr2p is achieved by the reduction, of the disulfide bond with glutathione by glutaredoxin.

ArsC from both the two Gram positive bacteria demonstrated phosphatase activity. The two enzymes have similar active sites. It has been found to hydrolyze the substrate of PTPase *in vitro*, suggesting a similar first step in their catalytic
mechanism (Bennet et al., 2001). Similar phosphatase activity is not exhibited by ArsC from *E. coli* or Acr2p from yeast.

Another bacterium, *Pseudomonas fluorescens* strain MSP3, which was isolated from seawater, contains chromosomally encoded arsenic resistance. The arsenic resistance operon (arsRBC) of strain MSP3 consists of 3 genes, *arsR* - encoding a repressor regulatory protein, *arsB*, encoding for a membrane efflux protein that pumps arsenic from the cell and *arsC*, encoding a small cytoplasmic polypeptide required for arsenate resistance only (not for arsenite resistance). *arsA* and *arsD* genes are missing from the chromosomally encoded *ars* operon of this bacterium (Prithivirajsingh et al., 2001)

The *arsC* phylogeny matched the established 16S rRNA phylogeny, with three major groups- the archaeal, bacterial and eukaryotic *arsC* genes (Jackson and Dugas, 2003). There were minor inconsistencies but otherwise the phylogeny formed expected patterns with separation of the subdivisions of Bacteria (e.g. Proteobacteria, Low G+C gram –Positives, Actinobacteria). The existence of non-orthologous *arsC* genes in the phylogeny is suggested to have arisen from multiple gene duplication events, convergent evolution or by horizontal gene transfer. The phylogeny of plasmid borne *arsC* genes shows that the origin may be paraphyletic, diverging into two very different *arsC* types: the Staphylococci and Enterobacteriales. The *arsC* phylogeny suggests that the arsenate reductase enzyme is an evolutionarily old enzyme, which leads to the supposition that the mechanism of arsenate resistance was present in ancestral organisms. The reducing environments of the Hadean era could have applied selective pressure to develop an arsenate resistance mechanism.
to protect phosphate-dependent cellular metabolism.

**E. Microbial respiration of Arsenic**

It is surprising that though known to be an essential toxin, there are microorganisms that use arsenic species for growth. Arsenate reduction can occur via a dissimilatory route, in which arsenate is used as an electron acceptor under anaerobic or micro aerobic conditions and reduction is coupled to the oxidation of acetate, lactate, pyruvate, glycerol and ethanol (Oremland and Stolz, 2003, Santini and Stolz, 2004). Arsenate respiring bacteria have been isolated from arsenic contaminated sites in United States and Australia. Isolated under strictly anaerobic conditions these bacteria have been enriched in anaerobic minimal medium supplemented with arsenate and organic carbon sources, (Newman et al., 1998, Oremland and Stolz, 2003).

The microbial transformation of arsenic oxyanions for energy generation can be exploited for detoxification purposes. Thermodynamic calculations using acetate or lactate as the electron donor, suggest that arsenate reduction is energetically favorable. Arsenate is electrochemically positive with an oxidation-reduction potential of +139 mV. The calculated thermodynamic values for arsenate reduction when lactate and formate (Figure 2) are the electron donors both illustrate that it is a energy yielding, proton consuming reaction.

Comparison of the calculated free energies for a number of electron acceptors using hydrogen as an electron donor illustrates that the reduction of arsenate is thermodynamically favorable yielding $-5.51\Delta G$ kcal/mol e$, higher than the energy yield from sulfate reduction. (Table 1)
HCO$_2^-$ + 3/2 H$^+$ + 1/2 HAsO$_4^{2-}$ + 1/2 H$_2$AsO$_4^-$ → H$_2$AsO$_3$ + HCO$_3^-$

($\Delta G^{0s}_{\text{pH}=7} = -82\text{kJ/mol of formate}$)

C$_3$H$_5$O$_5^-$ + 2H$^+$ + HAsO$_4^{2-}$ + H$_2$AsO$_4^-$ → 2H$_2$AsO$_3$ + HCO$_3^-$ + C$_3$H$_3$O$_2^-$

($\Delta G^{0s}_{\text{pH}=7} = -172\text{kJ/mol of lactate}$)

Figure 2: Thermodynamic reactions with formate or lactate as the electron donor and carbon source when arsenate is the terminal electron acceptor. (Niggemyer et al. 2001)

<table>
<thead>
<tr>
<th>THERMODYNAMIC REACTION</th>
<th>$\Delta G$ kcal/mol e$^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/4 O$_2$(g) + 1/2 H$_2$ --&gt; 1/2 H$_2$O</td>
<td>-23.55</td>
</tr>
<tr>
<td>1/2 MnO$_2$(s) + H$^+$ + 1/2 H$_2$ --&gt; 1/2 Mn$^+$ + H$_2$O</td>
<td>-22.48</td>
</tr>
<tr>
<td>1/5 NO$_3$ + 1/5 H$^+$ + 1/2 H$_2$ --&gt; 1/10 N$_2$(g) + 3/5 H$_2$O</td>
<td>-20.66</td>
</tr>
<tr>
<td>1/2 SeO$_4^{2-}$ + 1/2 H$^+$ + 1/2 H$_2$ --&gt; 1/2 HSeO$_3^-$ + 1/2 H$_2$O</td>
<td>-15.53</td>
</tr>
<tr>
<td>1/8 NO$_3$ + 1/4 H$^+$ + 1/2 H$_2$ --&gt; 1/8 NH$_4^+$ + 3/8 H$_2$O</td>
<td>-13.42</td>
</tr>
<tr>
<td>1/3 CrO$_4^{2-}$ + 5/3 H$^+$ + 1/2 H$_2$ --&gt; 1/3 Cr$^{3+}$ + 3 H$_2$O</td>
<td>-10.76</td>
</tr>
<tr>
<td>Fe(OH)$_3$ (am) + 2 H$^+$ + 1/2 H$_2$ --&gt; Fe$^{2+}$ + 3 H$_2$O</td>
<td>-10.4</td>
</tr>
<tr>
<td>1/4 HSeO$_3^-$ + 2 H$^+$ + 1/2 H$_2$ --&gt; 1/4 Se$^0$ + 3/4 H$_2$O</td>
<td>-8.93</td>
</tr>
<tr>
<td>1/2 H$_2$AsO$_4^-$ + 1/2 H$_2$ --&gt; 1/3 As$^0$ + 1/2 H$_2$O</td>
<td>-5.51</td>
</tr>
<tr>
<td>1/3 H$_3$AsO$_3$ + 1/2 H$_2$ --&gt; 1/3 As$^0$ + H$_2$O</td>
<td>-2.58</td>
</tr>
<tr>
<td>1/8 SO$_4^{2-}$ + 1/8 H$^+$ + 1/2 H$_2$ --&gt; 1/8 HS$^-$ + 1/2 H$_2$O</td>
<td>-0.10</td>
</tr>
</tbody>
</table>

Table 1: Comparison of free energies (in kcal/mol e$^-$) for various electron acceptors coupled to H$_2$ oxidation (Newman et al., 1998)
1. Diversity Of Arsenate Reducing Bacteria

The first reported bacteria to use arsenate as the terminal electron acceptor in anaerobic respiration when coupled with lactate was a new species of Eubacteria strain MIT -13. (Ahmann et al., 1994). A second bacterial strain that was cultured from a selenate contaminated marsh was SES-3. Both MIT13 and SES-3 were classified as a new genus Geospirillum but later were re-classified as two species of Sulfurospirillum belonging to the ε-Proteobacteria. MIT13 was designated as Sulfurospirillum arsenophilum while SES-3 came to be designated as S. barnesii. Though they are very closely related, MIT13 showed optimal growth at 10 mM arsenate while SES-3 required 5 mM arsenate. Eighteen novel species of prokaryotes, can respire using arsenate (Table 1) (Figure 3) (Oremland and Stolz, 2001).

Desulfitobacterium strain GBFH was the first bacterium isolated that has been found to couple the reduction of arsenate to the oxidation of formate for conserving energy (Niggemyer et al., 2001). It was isolated from anoxic sediments of Couer d’Alene Lake where it is suggested to contribute to the mobilization of arsenic by the reductive dissolution of iron hydroxides. D.frappieri and D hafniense also grow on arsenate when formate is the electron donor. All three anaerobes share similar growth and carbon utilization profiles but Desulfitobacterium strain GBFH is incapable of growing on nitrate. DNA/DNA hybridization analysis indicated they are probably strains of the same species (Niggemyer et al., 2001).
<table>
<thead>
<tr>
<th>Microbe</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Sulfurospirillum arsenophilum</em></td>
<td>ε - Proteobacteria</td>
</tr>
<tr>
<td><em>Sulfurospirillum barnesii</em></td>
<td>ε- Proteobacteria</td>
</tr>
<tr>
<td><em>Sulfurospirillum deleyianum</em></td>
<td>ε- Proteobacteria</td>
</tr>
<tr>
<td><em>Wolinella succinogenes BSA-1</em></td>
<td>ε- Proteobacteria</td>
</tr>
<tr>
<td><em>Chrysiogenes arsenatis</em></td>
<td>Deep branch Proteobacteria</td>
</tr>
<tr>
<td><em>Desulfitobacterium</em> sp.str.GBFH</td>
<td>Low G + C</td>
</tr>
<tr>
<td><em>Desulfotomaculum auripigmentum</em></td>
<td>Low G + C</td>
</tr>
<tr>
<td><em>Bacillus arsenicoselenatis</em> strain E1-H</td>
<td>Low G + C</td>
</tr>
<tr>
<td><em>Bacillus seleinitireducens</em> strain MLS-10</td>
<td>Low G + C</td>
</tr>
<tr>
<td><em>Bacillus</em> sp.. JMM4</td>
<td>Low G + C</td>
</tr>
<tr>
<td><em>Clostridium</em> sp. OhILAs</td>
<td>Low G + C</td>
</tr>
<tr>
<td><em>Bacillus</em> sp.str.HT-1</td>
<td>Low G + C</td>
</tr>
<tr>
<td><em>Desulfovibrio</em> sp. str. Ben –RB</td>
<td>δ – Proteobacteria</td>
</tr>
<tr>
<td>MLMS-1</td>
<td>δ – Proteobacteria</td>
</tr>
<tr>
<td><em>Pyrobaculum arsenaticum</em></td>
<td>Crenoarchaea</td>
</tr>
<tr>
<td><em>Pyrobaculum arsenicum</em></td>
<td>Crenoarchaea</td>
</tr>
<tr>
<td>SLAS-1</td>
<td>Halenoaerobacter</td>
</tr>
<tr>
<td><em>Shewanella</em> sp.</td>
<td>γ-Proteobacteria</td>
</tr>
<tr>
<td><em>Citrobacter</em> sp.str.TSA-1</td>
<td>γ-Proteobacteria</td>
</tr>
</tbody>
</table>

Table 2 : Novel Bacteria that respire arsenate (Oremland and Stolz, 2003, Santini and Stolz, 2004)
Figure 3: Phylogenetic tree of prokaryotes that transform arsenic (Oremland and Stolz, 2003)

- Arsenate respiring chemolithoheterotrophs
- Arsenite oxidizing chemolithoautotrophs
- Arsenite oxidizing chemolithoheterotrophs.
Chrysiogenes arsenatis gen. nov., sp. nov. (BAL-1T) was the first organism to be identified that is capable of growth using acetate as electron donor and carbon source. It was isolated from a reed bed at Ballarat Goldfields in Australia growing on arsenate (5 mM) and acetate (5 mM) with a doubling time of 4h. (Macy et al., 1996). C.arsenatis is a strict aerobic and is the first representative of the a deeply branching lineage of Bacteria as shown by the 16S rRNA sequence analysis. The new strain was capable of growing on various electron donors like pyruvate, lactate, succinate, malate and fumarate. The anaerobe was capable of growing on nitrate or nitrite when acetate was the electron donor. But C.arsenatis does not appear to grow on sulfate, thiosulfate and iron-oxide.

The two haloalkaliphilic bacteria Bacillus arsenicoselenatis and Bacillus selenitireducens were isolated from Mono Lake, CA. Bacillus selenitireducens strain MLS10 is a microaerophile using lactate as electron donor but can grow on glucose fermentatively (Figure 4). It grows at an optimum pH of 8.5 – 10. Phylogenetically the two isolates are placed within the proposed Bacillus alcalophilus group of the Bacillus clade (Switzer Blum et al., 1998).

Most recently a chemoautotroph has been isolated from the anoxic bottom water of Mono Lake, California. The isolate bacteria is MLMS-1 is a gram-negative, motile rod that couples the oxidation of sulfide to sulfate to the reduction of arsenate to arsenite (Hoeft et al., 2004).MLMS-1 belongs to the d-Proteobacteria subdivision of Bacteria which so far has only one other arsenate respirer Desulfovibrio Ben –RB.
Figure 4: *Bacillus selenitireducens* strain MLS10

A) Longitudinal section of MLS10. B) Whole mount (TEM) of a cluster of MLS10 cells (dark spots indicate elemental selenium) (Oremland et al., 2004)
2. **Respiratory Arsenate Reductase**

Respiratory arsenate reductase (Arr) has been characterized in three different species. The first Arr to be purified was from *C. arsenatis*. Arr from *C. arsenatis* is a heterodimeric molybdooenzyme located in the periplasmic space (Krafft and Macy, 1998). The enzyme is induced by arsenic and is arsenate specific.

*Shewanella* strain ANA-3 grows robustly in high concentrations of As(V) due to the resistance allowed by the *ars* operon, which has been confirmed by the aerobic reduction of arsenate (Saltikov *et al.*, 2003). It is the first arsenate respiring bacterium possessing the *ars* operon, and RT-PCR studies showed that the arsenate reductase enzyme used for As(V) respiration is different from the *arsC* used for arsenic resistance. Arr from ANA-3 was studied using a genetic approach. Mutagenesis studies showed that ArsB efflux system is not required for ANA-3 to respire on As(V) and that ArsC is not required for ANA-3 to reduce As(V) to As(III) when respiring As(V). The study provokes the question if As(V)-respiring microorganisms inhabiting natural systems require high-level As detoxification systems. Sequence analyses of the Arr operon indicate only 2 genes ArrA and ArrB. The presence of a TAT leader sequence in *arrA* suggests a periplasmic location for the enzyme.

Initial investigations of the arsenate reductase from the haloalkaliphilic gram-positive bacterium *Bacillus selenitireducens* revealed similar characteristics to ArrA from *C. arsenatis* (Table 3). The respiratory arsenate reductase Arr from MLS10 has been successfully purified and characterized (Afkar *et al.*, 2003). Unlike arsenate reductase from *C. arsenatis*, the enzyme was localized to the membrane fraction. The enzyme has two known heterologous subunits, a larger catalytic ArrA (110 kDa)
and the smaller ArrB (34 kDa) with an apparent $K_m$ for arsenate of 34 $\mu$M and $V_{\text{max}}$ of 2.5 $\mu$mol min$^{-1}$ mg$^{-1}$. Using degenerate primers the $arrA$ gene was cloned and sequenced. Metal analyses by inductively coupled plasma mass spectrometry (ICP-MS) revealed the presence of Molybdenum and Iron.

**Chrysiogenes arsenatis**

<table>
<thead>
<tr>
<th>Periplasmic protein - 123 kDa</th>
<th>Membrane anchored protein-150 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe-S cluster, Mo cofactor</td>
<td>Fe-S cluster, Mo cofactor</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m$ = 300 $\mu$M</td>
<td>$K_m$ = 34 $\mu$M</td>
</tr>
<tr>
<td>$V_{\text{max}}$ = 7013 $\mu$mol min$^{-1}$ mg$^{-1}$</td>
<td>$V_{\text{max}}$ = 2.5 $\mu$mol min$^{-1}$ mg$^{-1}$</td>
</tr>
</tbody>
</table>

**B. selenitireducens strain MLS10**

<table>
<thead>
<tr>
<th>Two subunits:</th>
<th>Two subunits:</th>
</tr>
</thead>
<tbody>
<tr>
<td>ArrA (84 kDa)</td>
<td>ArrA (110 kDa)</td>
</tr>
<tr>
<td>ArrB (29 kDa)</td>
<td>ArrB (34 kDa)</td>
</tr>
</tbody>
</table>

**Table 3: Comparison of Arsenate reductase from Chrysiogenes arsenatis and Bacillus selenitireducens strain MLS10**

The ArrA and ArrB subunits share 50% and 57% identity respectively with *C. arsenatis*. The N-terminal sequence determined for ArrA and ArrB revealed multiple cysteine residues. ArrA has an iron sulfur cluster binding motif (Cys–X$_2$-Cys-X$_3$-Cys) in a conserved 27aa sequence CQGCTAWCAVQVYRIDGRATKVRGNPN at the N–terminus, and a conserved 15aa sequence at the C-terminus KCYGQGHWAYGHIAS and an internal molybdenum binding domain (Figure 5). ArrB has an iron-sulfur cluster binding motif Cys-X$_2$-Cys-X$_2$-Cys-X$_3$-Cys.
Figure 5: Sequence comparison of ArrA from *Bacillus selenitireducens* strain MSL-10, *Desulfitobacterium hafniense*, *Wolinella succinogenes* and *Shewanella* sp. str. ANA-3.

The Fe-S binding site at the N terminus is boxed, the Mo-binding site is underlined, and the 15 amino acid C-terminus conserved sequence is shaded. (*) indicates identity and (:) indicates similarity.
The calculated pI was 5.12 and the amino acid sequence shares 47.1% identity and 78.8% similarity with ArrA from Shewanella sp., strain ANA-3; 56% identity and 60% similarity with a hypothetical protein from Desulfitobacterium hafniense. All arsenate reductase enzymes are members of the DMSO reductase family of mononuclear molybdenum enzymes (Santini and Stolz, 2004).

3. Microbial Arsenite Oxidation

Arsenite oxidation is also a significant process since the kinetics is slow in the absence of a surface catalyst. Thermus aquaticus and Thermus thermophilus, common inhabitants of terrestrial hot springs and thermally polluted domestic and industrial waters, have been found to rapidly oxidize arsenite to arsenate. Considering that arsenate is less toxic than arsenite, the ecological role of arsenite oxidation by these organisms is likely detoxification. (Gihring et al., 2001)

Since the first report in 1918 of arsenite oxidation by bacteria a small number of microorganisms having this metabolic capability have been isolated. These include the heterotrophs Pseudomonas putida and Alcaligenes faecalis as well as the chemolithoautotrophic arsenite oxidizers Pseudomonas arsenitoxidans and “NT-26”. Chemolithoautroph arsenite oxidizers utilize arsenite as the electron donor and oxygen as the terminal electron acceptor. Five members of α-Proteobacteria and one member of γ-Proteobacteria are known to grow by chemolithoautotrophic oxidation of arsenite (Figure 3). The bacterium P.arsenitoxidans was isolated from a gold-arsenic deposit. To date, only two arsenite-oxidizing bacteria have been studied in detail with respect to their arsenite oxidation abilities. These include the chemolithoautotrophic arsenite-oxidizer NT-26 and the heterotrophic arsenite-oxidizer Alcaligenes faecalis. NT-
26 and *A. faecalis* are phylogenetically unrelated as NT-26 is a member of the α-
*Proteobacteria* and *A. faecalis* is a member of the β-*Proteobacteria* (Vanden Hoven *et al.*, 2004)

The arsenate oxidase from *Alcaligenes faecalis* has been purified and characterized (Anderson *et al.*, 1992) and X-ray diffraction analysis of the enzyme has been performed. It belongs to the DMSO reductase family like Arr, a molybdoenzyme. The arsenate oxidase is localized to the inner membrane and uses cytochrome- 
<sup>c</sup> as the electron acceptor. Arsenate oxidase consists of two subunits – a 88 kDa subunit (encoded by the *asoA* gene) containing the molybdenum in conjunction with two pterin cofactors, and a smaller 14 kDa subunit (product of the *asoB* gene) with a Rieske 2Fe–2S cluster. Unlike the other DMSO reductase family enzymes with the molybdenum cofactor linked to a cysteine or serine residue, the molybdenum in AsoA is not directly linked to the protein by covalent bonds but may be coordinated by Ala199. The cells cannot grow with arsenite as an electron acceptor since energy is not released in this process of detoxification, but aerobic growth on arsenite is energetically favorable, since the oxidation of arsenite is an exergonic reaction. Transposon mutagenesis of the arsenate oxidase gene did not hinder the growth of *A. faecalis* on arsenite. (Anderson *et al.*, 1992)

The first arsenite oxidase to be fully characterized at the molecular and biochemical level is from NT-26. (Santini and vanden Hoven, 2004). The enzyme has two heterologous subunits – AroA (98 kDa) and AroB (14 kDa). The periplasmic enzyme Aro, has been purified by high-performance hydrophobic interaction and gel filtration chromatography. The *aroA* gene was characterized by insertion
mutagenesis. The N-terminal of Aro has a characteristic conserved cysteine motif (Cys-X2-Cys-X3-Cys), which places the enzyme in the DMSO reductase family of molybdoenzymes.

AroB is the first protein that is a periplasmic protein with a Rieske protein. The arsenite oxidase Aro from NT-26, thought similar in function to the Aso, has a $\alpha_2\beta_2$ subunit configuration as opposed to $\alpha_1\beta_1$ in Aso. Unlike the oxidase from \textit{A.faecalis}, aroA mutants were not capable of chemolithoautotrophic growth demonstrating that the enzyme is involved in energy generation processes. The mutants did not have active enzyme. Interestingly, NT-26 is resistant to high amounts of arsenate (up to 100 mM) and contains a homologue of the \textit{arsC} gene.

\textit{Thermus} HR13 is a facultative anaerobe and uses As(V) for respiration in oxygen deficient environment such as in Growler Hot Spring. When cultured with arsenate as the sole electron acceptor cell densities doubled in magnitude. \textit{Thermus} species have been associated with the mobilization of arsenic in the thermal environments though with conditions which allow precipitation of arsenic sulfides it may lead to arsenic removal from geothermal waters (Gihring and Banfield, 2001).

The 16S rRNA sequences indicate that the arsenate respirers belong to phylogenetically diverse groups and therefore a signature sequence for arsenate reductase is not available which can be used to detect other arsenate respirers. The development of a biochemical probe for arsenate reducing bacteria in the environment will greatly enhance the current knowledge of the microorganisms and their biochemistry and putting the information to use in the development of novel bioremediation methods. The 16S rRNA sequence provides a framework for the study
of the abundance and diversity of the microbes, it only provides information to characterize bacterial communities based on phylogeny and ancestry. The 16S rRNA does not reveal the physiology or biochemical processes of the microorganism.

II. Specific Aims and Experimental Approach

A. Hypothesis 1

*ArrA* from *Bacillus selenitireducens* strain MLS10 can be used to develop a biochemical probe to identify arsenate respiring bacteria

The functional gene of dissimilatory sulfite reductase *dsrAB* in conjunction with 16S rRNA gene has been used to study the phylogenetic and functional diversity of sulfate reducing bacteria in the Guaymas Basin, a hydrothermal vent where petroleum undergoes microbial degradation. (Dhillon *et al.*, 2003). The *dsrAB* functional probe indicated the presence of major clades of sulfate reducing bacteria in the Guaymas sediments that were previously undetected by the 16S rRNA gene. Such studies accentuate the necessity of a reliable functional molecular or biochemical probe to analyze microbial communities that will circumvent PCR and cloning biases.

The 16s RNA sequences for the known arsenate respiring bacteria indicate that they belong to phylogenetically diverse groups which makes identification of potential arsenate respiring bacteria difficult with a signature phylogenetic sequence. Molecular probes targeting genes that encode the enzymes essential to arsenate reduction are required to detect the activity of arsenate respiring bacteria.
This study will aim to construct a biochemical probe to detect bacteria that are actively growing by respiring arsenate. To this end, the 110kDa subunit of the enzyme Arsenate reductase (ArrA) from *Bacillus selenitireducens* strain MLS10 will be used. *B. selenitireducens* strain MLS10 is a gram positive anaerobic bacteria that is able to reduce arsenate (AsV) to arsenite (AsIII). It is a non motile gram positive rod with low G + C content. The cells are 2 – 6 µm long and 0.5 µm wide. It is a facultative anaerobe, which uses selenite, arsenate, nitrate, TMAO and fumarate as electron acceptors. Electron donors include lactate, glucose, and pyruvate (Blum *et al.*, 1998).

Multiple sequence alignment of the amino acid sequence of the arsenate reductase from MLS-10 with *Wolinella succinogenes*, *Shewanella sp* strain ANA-3, *Des. hafniense* reveals two conserved motifs in ArrA. We propose to raise polyclonal antibodies to the conserved sequence at the C-terminus of the 110kDa subunit and then use it to probe for bacterial, which have similar enzymes. The antibody developed by peptide synthesis and immunization of rabbit serum will help to identify other bacteria belonging to same evolutionary branch having similar mechanisms for respiration.

**B. Hypothesis 2**

**Arsenate reductase (Arr) in *B. selenitireducens* is regulated by Arsenic**

*B. selenitireducens* can use different terminal electron acceptors besides arsenate, including nitrate, selenite, fumarate, and trimethylamine oxidase (TMAO). It is unclear if the respiratory arsenate reductase of MLS10 is constitutively expressed or induced by the presence of arsenate. This study aims to determine the relative amount of Arr present in cells grown on different
terminal electron acceptors.

The cells of MLS10 will be grown on media containing different terminal electron acceptors. Cell lysates will be prepared by sonication and run on SDS-PAGE. The presence of the enzyme will be determined using the gel assay and western blot analysis (Stolz et al., 1990; Stolz et al., 1997). Arr activity will be quantitatively determined using the spectrophotometric assay using reduced methyl viologen as the artificial electron donor. The results would indicate if Arr from B. selenitireducens is constitutively expressed as in S. barnesii or induced by arsenic as in C. arsenatis.

C. Hypothesis 3

Growth conditions and purification procedure affect activity of the arsenate reductase enzyme

The 110kDa catalytic subunit will be extracted from the membrane fraction of the cell lysate and be purified using Ion Exchange Chromatography and Gel Filtration Chromatography for further characterization and biochemical analyses that will provide insight into the physiology, metabolic capabilities and evolutionary significance of bacteria adapted to extreme environments.

III. MATERIALS AND METHODS

A. Cell Culture and Harvesting

Cells of Bacillus selenitireducens strain MLS10 were grown at 30°C in a mineral salt medium as previously described (Switzer Blum et al., 1998) (Appendix 1)
containing sodium arsenate (10 mM) as the terminal electron acceptor and sodium lactate (20 mM) as the electron donor and (0.5%) yeast extract. The pH of the media was adjusted to 9.8. The medium was transferred into Wheaton bottles (125 ml) and degassed (10 min) with N\textsubscript{2} and CO\textsubscript{2} (80:20, v/v). The headspace in the bottle was degassed for 5 min before the bottles were sealed with crimps and autoclaved. The medium was inoculated with 10\% v/v of MLS10 suspension. For large-scale cultivation, 14L of media was prepared (pH 9.8), degassed for 20 min and autoclaved. The fermentor was inoculated with 1L MLS10 suspension and allowed to grow at 30\(^{\circ}\)C. Just after inoculation, sodium dithionite (1.25 g /10 ml DI water) was added to maintain anaerobic conditions (New Brunswick, NJ, USA).

Cells of MLS10 were grown separately in the basal medium with different terminal electron acceptors, mainly Arsenate (10 mM), Nitrate (20 mM), Fumarate (20 mM), Selenite (20 mM), and Dimethylsulfoxide (DMSO) (20 mM). The electron acceptors were added to the medium after autoclaving from individually degassed and autoclaved 100 mM stock solutions.

Cells of *B. selenitireducens* strain MLS10 were harvested at the late log phase after approximately 48 hrs growth, by centrifugation at 9000rpm in a 50.2 Ti rotor using a R-5B centrifuge, for 20 min at 4\(^{\circ}\)C and resuspended in 5 mL of Buffer A (10 mM Tris-HCl buffer, (pH 8.0) 1 mM EDTA, 10 \(\mu\)M phenylmethylsulphonylfluoride (PMSF)) and stored at -20\(^{\circ}\)C.

**B. Preparation of cell extracts**

The whole cell pellet that had been harvested and pooled from the 14 L fermentors was resuspended in 5 ml of Buffer A. The suspension was then sonicated
(150W, 50/60 Hz) for 10 min on ice. To avoid the risk of denaturing the cells from the resultant heat, sonication was done at intervals of 30 sec, allowing the cells to cool each time. The resulting cell suspension was centrifuged at 7500 x g for 15 min to remove the whole cells and cell debris. DNase (1mg/10 ml) and RNase A were added to the cell lysate that was then centrifuged at 200,000 x g for 2 hrs to separate the soluble and membrane fractions. The supernatant (soluble fraction) was decanted and stored at -20 °C. The resulting pellet (membrane fraction) was resuspended in 50 ml of buffer A, and Triton X-100 was added to give a final concentration of 2% (wt/vol, CMC 0.24 mM). After the suspension was gently stirred for 2 hrs at 4°C, the solubilized membrane protein centrifuged at 100,000 x g for 1 hr. The solubilized protein was stored at -20°C and insoluble fraction was saved for further treatment.

**C. Protein Purification**

All purification processes were carried out under aerobic conditions at 4°C using Biologic LP (Bio-Rad, CA) (Afkar et al., 2003). The supernatant with the solubilized Arr was applied to a DEAE-Toyopearl ion exchange column (3 x 14cm) equilibrated with Buffer A containing 1% Triton X-100 (w/v) and eluted with NaCl gradient (0-0.5 M) in Buffer A. The flow rate was set at 1.5 ml/min and the protein elution was monitored by measuring the absorbance at 280 nm. Fractions with enzymatic activity were pooled and concentrated by ultra filtration using Amicon Ultra-15 centrifugal filter devices (30,000 NMWL) at 7,500 rpm for 15 min. The concentrated fraction was then subjected to a second DEAE–Toyopearl ion exchange column (1.5 cm x 11.5 cm) also eluted with a linear gradient of NaCl (0-0.5 M) in Buffer A containing 1% Triton X-100.
The DEAE fractions with Arr activity were pooled and loaded onto a Sephacryl S-300-HR (SIGMA) gel filtration column (1.5cm x 75cm). The resin was packed efficiently to ensure uniform resin composition through the entire length of the column and equilibrated with Buffer A containing 0.25 M NaCl. The column was calibrated with the gel filtration molecular weight standards (50mg total protein) (Appendix 2). The gel filtration column was set at a flow rate of 0.6 ml/min for 650 min and the protein elution was monitored by measuring absorbance at 280 nm. The resin was stored in 20% ethanol and regenerated with 6 M urea after repacking the column. The fractions containing pure active Arr were pooled and the final protein concentration determined by the Lowry method (Lowry et al, 1951).

D. Antibody Production and Affinity Purification

The conserved sequence at the C-terminus (N-KCYGQGHWAYGHIAS-C) of the catalytic subunit ArrA of Arr from MLS10 was synthesized by Sigma Genosys, TX. The synthetic peptide ArrA-1 was used to raise a polyclonal ArrA-1 antibody conjugated post synthetically with keyhole lympht hemocyanin (KLH) in two rabbits (#11393, #11394) (Appendix 3). The serum immunizations were given once every week for three weeks and ~30ml of serum was drawn from the rabbit. 10.3mg of the synthetic peptide (IgG purity) of ArrA-1 and the preimmune and antiserum (~30ml ea.) from three consecutive immunizations per rabbit were acquired from Sigma Genosys, TX. The serum was affinity purified with IgG (ligand) using the Montage® Antibody Purification Kit with PROSEP®-A (Millipore, MA).

The serum from #11394 was affinity purified using 1gm Sepharose 4B (Sigma) with ArrA-1 as the ligand. The gel was prepared in 50 ml of 1 mM HCl, spun at low
speed and supernatant was discarded. This was repeated twice for 15 min each. 2.5mg of the synthetic peptide ArrA-1 (ligand) was dissolved in 5ml coupling buffer (0.1M NaHCO₃ pH 8.3, containing 0.5M NaCl) and mixed with the gel by gentle stirring for 2hrs followed by washing once with 0.1M acetate buffer (pH 4.0) containing 0.5M NaCl and then with 0.1M Tris HCl buffer (pH 8.0) containing 0.5M NaCl each time discarding the supernatant after a low speed spin. The column was prepared with the washed gel in PBS. 8ml of filtered serum was diluted with 50ml PBS, and applied to the affinity column. The column was washed with 20ml each of PBS, Tris buffer pH 8.0 (50 mM Tris-HCl, pH 8.0; 0.1% Triton X-100; 0.5 M NaCl), Tris buffer, pH 9.0 (50 mM Tris-HCl, pH 9.0; 0.1% Triton X-100; 0.5 M NaCl), in that order and finally with 20ml Sodium phosphate buffer pH 6.3 (50 mM Sodium Phosphate, pH 6.3; 0.1% Triton X-100; 0.5 M NaCl ). The bound antibody was eluted with Glycine buffer, pH 2.5 (50 mM glycine-HCl, pH 2.5; 0.1% Triton X-100; 0.15 M NaCl) and the fractions were eluted into tubes containing 4 ml 1M Tris-HCl (pH 9.0). The column was washed with PBS and the purified antibody was desalted using a Sephadex G-25 column (gel preparation similar to Sepharose 4B) with PBS as the desalting buffer.

### E. Protein Concentration Assay

Bovine serum albumin (BSA) standards (0, 5, 25, 50, 75, 100 µl) were prepared with 2% SDS to make up to 200 µl. 200 µl of 2% SDS served as blank. 5-10µl of protein samples in 2% SDS made up to 200 µl was vortexed. The standards and samples were denatured at 90°C for 10 min in a sand bath and incubated for 10 min in 1ml of
Lowry reagent I after vortexing. 100 µl of Lowry reagent II was added to the standards and the protein samples and allowed to sit for 30 min. At the end of the 30 min, the OD for the standards and the protein sample was determined spectrophotometrically using a Lambda 2 UV/VIS spectrophotometer (Lowry et al., 1951) (Appendix 4)

F. SDS - PAGE and Western Blot Analysis

SDS- Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed as described by Laemmli (Laemmli, 1970). 0.5 mm thick pre-cast mini Ready Gel Tris-HCl gels (7 x 8 cm x 0.5 mm) with 12% Tris (Bio-Rad, CA) were used for mildly denaturing and denaturing gel electrophoresis. 20-30 µg of protein was diluted with Laemmli sample buffer to make up to 20 µl. The samples were denatured by heating at 95°C for 5 minutes. Low protein concentration samples were precipitated with 72% tricholoroacetic acid (10% final concentration), rinsed thoroughly before heating. The samples were vortexed and microfuged for 10 min before loading onto the gel. 4 µl of Pre-stained Broad Range Molecular weight markers (Bio-Rad, CA) was loaded in lane1 of the gel. The gel was run at constant voltage (150 V) for 1 hr in Tris-Glycine-SDS electrophoresis buffer in a Mini-PROTEAN 2 Electrophoresis Module (Bio-Rad, CA). After electrophoresis, the gel was stained in 0.05% Coomassie Brilliant Blue G-250 for 1 hr and destained for 1 hr using glacial acetic acid (7%) and methanol (5%). (Appendix 5)

Western Blot using polyclonal antibody raised against the synthetic peptide ArrA-1 was performed as follows. The denatured protein samples were run on a 12% Tris-HCl pre-cast mini Ready Gel Tris-HCl gel at 150V for 1 hr in a Mini-PROTEAN 2 Electrophoresis Module (Bio-Rad, CA). The gel and the nitrocellulose
membrane were equilibrated in trans-blot buffer (Appendix 6) for 15 minutes. The electro blot unit (Mini Trans-Blot Electrophoresis Transfer Cell, Bio-Rad, CA) was filled with 800 ml of trans-blot buffer, and a small stirring bar and the “cooling unit” were installed in the unit. The gel holder cassette was carefully assembled to ensure there were no air bubbles between the gel and the membrane and then placed in the transblot unit buffer tank. The unit was run at constant current 250 mA for 2 hr with stirring after which the nitrocellulose membrane was stained with 20% PonceauS (SIGMA Chemical, St. Louis, MO) to ensure efficient transfer of proteins. The blot was treated with the blocking reagent “Killer Filler” for 1 hr to block the nonspecific reactive sites. The blocking solution was poured off and the blot was incubated with the anti-ArrA antibody (diluted in Killer-Filler 1:1,000) overnight. The primary antibody solution was saved and the blot was washed with Tris buffered saline (TBS) for 10 min followed by Tween 20- Tris Buffered Saline (TTBS) for 10 min with gentle shaking. This was repeated thrice after which the blot was incubated with secondary antibody - Goat anti-rabbit whole IgG conjugated to Alkaline Phosphatase AP, (SIGMA St.Louis, MO) (diluted in Killer-Filler 1:1000) for 2 hr with gentle shaking at room temperature. The blot was washed thrice with TBS and TTBS, equilibrated with carbonate buffer (pH 9.8) for 10 min, and developed with 50 ml of Carbonate buffer containing (freshly prepared) AP substrates 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP, p-toluidene salt) and Nitro Blue Tetrazolium (NBT) (Appendix 6) with gentle shaking. Once the blue reaction product was formed the blot was washed several times in tap water to stop the reaction.
G. Enzyme Linked ImmunoSorbent Assay (ELISA)

The antibody titer of the three bleeds of anti-ArrA-1 sera and optimal antibody and antigen dilution were determined using the Indirect ELISA. The first column of wells of a 96 well NUNC micro plate, used as blank, was filled with 200 µl PBS buffer without antigen. The antigen ArrA-1 (synthesized peptide) was diluted in PBS buffer 1:1000 from a 1mg/ml stock solution. The rest of the columns were filled with 200 µl of diluted antigen and the micro plate was incubated at 4°C overnight in a moist chamber to prevent the plate from drying out. The micro plate was washed five times thoroughly with PBS to remove the excess antigen. Then the plate was incubated with 200µl blocking solution Blotto (5% non fat dried milk in PBS buffer) for 1 hr to block all the uncovered reactive sites. The primary antibody was diluted 1:1000 in Blotto. 200 µl of diluted primary antibody solution was added to the second column. The rest of the columns were filled with 100µl of PBS solution. Starting with column 2, the primary antibody was serial diluted across the micro plate by removing 100µL from each well and adding it to the next column. After incubation in the primary antibody solution for 2 hrs at RT, the micro plate was washed five times thoroughly with PBS buffer. The micro plate was incubated in 200µl of secondary antibody Goat anti-rabbit whole IgG conjugated with Horseradish Peroxidase diluted 1:1000 in Blotto for 2 hr at RT. After one hour the micro plate was washed with PBS buffer and developed using 100µl of the substrate mixture, containing 9 ml of TMB solution A mixed with 1ml of TMB solution B (Bio-Rad, Hercules, CA). At the end of five minutes the micro plate was read at 665nm using a micro plate reader after which 100 µl of 1N H₂SO₄ was added to all wells and the micro plate was read at 450 nm. (Appendix 7)
H. Enzyme Activity Assay

To detect Arr activity in non-denaturing PAGE, the gel was immersed in Tris buffer (50 mM, pH 8.0) containing 10 mM methyl viologen (artificial electron donor) reduced with sodium dithionite (0.1%). (Appendix 8) The gel was kept covered for 2-4 minutes to prevent oxidation. The violet-stained gel was rinsed with DI water and exposed to 5 mM sodium arsenate with minimal contact with air. The gel in reduced methyl viologen was allowed one complete oxidation cycle before exposing to electron acceptor again. Arsenate reductase enzyme activity appeared as a clear band of oxidized MV.

Spectrophotometric detection of the Arr activity was done using a Perkin-Elmer Lambda 2 Dual Beam spectrophotometer. All the solutions were made in Tris buffer (pH 8.0). Freshly prepared methyl viologen solution was first degassed for 10 min, quickly reduced with 0.1% sodium dithionite and immediately sealed with rubber stopper. The reduced solution was degassed for an additional 10 min and the head space was also degassed for 5 min.

The protein fraction (~25-50 µg), electron acceptor (5 mM arsenate) and Tris buffer (50 mM, pH 8.0) degassed for a few minutes in a sealed quartz cuvette. The reduced methyl viologen was carefully syringed into the cuvette without introducing air bubbles. The activity was measured at 600nm for a period of 5min. The reference cuvette did not contain the enzyme. Negative control for this experiment was protein sample reduced with rMV in the absence of the electron acceptor. Enzyme activity was calculated as methyl viologen (mM) oxidized min⁻¹ mg⁻¹ protein using the extinction coefficient of 13.1 mM⁻¹ cm⁻¹ (Afkar et al., 2003).
IV. RESULTS

A. Development of a Biochemical Probe for Arsenate Respiring Bacteria

The respiratory enzyme arsenate reductase from *Bacillus selenitireducens* strain MLS10, may provide for a potential biochemical probe for arsenate respiring bacteria. Polyclonal antibodies raised against a 15 amino acid conserved sequence from the catalytic subunit of the Arsenate reductase enzyme was successfully shown to detect Arr in Western blots (Figure 6).

After preliminary tests, the antibody from the second bleed of rabbit #11393 was tested for specificity and sensitivity against MLS10 cell fractions, (e.g. soluble cytoplasm, membrane fraction, Triton X-100 solubilized protein). Purified native ArrA and recombinant ArrA from different projects were also used to test the reactivity of the antiserum (Figure 6). The blot showed conspicuous bands at the 110kDa in all the native fractions and the two smaller protein bands, corresponding to the oxidized bands in the zymogram, also reacted with the antiserum. The purified native ArrA and recombinant ArrA also exhibited significant reaction with the antiserum. The antiserum from the second bleed of rabbit #11394 was IgG affinity purified and utilized for the subsequent cross reaction experiments.

Different arsenate respiring bacteria probed with the antibody were *Bacillus arsenicoselenatis* strain E1-H, MLMS-1, *Bacillus* sp. strain JMM-4, SLAS, *Clostridium* sp. strain OhILAs, *Sulfurospirillum barnesii* strain SES-3, *Sulfurospirillum deliyianum* and *Sulfurospirillum arsenophilum* strain MIT-13. These bacteria are phylogenetically diverse which enables efficient testing of the biochemical probe.
Figure 6: Western Blot results using polyclonal antibodies to ArrA-1 from bleed 2 of rabbit #11394.

A) Coomassie stained gel of MLS10 fractions  B) Zymogram showing the activity in the cell lysate, soluble cytoplasm and membrane fraction.  C) Western blot showing reaction of antibodies to bands at 110 kDa, 63 kDa and 58 kDa. The recombinant ArrA is seen as a faint band of lower molecular weight that ArrA. Lane 1, Prestained molecular markers, lane 2, cell lysate, lane 3, soluble cytoplasm, lane 4, membrane fraction, lane 5, Purified ArrA, lane 6, recombinant ArrA. The molecular standards are shown on the side of the gel.
When the IgG affinity purified antibody was used for immunoblotting, the blot showed several bands in the positive control in addition to the expected 110kDa band. Similar bands were seen in all the cell lysates of the different arsenate respiring bacteria (Figure 7). E1-H, which belongs to the same clade as MLS10 had a band of approximately the same molecular weight as ArrA. SLAS showed two conspicuous bands. The OhILAs cell lysate there was one band with approximately the same molecular weight as ArrA. The three bacteria from the *Sulfurospirillum* clade did not appear to react with the ArrA antibody.

On testing the preimmune of rabbit #11394 in immunoblotting of MLS10 fractions and the cell lysates of the different anaerobes, similar non-specific bands appeared as observed earlier with the IgG–affinity purified antibody (Figure 8). This indicated that the antibody required further purification. Equal amount of protein was loaded in all lanes (Figure 9a). The serum from affinity purification using the synthetic peptide as the ligand ArrA-1, was used for immunoblotting at a dilution of 1:1000 (Figure 9b). Surprisingly, only MLS10, E1-H, and OhILAs, appeared to react with the ArrA-1 antibody. The chemolithoautotroph MLMS-1 obtained from Mono Lake, California, and the *Bacillus* sp. strain JMM-4 from an Australian gold mine did not appear to react with the antibody. The possibility of low concentration of protein loaded onto the lane, was invalidated by the membrane stained with PonceauS after transfer and the Coomassie stained gel of the denatured cell lysate, which showed appreciable levels of protein. OhILAs was the only other arsenate respiring bacteria that reacted with the ArrA-1 antibody.
Figure 7: Western blot using IgG affinity purified polyclonal antibodies.

The positive control MLS10 shows bands additional to ArrA, suggesting non specific cross reaction. SLAS and OhILAs have conspicuous bands. Lane 1, Prestained Molecular markers, lane 2, *B. selenitireducens* MLS10, lane 3, *B.arsenicoselenatis* E1-H, lane 4, MLMS1, lane 5, *Bacillus* sp. strain JMM4, lane 6, SLAS, lane 7, *Clostridium* sp. strain OhILAs, lane 8, *S.barnesii* strain SES3, lane 9, *S.deliyianum*, lane 10, *S.arsenophilum* strain MIT13.
Figure 8: Western blotting results of preimmune used as the primary antibody. Control was cell lysate from MLS10. The blot shows nonspecific reaction with the cell lysate of MLS10. Lane 1, Prestained Molecular marker, lane 2, *B. selenitireducens* MLS10, lane 3, *B.arsenicoselenatis* E1-H, lane 4, MLMS1, lane 5, *Bacillus* sp. strain JMM4, lane 6, SLAS, lane 7, *Clostridium* sp. strain OhILAs, lane 8, *S.barnesii* strain SES3, lane 9, *S.deliyianum*, lane 10, *S.arsenophilum* strain MIT13.
Figure 9: Western blot results using ArrA-1 affinity purified antibodies.

A) Coomassie stained PAGE gel of cell lysate from different arsenate respiring bacteria. B) Western blot showing the highly specific reaction of ArrA-1 purity Ab. A single band is seen at 110 kDa in MLS10, E1-H and OhILAs. Lane 1, Prestained Molecular markers, lane 2, B. selenitireducens MLS10, lane 3, B.arsenicoselenatis E1-H, lane 4, MLMS1, lane 5, Bacillus sp. strain JMM4, lane 6, SLAS, lane7, Clostridium sp. strain OhILAs, lane 8, S.barnesii strain SES3, lane 9, S.deliyianum, lane10, S.arsenophilum strain MIT13.
Figure 10: Arsenate reductase activity in various arsenate respiring bacteria.

Conspicuous arsenate reductase activity was seen in MLS10, SLAS and OhILAs.

Lane 1, Prestained Molecular markers, lane 2, B. selenitireducens MLS10, lane 3, B.arsenicoselenatis E1-H, lane 4, MLMS1, lane 5, Bacillus sp. strain JMM4, lane 6, SLAS, lane 7, Clostridium sp. strain OhILAs, lane 8, S.barnesii strain SES3, lane 9, S.deliyianum, lane 10, S.arsenophilum strain MIT13.
There was a distinct band at ~110kDa in the lane containing the cell lysate from OhILAs. The three bacteria belonging to the *Sulfurospirillum* clade did not appear to react with the ArrA-1 antibody.

The cell lysates of the various arsenate respirers were subjected to a qualitative activity assay to correlate the results from the western blot with arsenate reductase activity. The zymogram (Figure 10) showed conspicuous arsenate reductase activity in cell lysate of SLAS (lane 6) and also in cell lysate of OhILAs (lane 7). On repeated trials the SLAS cell lysate revealed similar enzyme activity in mildly denaturing electrophoresis but did not appear to react immunologically with the ArrA-1 antibody. Further analysis of arsenate respiring SLAS is required to characterize the active protein bands. E1-H, which is immunoreactive with ArrA-1 antibody, revealed no activity in the zymogram as did MLMS-1 and JMM4.

**B. Determination of the ArrA Antibody Titer**

The serum containing the antibodies to the synthetic peptide ArrA-1 was titrated against a constant antigen dilution and constant antigen dilution (1:1000). The three bleeds after the three consecutive immunizations were titrated with the preimmune as the control. The preimmune showed negligible reaction with the antigen suggesting little nonspecific reaction. Among the three bleeds of sera from both the rabbits, the antiserum from the first and the third bleed had a relatively low titer of antibodies. The second bleed from rabbit #11394 appeared to have the highest titer of antibodies for the antigen (Figure 11).
Figure 11: Antibody titer using antisera from preimmune and three bleeds of two rabbits (#11393, #11394).

The antibody from the bleed 2 of rabbit #11394 shows higher titer of antibody. The assay was done in duplicate.
Affinity purified serum from the second bleed was titrated against a constant antigen dilution (1:500). IgG affinity purified anti-ArrA-1 and the synthetic peptide ArrA-1 affinity purified serum were serial diluted starting with the antibody dilution of 1:1000. The optimum antibody titer for the IgG affinity purified ArrA antibody was 1:10,000 while the optimum antibody titer for the ArrA-1 purified antibody was 1:1000. The ArrA-1 affinity purified serum was four times as diluted as the IgG purified antibody. The O.D. of the IgG pure serum was 2.86 at 280 nm while that of the ArrA-1 pure antiserum was 0.77 (Figure 12).

The synthetic peptide ArrA-1 was titrated against a constant primary antibody dilution (1:10,000) and constant secondary antibody dilution (1:1000). The primary antibody used was IgG affinity purified ArrA antibody. The antigen was serial diluted starting with 1:500 dilution. The results from the assay demonstrated that the optimum dilution for the antigen was 1:500 (Figure 13).

Serial dilution of antigen and antibody was done to assay the specific cross reaction between antigen and the anti-ArrA-1 antiserum. The antigen used was the synthetic peptide ArrA-1 at a dilution of 1:500. The ArrA-1 affinity purified antisera at 1:100 dilution was used for the cross titer assay. The results from the assay revealed that the antibody is most specific at 1:100 when the antigen is at a dilution of 1:400 (Figure 14).
Figure 12: Antibody titer of IgG affinity purified antibody and ArrA-1 affinity purified antibody.

The ArrA-1 affinity pure antibody is most specific to the antigen (ArrA-1) at 1:1000 dilution. The assay was performed in triplicate.
Figure 13: Antigen titer of the ArrA-1 synthetic peptide

The optimal antigen dilution is 1:500 against constant dilution of primary antibody (1:10,000) and secondary antibody (1:1000).
Figure 14: Optimal antibody titer for optimal antigen dilution.
C. Detection of Arr from MLS10 under different growth conditions

MLS10 cells grown in media containing different terminal electron acceptors were harvested and the cell lysate was used for Western blot. The media was inoculated with 10% v/v MLS10 cells growing in 10 mM arsenate and transferred twice before harvesting. The media containing nitrate, selenate and DMSO favored the growth of MLS10 while growth in fumarate, selenite, and thiosulfate was greatly diminished. The luxuriant growth of MLS10 by respiration of selenate (20 mM) produced the characteristic red elemental selenium during the late log phase. Previous studies with MLS10 have demonstrated that cells were capable of growing on selenite by non biological reduction of selenate during autoclaving.

The qualitative enzyme activity assay supported the presence of active enzyme in all cell lysates of MLS10 grown in different terminal electron acceptors. (Figure 15 A). But interestingly the cell lysate from cells grown in arsenate showed two bands of activity while the rest of the lanes had a single active band also in the region of 58kDa corresponding to the lower of the two protein bands displaying activity in the arsenate grown cells. Cells grown in nitrate appeared to show maximum activity, greater than the control. Fumarate and selenate grown cells also showed comparable activity, but selenite, DMSO and thiosulfate grown cells did not show any activity.

Immunoblotting with IgG affinity purified antibody was performed on cell lysate of MLS10 grown in media containing nitrate, fumarate, selenate, selenite, DMSO and thiosulfate as terminal electron acceptors. MLS10 cell lysate
Figure 15: Detection of Arr from MLS10 grown on different terminal acceptors.

A) MLS10 cell lysate from different Terminal Electron Acceptors. The gel shows the reduced protein concentration in selenite and thiosulfate grown cells. All lanes were uniformly loaded. B) Activity assay of cell lysate from Arsenate and nitrate grown cells exhibit activity. C) Western blot of MLS10 cell lysate with IgG purified antibody. Lane 1, Prestained molecular markers, lane 2, cell lysate of MLS10 in arsenate, lane 3, nitrate, lane 4, fumarate, lane 5, selenate, lane 6, selenite, lane 7, DMSO, lane 8, thiosulfate.
from cells grown in medium containing arsenate was used as the positive control.

These results suggested that Arr in MLS10 was constitutively expressed (Figure 15 B). All the cells grown in the different terminal electron acceptors had a band at the 110kDa region and also two bands of lower molecular weight. The cell lysates from selenite and thiosulfate appeared to have faint bands in the blot, but that may be attributed to the low protein concentration levels due to growth yield as confirmed by the Coomassie stained gel (Figure 15 C).

To confirm the constitutive expression of the respiratory Arsenate reductase in MLS10 and eliminate the possible carry over of residual arsenate in the inoculums, further tests were done. MLS10 was cultured in medium containing nitrate as the terminal electron acceptor. After three successive transfers in the nitrate media, the cells from the third transfer were used as the inoculum. MLS10 was grown in media containing nitrate, fumarate, selenite and DMSO. The cells growing in media containing the individual electron acceptors, were then transferred twice and used as inoculum for 1L cultures that were then harvested and sonicated under ice to extract the cell lysate. The cell yields from cultures containing fumarate and selenite were significantly low, and had to be concentrated by speed vacuum.

The qualitative enzyme activity assay detected arsenate activity in MLS10 cells grown on arsenate, nitrate, and DMSO. (Figure 16 A). MLS10 cell lysate from arsenate grown cells showed two oxidized bands at 63kDa and 58kDa while the activity in nitrate and DMSO grown cells was conspicuous at ~58kDa.
A

B

110 kDa

58 kDa

110 kDa
Figure 16: Detection of Arr in cells of MLS10 grown on different terminal electron acceptors using cells grown on nitrate as inoculum

A) Zymogram showing activity in MLS10 grown in arsenate, nitrate and DMSO

B) Coomassie stained gel of denatured cell lysates of MLS10 grown in different TEAs. C) Western blot with single band at 110 kDa in MLS10 grown on arsenic.

Lane 1, Prestained molecular markers, lane 2, cell lysate of MLS10 in arsenate, lane 3, nitrate, lane 4, fumarate, lane 5, selenite, lane 6, DMSO
<table>
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<th>Terminal Electron Acceptor</th>
<th>Activity µmoles min⁻¹</th>
<th>Specific Activity µmoles min⁻¹ mg⁻¹</th>
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<tr>
<td>Arsenate</td>
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<td>5.49 x10⁻¹</td>
</tr>
<tr>
<td>Nitrate</td>
<td>2.74 x10⁻²</td>
<td>2.74 x10⁻¹</td>
</tr>
<tr>
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</tr>
<tr>
<td>DMSO</td>
<td>1.79 x10⁻²</td>
<td>3.58 x10⁻¹</td>
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Table 4: Activity of Arr from MLS10 grown in different terminal electron acceptors.

Cell lysate of MLS10 grown in basal medium containing lactate (20 mM) as the electron donor and one of the terminal electron acceptors (arsenate, nitrate, fumarate, selenite, DMSO) was used for the activity.
The cells grown in arsenate, appeared to have conspicuous activity whereas cells grown in nitrate, and DMSO appeared to have comparably reduced activity. Fumarate and selenite grown cells showed negligible activity that can be attributed to the low protein concentration, which was validated by the Coomassie stained gel. (Figure 16 B).

When the cell lysate fractions were tested against ArrA-1 affinity-purified antibody (1:500) by Western blotting, the immunoblot revealed that the expression of Arr appeared to be upregulated by arsenate (Figure 16 C). MLS10 cells grown in arsenate were used as control. The blot revealed a single ArrA band in MLS10 grown in arsenate, faint bands in nitrate and DMSO. No bands were seen in lanes containing cell lysates from fumarate and selenite.

The enzyme activity for cell lysate of MLS10 grown in different terminal electron acceptors was calculated using the spectrophotometric enzyme assay (Table 4). The specific activity of Arr in cells grown in nitrate is almost the same as cells grown in arsenic while activity of Arr from DMSO grown cells have only 1/3 the activity of the arsenate grown cells. Activity was not detectable in MLS10 cells grown in fumarate and selenite, which may be attributed to the very low protein concentrations in the cell lysates. The enzyme activity assay supports the results from zymograms and western blotting that Arr is constitutively expressed but upregulated by arsenic.

**D. Purification of Arr**

Arr activity was found both in the soluble and membrane fraction. The sonication process may have released the membrane anchored enzyme into the
soluble cytoplasm. To ensure minimal loss of protein between subsequent stages in the purification process effectively, the enzyme was recovered from the soluble supernatant by ultra filtration and pooled for purification by anion exchange chromatography and gel filtration technique. The membrane fraction of the cell lysate was solubilized using 2% Triton X-100 and the solubilized protein fraction was separated from the insoluble pellet. Enzyme activity was not detected in the insoluble pellet after ultracentrifugation. Cell fractions harvested from several 14L fermentors were pooled to maximize the amount of desired protein for purification.

The soluble fraction was loaded directly onto the DEAE- Toyopearl column. The solubilized arsenate reductase was eluted from the DEAE- Toyopearl column by anion exchange using linear gradient (0- 0.5M) of NaCl. The unbound protein eluted with the Buffer A while the bound protein was eluted along with the linear gradient of NaCl. The absorbance peak corresponded to the golden yellow fractions containing the active enzyme, which were pooled and concentrated by ultra filtration using a 30,000 NMWL filter device and later reconstituted in 5ml of Buffer A. There was no apparent loss of protein in the filtrate.

The concentrated protein was loaded on a second DEAE column again using a linear gradient of 0.5M NaCl. The initial absorbance peak (Figure 17) extending between fractions 5-12 indicated the unbound proteins and the second peak extending between fractions 27-33 (~55 min, 80ml) corresponded to the elution of the bound Arr. The active enzyme fractions from anion exchange chromatography of the soluble fractions and the Triton X-100 solubilized protein
Figure 17: Elution profile of Arr from ion exchange chromatography

Resin: DEAE-Toyopearl
Flow Rate – 1.5 ml/min
fractions were pooled for further purification by gel filtration yielding a final protein concentration of 120 mg in 60ml.

A mixture of molecular weight standards containing 50 mg total protein (Gel Filtration Molecular Weight Standards Kit, SIGMA) were used to calibrate the size exclusion column before applying the arsenate reductase protein sample (Appendix 9) 10ml (2mg/ml) subsets of the pooled DEAE fractions were applied to the gel filtration column with the addition of 2ml of 200 mM NaCl. The chromatograms showed a single absorbance peak that corresponded to the fractions containing active arsenate reductase enzyme (Figure 18). The enzyme was eluted at fractions 65-75 (~220ml) after 350 min. The presence of Arr was confirmed by PAGE and qualitative activity assay. Similarly all the subsets of the DEAE pooled fractions were applied to the Sephacryl S-300 HR column and “purified” by gel filtration chromatography. The fractions corresponding to the absorbance peak were pooled separately for enriched fractions and the rest of the fractions corresponding to the slopes of the absorbance peak were pooled. The activity assay confirmed the presence of active enzyme in all the representative samples while SDS-PAGE analysis revealed distinctly stained bands of subunits ArrA and ArrB. (Figure 19 A, B). Fractions containing Arr from six gel filtration elutions were pooled and the final protein amount was 12.71mg.

When concentrated to remove excess buffer, the protein concentration was significantly reduced to 3mg. It is hypothesized that concentration using the ultra filters decreased the total protein concentration of the membrane protein for two reasons. MLS10 is a haloalkaliphile requiring high concentration of salt for optimal growth.
Figure 18: Elution profile of Arr from gel filtration chromatography
Figure 19: Arr from Size exclusion Chromatography.

A) Coomassie stained SDS-PAGE gel of RAsR purified by gel filtration chromatography. ArrA and ArrB with the conspicuous band of 63kDa. B) Zymogram showing the presence of active Arsenate reductase purified by Gel Filtration chromatography. The two bands of activity are 63kDa (upper) and 58kDa (lower) Lanes 1, Prestained Molecular markers, lanes 2 –7, ArrA enriched fractions from subsets 1-6, lanes 8-10, pooled fractions from subsets 2, 3, 4.
The ultra filtration procedure is also a desalting procedure, which might have greatly reduced the salt below the optimum level required for the stability of the enzyme. The enzyme might also have bound tightly to the membrane filter and was not released, during the concentration procedure.

Figure 20 shows the enzyme during the subsequent stages of purification. The bulk of the proteins have been removed by ion exchange chromatography. The two known subunits of the respiratory arsenate were abundant in the gel filtration fractions (lane 7) (Figure 20 A). Additional protein bands, however were present, indicating impurities or denaturation of the enzyme. Further processing is necessary to achieve electrophoretically pure arsenate reductase enzyme.

All the cell fractions were tested for arsenate reductase activity by the qualitative enzyme assay (zymogram) using non-denatured protein samples. The enzyme activity did not appear in the region of ArrA, the larger catalytic subunit of the enzyme (110kDa). With the exception of the whole cell of MLS10, all the other cell fractions tested, showed arsenate reductase activity was seen as two separate bands of oxidized methyl viologen (Figure 20 B).

Similarly the two protein bands were conspicuous in Coomassie-stained PAGE gels of fractions enriched with arsenate reductase activity. The molecular weight of each of the two active bands was calculated to be of 63kDa and 58kDa. This may be attributed to the difference in the mobility of the protein within the gel matrix affected by the net charge of the protein or the gel may have partially disrupted the protein complex resulting in the two active protein bands
Figure 20: Arr at different stages of purification

A) Coomassie stained SDS-PAGE gel showing sequential purification of RAasR from MLS10  
B) Zymogram of Arsenate reductase activity at various stages of purification. Arrow indicates the Arsenate reductase activity. Lane 1, Prestained Molecular markers, lane 2, MLS10 whole cell, lane 3, cell lysate, lane 4, soluble cytoplasm, lane 5, membrane fraction, lane 6, Triton X-100 solubilized protein, lane 7, ion exchange chromatography fraction, lane 8, gel filtration fraction.
Western blotting with antibody raised to the synthetic peptide confirmed the sequential enrichment of Arr. The whole cell, cell lysate, soluble cytoplasm, membrane fraction, solubilized protein, ion exchange chromatography eluent, size exclusion chromatography eluent tested by immunoblotting appeared to have one conspicuous band that was immunologically reactive with the anti-ArrA antiserum. This band was in the 110 kDa region in all the fractions tested. The two bands, which demonstrated enzyme activity in the zymogram, appeared as faint bands in the blot (Figure 21).

The total activity and specific activity of Arr from various cell fractions, were calculated using the spectrophotometric enzyme assay using reduced methyl viologen (10 mM) as the artificial electron donor and sodium arsenate (5 mM) as the electron acceptor (Table 5). Except for the ion exchange chromatography and size exclusion chromatography fractions, all the fractions tested were from a single harvest of a fermentor (14L). The total enzyme activity and the specific activity were calculated using the extinction coefficient for reduced MV (13.1 mM⁻¹ cm⁻¹). The enzyme activity observed with the whole cell was equal to the activity in cell lysate. This indicates the protein may be very loosely anchored to the membrane and oriented away from the cytoplasm. The whole cell harvest may have dislodged the loosely anchored protein making it more accessible to the electron donor. This orientation of the enzyme might enable effective arsenate reduction without requiring transport of the toxic arsenate into the cell. It also eliminates the need for active transporters to pump out the more toxic and mobile arsenite, which is usually the case in arsenic resistance.
Figure 21: Western blot of ArrA from different cell fractions.

A single band at 110kDa region with ArrA-1 affinity pure antibody.

Lane 1, Prestained Molecular markers, lane 2, MLS10 whole cell, lane 3, cell lysate, lane 4, soluble cytoplasm, lane 5, membrane fraction, lane 6, Triton X-100 solubilized protein, lane 7, ion exchange chromatography fraction, lane 8, gel filtration fraction.
<table>
<thead>
<tr>
<th>MLS10 Cell Fractions</th>
<th>Activity $\mu$moles min$^{-1}$</th>
<th>Total Protein mg</th>
<th>Total Volume ml</th>
<th>Total Activity $\mu$moles min$^{-1}$</th>
<th>Specific Activity $\mu$moles min$^{-1}$ mg$^{-1}$</th>
</tr>
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<tbody>
<tr>
<td>Whole Cell</td>
<td>1.59 x10$^{-1}$</td>
<td>342</td>
<td>37.5</td>
<td>238</td>
<td>1060</td>
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<tr>
<td>Cell Lysate</td>
<td>1.09 x10$^{-1}$</td>
<td>341</td>
<td>37.5</td>
<td>204</td>
<td>1135</td>
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<tr>
<td>Soluble Cytoplasm</td>
<td>6.14 x10$^{-2}$</td>
<td>189</td>
<td>47.5</td>
<td>50</td>
<td>217</td>
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<tr>
<td>Membrane Fraction</td>
<td>1.0 x10$^{-1}$</td>
<td>56</td>
<td>7.6</td>
<td>63</td>
<td>719</td>
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<tr>
<td>TritonX-100 Soluble Protein</td>
<td>1.29 x10$^{-1}$</td>
<td>28</td>
<td>15</td>
<td>48</td>
<td>671</td>
</tr>
<tr>
<td>TritonX-100 Insoluble Protein</td>
<td>1.49 x10$^{-2}$</td>
<td>8</td>
<td>5</td>
<td>0.0025</td>
<td>0.5</td>
</tr>
</tbody>
</table>

| Pooled Fractions      |                               |                  |                |                                     |                                             |
| DEAE – Column Fraction| 2.49 x10$^{-2}$               | 120              | 60             | 74                                  | 1867                                        |
| Gel Filtration Fraction| 5.34 x10$^{-2}$                | 12               | 12             | 42                                  | 2848                                        |

Table 5  Enzyme activity of Arr in different cell fractions of MLS10
The total activity decreased from whole cells to protein sample from gel filtration and as expected there was a marked increase in the specific activity from whole cells to protein eluted after gel filtration. More than 40% of the total activity of the cell lysate was lost after separation of the membrane fraction from the soluble fraction while the total activity of the soluble fraction (25%) was comparable to that of the membrane fraction (30%). The specific activity for the membrane fractions shows that Arr is localized to the membrane (64%) with soluble cytoplasm containing only 14% of specific activity of the cell lysate. The size exclusion fraction had an activity of 5.34 x10^2 µmoles min^{-1}, and a specific activity of 2.84x10^3 µmoles min^{-1}mg^{-1}.

The concentrated enzyme was used for EPR studies to analyze the metal ion centre of the catalytic subunit of Arr and also for metal analyses to identify metal ion complexes in the arsenate reductase enzyme. The EPR analysis of the metal centre of the enzyme produced a strong copper signal (data not shown). There was no detectable molybdenum signal. The metal analyses (Metal Analyses Facility, University of Georgia) as shown from ICP-MS results of the concentrated and pooled arsenate reductase enzyme from gel filtration chromatography indicates the presence of tungstate (0.29 mg/L) and copper (1.82 mg/L), while the molybdenum concentration was significantly lower (0.07 mg/L, 0.0028 mM) than the expected value (1 molecule of Molybdenum cofactor per reductase) (Table 6) As was present at 0.48 mM in the sample, while Fe content was 0.074 mM.
<table>
<thead>
<tr>
<th>METAL</th>
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<tbody>
<tr>
<td>Ag</td>
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</tr>
<tr>
<td>Al</td>
<td>0.108192</td>
</tr>
<tr>
<td>As</td>
<td>36.41025</td>
</tr>
<tr>
<td>B</td>
<td>0.070321</td>
</tr>
<tr>
<td>Ba</td>
<td>0.083576</td>
</tr>
<tr>
<td>Be</td>
<td>0</td>
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<tr>
<td>Ca</td>
<td>3.353578</td>
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<tr>
<td>Cd</td>
<td>0.424781</td>
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<td>Co</td>
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<td>Cr</td>
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<td>Fe</td>
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<td>Mg</td>
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<td>9.548023</td>
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<td>Sr</td>
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<td>U</td>
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<tr>
<td>W</td>
<td>0.294496</td>
</tr>
<tr>
<td>Zn</td>
<td>1.121357</td>
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</tbody>
</table>

**Table 6: Metal analysis of the Arr pooled and concentrated from Gel filtration chromatography.**

3mg of Arr (in 500 µl) from gel filtration chromatography was used for metal analyses.
V. DISCUSSION

A. Arr from MLS-10 as a Biochemical Probe

The respiratory arsenate reductase enzyme from MLS10 has potential to be used as a biochemical probe to detect arsenate respiring bacteria in the environment. The results of the study on detection of Arr in MLS10 suggests that the protein is constitutively expressed and is upregulated by arsenic.

The polyclonal antibodies raised to the conserved amino acid sequence at the C-terminus of ArrA, has been affinity purified and though it has a low titer (1:400), the Western blotting results show that, the ArrA-1 purity antibody is highly specific for arsenate reductase. It therefore may be used for immunolocalisation experiments and for the in situ detection of arsenate reductase in environmental samples.

The apparent constitutive expression of Arr affects its ability to serve as an effective biochemical probe. The constitutive expression of arsenate reductase in the cell may lead to false positives. While the bacteria may be expressing the protein, it does not provide information regarding the physiology and metabolism of the bacteria in the environment. Yet it may prove to be useful for preliminary analysis of environmental samples for the detection of uncultured arsenate respiring bacteria.

The antibody did not recognize the active band from SLAS. The DMSO family of molydoenzymes have similar molecular weight but show diverse metabolic capabilities. A single enzyme is capable of using various electron acceptors and donors, therefore the activity seen in SLAS may have occurred from an enzyme, which was not Arr, but a molybdoenzyme utilizing arsenate as the electron acceptor.
The results of Western blot analysis showed that Arr is not an effective probe for the *Sulfurospirillum* clade belonging to the ε-Proteobacteria. The antibody did not appear to react with known arsenate respirers *Sulfurospirillum barnesii* strain SES-3, *Sulfurospirillum deliyianum*, *Sulfurospirillum arsenophilum* MIT-13. Interestingly, a previous study with *S. barnesii* strain SES-3 indicated that the arsenate reductase was constitutively expressed and the antibody did not react with the Gram positive bacteria. This suggests that there might be more than one type of Arr.

Regulation of expression, post translational modifications, translocation of the holoenzyme, location in the cell, orientation of the enzyme within the cell and relative affinity for the electron acceptor are factors that affect the proper identification of the target enzyme. Nevertheless the antibodies developed in this study may yet provide a useful tool in detecting MLS10 and closely related bacteria, which are involved in arsenic mobilization.

**B. Purification of Arsenate reductase**

The purification of the respiratory arsenate reductase using the previously published techniques (Afkar *et al.*, 2003) did not yield electrophoretically pure enzyme. The size exclusion fractions, from the last step of the purification process suggested impurities as visualized by the Coomassie stained SDS-PAGE gels. The additional bands imply further processing of the sample is required to achieve purification.

The results of the purification process suggest modification of certain parameters. First, EDTA added to the Buffer A is known to inactivate
metalloproteins at a concentration of 10 μM. It is suggested that all buffers made for purification of Arr be devoid of EDTA in their composition. Secondly the non-ionic detergent used for solubilization of the membrane fraction appears to hinder with the adsorption spectrum and produces false positive signals. On concentration of protein samples, TritonX-100 formed an extremely viscous suspension affecting the successful measurement of protein concentration. It could not be separated easily from the protein suspension and might hinder with biochemical characterization of Arr. An alternative detergent commonly used for protein purification is CHAPS. Replacement of 1%TritonX-100 with 0.5% CHAPS for anion exchange and size exclusion column chromatography seemed to be more effective (data not shown).

The presence of unknown protein bands in addition to ArrA and ArrB visualized in the lane containing purified Arr after gel filtration may be attributed to low resolution of the size exclusion chromatography or intervention by excess of Triton X-100 and NaCl after passage through the second DEAE column. A dialysis step in Tris –HCl buffer is advocated, for the removal of excess NaCl and detergent after the second anion exchange column elution, before loading on to the gel filtration column. To ensure higher resolution of the gel filtration chromatography, the resin suggested for future use is Sephacryl S-200 HR (SIGMA). The impurities may also be degradation products resulting from changes in pH inside the columns.

The EPR and metal analyses results did not indicate the presence of molybdenum but showed a strong copper signal. The high amount of copper is very perplexing, since copper available to the cells is only from the trace elements added
to the media. It may have arisen as impurity during the purification of the enzyme. The presence of tungstate at high levels may be explained by the sodium tungstate ($9 \times 10^{-6}$ g/L) that is amended to the media (Switzer Blum et al., 1998). Tungstate is a competitor for molybdenum at the metal ion center of the catalytic subunit of molybdoenzymes.

In earlier studies with DMSO reductase from *Rhodobacter sphaeroides* f. sp. *denitrificans*, it was found that induction of the enzyme was inhibited by tungstate competing for the guanine dinucleotide binding site of molybdenum. (Yoshida et al., 1991). It was shown in DMSO reductase, that the binding of molybdenum is required for the processing of the precursor form into the mature protein. The translocation of the protein to the periplasm requires the molybdenum cofactor. Under conditions of limiting molybdenum supply, the precursor form accumulated in the cytoplasm or cytoplasmic membranes. The enzyme activity increased steadily with addition of the molybdenum.

The activity seen in proteins of molecular mass lower than the catalytic subunit of the arsenate reductase enzyme is not clearly understood. The consistent activity in zymograms seen as two oxidized bands at 63 kDa and 58 kDa respectively as opposed to the 110 kDa ArrA, may be due to the requirement for molybdenum for processing into the mature protein and its translocation to the cytoplasmic membrane. Alternatively, the activity at the lower molecular weight proteins might indicate a requirement of ArrB subunit for arsenate reductase activity. N-terminal sequencing of the two protein bands from a previous experiment (unpublished data) showed both partial ArrA and ArrB sequences. This indicates that the Fe-S cluster containing
ArrB subunit may be involved in the channeling of electrons to the catalytic subunit and is therefore essential for arsenate reductase activity. The two bands also appeared to react with the antibody to Arr in all fractions tested indicating that the activity is indeed from Arr and not random proteins in the crude fractions that oxidize rMV. Interestingly, MLS10 cells grown in different terminal electron acceptors, activity was seen only in the 58 kDa protein.

It can be inferred that Arr, which belongs to the DMSO reductase family of molybdoenzymes, may similarly be inhibited by tungstate. It has been previously reported that tungstate inhibits arsenate reduction though the activity was not affected by molybdate, nitrate and phosphate (Dowdle et al., 1996). The excess tungstate present in the purified enzyme, hinders the further biochemical characterization like extended X-ray absorption fine structure (EXAFS) and crystallography required to study the metal binding site and oxidation states of molybdoproteins.

VI. FUTURE DIRECTIONS

The polyclonal antibodies developed against the C-terminus conserved sequence of Arr from B. *selenitireducens* MLS10 has been purified. It’s efficiency as a probe to detect arsenate respiring bacteria has been tested and Arr antibody appears to have great potential to function as a functional probe. It can be utilized for *in situ* hybridization to detect arsenate respirers in water samples without the requirement for pure cultures.

The expression of respiratory arsenate reductase can be further characterized by studying mRNA expression. The upregulation can also be quantified appropriately by assessing mRNA levels in cells grown in different terminal electron acceptors.
Future directives for Arr characterization involve immunolocalisation, crystallography to study the metal binding center, and Extended X-ray Absorption Fine Structure (EXAFS) to determine the coordination environment of the molybdenum ion centre of the enzyme.

Given below are the suggestions that will be of help in the future experiments addressing the three hypotheses:

**Culture media**

- Eliminate tungsten from the media.
- Amend the media with sodium molybdate.

**Purification procedure**

- The supernatant from the whole cell harvest should be tested for enzyme activity.
- EDTA to be eliminated from all buffers. It is a chelating agent and can inactivate the enzyme by binding with the metal.
- The non-ionic detergent Triton X-100 should be replaced by the zwitter-ionic detergent CHAPS (0.5%). Triton X-100 strongly absorbs at 280nm. The detergent can be easily removed by dialysis and is non-denaturing.
- Dialysis should be performed between each chromatography step to remove excess salt, which will affect the protein binding to the DEAE column. Excess salt and detergent in the sample also affect its flow in the gel filtration column. Dialysis should be performed using the dialysis tubing rather than the Amicon Filter Centrifuge tubes.
• Sephacryl S-300 HR resin should be replaced by Sephacryl S-200 HR for higher resolution.

• The resins – DEAE-Toyopearl and Sephacryl S-200 HR should be thoroughly rinsed with the washing buffer, regenerated in ethanol and equilibrated with the running buffer to avoid contaminants which may affect binding, flow, and purity of the sample.

• The gel filtration fraction should be concentrated using Speed vacuum and further processed by Anion Exchange Chromatography.

Zymogram / Qualitative Enzyme Activity Assay

• Use lower concentration of the electron acceptor (sodium arsenate). The Km for MLS10 is 34 µM arsenate.

• Use a non-arsenate respiring bacteria that can serve as a negative control

• Test the same fractions with different electron acceptors like nitrate, nitrite, fumarate, arsenite, selenite, DMSO.

Spectrophotometric / Quantitative Enzyme Activity Assay

• A non-arsenate respiring bacteria should be tested for oxidation of reduced methyl viologen as a negative control.

• The reaction mixture in the reference cuvette should contain the protein sample and not the electron acceptor.

• The sample cuvette should contain the protein sample and the electron acceptor.

• Sodium dithionite can be replaced by titanium citrate to reduce methyl viologen.
### VII. Appendices

#### A. Appendix 1: BASAL SALT MEDIUM

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<th>CONSTITUENTs</th>
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<td>$NaCl$</td>
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<td>$(NH_4)_2SO_4$</td>
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<td>$MgSO_4.7H_2O$</td>
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<tr>
<td>$Na_2CO_3$</td>
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<td>Cysteine-HCl</td>
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<tr>
<td>$Na_2WO_4$</td>
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<td>Yeast extract</td>
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<td>$NaMoO_4$</td>
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**Additions**

- Trace elements: 15ml
- Vitamin mix: 15ml

### Vitamin Mix

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<thead>
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<th>mg/L</th>
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<td>Thiamine</td>
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<td>Vitamin</td>
<td>Concentration (g/L)</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------------</td>
</tr>
<tr>
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<tr>
<td>Pantothenic Acid</td>
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</tr>
<tr>
<td>p-Aminobenzoic acid</td>
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</tr>
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<td>Thiococ Acid</td>
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</tr>
<tr>
<td>Vitamin B12</td>
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**Mineral Mix**

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<th>Mineral</th>
<th>Concentration (g/L)</th>
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<td>Manganese Chloride</td>
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B. Appendix 2 : GEL FILTRATION STANDARDS

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<th>Protein Standards</th>
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<tr>
<td>Blue Dextran</td>
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<tr>
<td>Alcohol dehydrogenase</td>
<td>150</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>66</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>29</td>
</tr>
</tbody>
</table>

C. Appendix 3 : IMMUNIZATION SCHEDULE

WEEK

0  Pre- Immune
1  Bleed 1
2  Bleed 2
3  Bleed 3

D. Appendix 4: LOWRY REAGENTS

2 %   Sodium Dodecyl Sulfate (Lauryl Sulfate
1 mg/ml Bovine Serum Albumin (BSA) in 2% SDS

First Lowry Reagent

2.0 %   Sodium Carbonate in 1M Sodium Hydroxide
1.0 %   Copper Sulfate (CuSO₄)
0.5 %   Sodium Potassium Tartrate

Second Lowry Reagent

1:1   Folin Phenol reagent diluted in distilled water
E. Appendix 5 : SDS - PAGE

Electrophoresis Buffer (mildly denaturing)

25 mM Tris HCl pH 8.3
192 mM Glycine
0.1 % Sodium Dodecyl Sulfate (SDS)

Sample Buffer (Bio-Rad #161-0737)

62.5 mM Tris pH 6.8
25 % Glycerol
2 % Sodium Dodecyl Sulfate (SDS)
0.01 % Bromophenol Blue
5 % β- Mercaptoethanol

Broad Range Prestained Molecular Weight Standards (Bio-Rad # 161-0318)

<table>
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<tr>
<th>PROTEIN STANDARDS</th>
<th>Molecular Weight (Daltons)</th>
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<tr>
<td>Myosin</td>
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<td>β- galactosidase</td>
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<td>Ovalbumin</td>
<td>54,604</td>
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<td>Carbonic anhydrase</td>
<td>37,390</td>
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<td>Soyabean trypsin inhibitor</td>
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<td>Lysozyme</td>
<td>20,366</td>
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<tr>
<td>Aprotinin</td>
<td>7,036</td>
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</table>
SDS-PAGE Staining Solution

50 % Methanol
10 % Acetic acid
0.05 % Brilliant Blue G-250 / R-250

Destaining Solution

7 % Methanol
5 % Acetic acid

F. Appendix 6: WESTERN BLOT

Transfer Buffer pH 8.3:

39 mM Glycine
48 mM Tris base
0.037 % Sodium Dodecyl Sulfate (SDS)
20 % Methanol

Tris Buffered Saline (TBS)

9 g/L Sodium Chloride
50 ml/L Tris HCl (1 M)

Tween-20 – Tris Buffered Saline (TTBS)

9 g/L Sodium Chloride
50 ml/L Tris HCl (1 M)
0.5 ml Tween 20
Phosphate Buffered Saline pH 7.4

Solution A
16 gm Sodium Chloride
0.4 gm Potassium Chloride
2.3 gm Sodium Phosphate (dibasic)
0.4 gm Potassium Phosphate (monobasic)
2 L Deionized water

Solution B
0.2 gm Calcium Chloride CaCl$_2$.2H$_2$O
0.3 gm Magnesium Chloride MgCl$_2$.6H$_2$O

Add solution A and solution B. Adjust pH to 7.4

Blocking Solution (Killer-Filler) pH 7.4

0.1 N Sodium Hydroxide
A) 10 gm Casein in 200ml of 0.1 N Sodium Hydroxide. Boil.
B) 10 gm Bovine Serum Albumin (BSA) in 1800 ml PBS

Add A and B. Adjust pH with HCl. To this solution add:
0.2 gm Phenol Red
3.6 gm Sodium Azide (27.7 ml)

Developing Solution

Carbonate buffer pH 9.8 :
8.4 g/L Sodium Bicarbonate
0.2 g/L Magnesium Chloride
Solution 1
7.5 mg 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP)
0.5 mL Dimethyl formamide (DMF)

Solution 2
15 mg Nitro Blue Tetrazolium (NBT)
0.35 ml Dimethyl formamide (DMF)
0.15 ml dH2O

Mix solution 1 and solution 2 in 50 ml of Carbonate buffer prior to developing and keep out of direct light.

G. Appendix 7: ELISA

Blocking Solution (PBS-Blotto)
5 gm Non fat dried Milk in 100ml PBS

Developing Solution
TMB Peroxidase EIA Substrate Kit (Bio-Rad, CA)

Solution A
9 ml 3,3',5,5'-Tetramethylbenzidine in aqueous Dimethyl Formamide (DMF)

Solution B
1 ml Hydrogen peroxide solution

Mix solution A and Solution B

H. Appendix 8: ENZYME ACTIVITY ASSAY

50 mM Tris HCl (pH 8.0)
10 mM Methyl Viologen (1,1’ Dimethyl 4,4’ Bipyridinium dichloride)
5 mM Sodium arsenate
0.1% Sodium dithionite

I. Appendix 9: CALIBRATION OF THE SIZE EXCLUSION COLUMN

[Graph showing calibration curves for various substances with absorbance at 280 nm against volume in ml.]

- Resin – Sephacryl S-300-HR
- Sample -50mg
- Flow rate- 1ml/min
- Void volume – 150ml
VIII. REFERENCES


47. Santini and Stolz Prokaryotic Arsenate and Selenate Respiration. (unpublished)


