

PROTEOMICS AND IN VIVO LABELING OF PROTEIN THIOLS  
IN SULFOLOBUS SOLFATARICUS DURING  
EXPOSURE TO ANTIMONY

by

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## DEDICATION

I dedicate this thesis to my son Vuyisile Ditumisho Mathabe Toli for persevering with me from the beginning until the end He is a good child through and through and I hope that one day when (Not if) he completes his own PhD, he will know and appreciate that everything I do, is so that he can have a brighter future than mine and follow a less turbulent pathway that I as a black South African had to follow. Salome Mathabe, for supporting all my pursuits and for preaching the gospel of education from a very young age. My grandparents, though late, raised me to be a stubborn determined woman.

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## ABSTRACT

Antimony (Sb) has a long history in both the chemical and social literature. As a metalloid it is often found in the environment with Arsenic (As). Extended exposure in humans causes heart disease, lung disease, diarrhea, severe vomiting and ulcers. In plants, it inhibits early crop growth. A large body of data on bacterial response and mechanisms for detoxification also exists. In contrast, knowledge about how archaeal species respond to Sb is much less extensive. The model crenarchaeal organism *Sulfolobus solfataricus*, can survive in environments with high antimony concentrations, and the genetic and biochemical mechanisms responsible for antimony tolerance have yet to be reported. As a first step in bringing to light the biological response of *S. solfataricus* to antimony, a set of proteomic and chemical tagging experiments were undertaken. Two-dimensional differential gel electrophoresis (2D-DIGE) showed a limited response from intracellular and membrane proteins with respect to their abundance. In contrast, chemical targeting of cysteine residues revealed that extensive oxidation had occurred to both cytosolic and membrane proteins upon exposure to antimony. To remove any possible experimental artifacts that could alter the oxidation state of protein thiols, a method for labeling cytoplasmic proteins in live *S. solfataricus* cells was developed. This method used the recently described Z-dye probes for quantitative comparisons. Together, our results suggest that Sb response is primarily focused on a general stress factors likely stemming from oxidative damage to proteins. No evidence for a specific transport or bioconversion was present. Cysteine residues in membrane proteins displayed the most significant oxidative changes. The demonstration that chemical biology approaches can be applied to prokaryotic cells, even those growing at extremes of temperature and Ph, should have broad appeal for microbiologists well beyond those investigating archaea

## CHAPTER 1

## INTRODUCTION

Heavy Metals

Heavy metal(s) are prevalent pollutants of great concern as they are non-degradable and thus obstinate. Common sources of heavy metal pollution include, discharge from industries such as electroplating, plastics manufacturing, fertilizer producing plants and waste streams generated by mining and metallurgical processes (Zouboulis, Lazaridis, Karapantsios, & Matis, 1997). Even though some metalloids are essential trace elements, most are toxic at high concentrations to all forms of life. The primary routes of exposure for humans are ingestion and inhalation. Working in or living near an industrial site which utilizes these metals and their compounds increases ones risk of exposure, as does living near a site where these metals have been improperly disposed. The impact of metals in the environment extends well beyond animals and plants. The composition and activities of microbial communities can also be altered (Liao & Xie, 2007). Two heavy metals of primary interest to human health and environmental fate are arsenic (As) and antimony (Sb). Both occur widely in soil and aquatic systems and are released by industrial processes. They are Group 15 elements in the Periodic Table, with Sb positioned directly below As. Sb and its compounds are considered hazardous pollutants by the US Environmental Protection Agency (USEPA, 1979) and the Council of the European Communities (CEC, 1976). Indeed the EPA drinking water standard for Sb

is 0.006 mg/L, which is lower than As, 0.01 mg/L. This reflects antimony's overall toxicity than As.

Inorganic arsenic is a known is a heavy metal that has been known to be poisonous for centuries. It is a carcinogen and can cause cancer of the skin, lungs, liver and bladder (Smith et al., 1992). Ingestion arsenic levels as low as 0.00017 mg/L can cause arsenicosis. Arsenicosis is a serious public-health problem that affects millions of people in Asian countries, including Bangladesh, China, India, Cambodia, Lao PDR, Mongolia, Myanmar, Nepal, Pakistan, and Viet Nam. It is mainly caused by drinking water from pump-wells contaminated by high levels of As, and in some areas of China, by eating food dried by burning As-rich coal. Chronic As poisoning can impair several systems of the body, giving rise to skin lesions with hyperpigmentation, depigmentation, and hyperkeratosis, As-related cancers of the skin, lung, and bladder (Sun, 2004; Yoshida, Yamauchi, & Fan Sun, 2004).

To a developing child, it can have complex consequences such as cognitive delays, reduced IQ, mental slowing or even poor memory (Chattopadhyay, Bhaumik, Nag Chaudhury, & Das Gupta, 2002). As is not the only metal with these toxicity effects. Sb will potentiate lung, heart, and gastrointestinal diseases. The effects on health are inducement of vomiting and eye and mucous membrane irritation.

In microorganisms, heavy metals generally exert toxicity by blocking essential functional groups, displacing essential metal ions or modifying the active conformations of biological molecules. However, at relatively low concentrations, some heavy metal ions are essential for microorganisms since they provide vital cofactors for metallo-

proteins and enzymes. Microorganisms can survive in heavy-metal polluted environments and have evolved mechanisms of resistance to toxic metal species. These mechanisms include, but are not limited to: metal exclusion by permeability barriers, active transport of the metal away from the cell, intracellular sequestration of the metal by protein binding, extracellular sequestration of the metal and reduction in metal sensitivity of cellular targets. The detoxification mechanisms may be directed against a single metal or may work on a group of chemically related metals (G. M. Gadd & Griffiths, 1978). A variety of detoxification mechanisms have been described to date. Numerous microorganisms have specific genes for resistance to toxic compounds containing heavy metals (Silver & Ji, 1994). The intake and subsequent efflux of heavy metal ions by microbes normally includes a redox reaction involving the metal. As such, some bacteria even use them for energy and growth (Wackett, 2000). Bacteria that are resistant to heavy metals also play an important role in biogeochemical cycling of those metal ions. Also, since the oxidation state of a metal ion may determine its solubility, many scientists have been attempting to use microbes that are able to oxidize or reduce metals in order to remediate metal contaminated sites. Thus, it is worthwhile to take note that although some heavy metals are important and essential trace elements and others are toxic above low concentrations, some microbes have adapted to tolerate the presence of metals or even to use them as a source of energy.

The heavy metals constitute a group of about 40 elements with a density greater than  $5 \text{ g/cm}^3$  (J. Gadd & Griffiths, 2013). Even though many of them are essential for growth, they are also reported to have comprehensively toxic effects on cells, as a result

of their ability to denature protein molecules. Heavy metal ions form strong bonds with the carboxylate anions of the acidic amino acids or SH groups of cysteine, disrupting ionic bonds and disulfide linkages. That inhibits protein folding (Kägi & Hapke, 1984; Vallee & Ulmer, 1972).

It is evident that metal toxicity can be heavily influenced by environmental conditions. Binding of metals to organic materials, precipitation, complexation, and ionic interactions are all important phenomena that must be considered carefully in laboratory and field studies. It is also obvious that microbes possess a range of tolerance mechanisms, most featuring some kind of detoxification.

#### Antimony in the Environment

Antimony (Sb) is a naturally occurring metalloid which can occur in several oxidation states. (-III, 0, III, V) however, it is mainly found in two oxidation states (III and V) in environmental, biological and geochemical samples (Hamamura, Fukushima, & Itai, 2013). According to the classical classification of Goldschmidt, Sb is a strong chalcophile element and as such, it occurs a lot in nature as  $\text{Sb}_2\text{S}_3$  (stibnite, antimonite) and  $\text{Sb}_2\text{O}_3$  (valentinite), which is a transformation product of stibnite. These compounds with Sb are commonly found in ores of copper, silver, and lead. It is also a common component of coal and petroleum. Sb-containing minerals occur in association with metal deposits throughout the world. When released from mineral forms, it exists primarily as antimonite [Sb(III)] and 4ldg.4an4e [Sb(V)] (Porquet & Filella, 2007). Currently, China is the largest producer of Sb, with over 80% of the world's supply of Sb coming from

mines in the south west of China. In other parts of the world, it is produced in USA, South Africa, Russia and Bolivia, (Figure 1).

Antimony has been used since the 18<sup>th</sup> century. It is known for its ability to dissolve many other metals including gold (Shotyk, Cheburkin, Appleby, Fankhauser, & Kramers, 1996). It also finds use as a therapeutic agent to treat problematic tropical diseases. It has been used to treat *Leishmania donovani* (Singh et al., 2004) and AIDS (Haldar, Sen, & Roy, 2011). Pentostam and Glucantime, pentavalent Sb-containing drugs, are widely used in the treatment of these diseases. Less information is available on the transformation and transport of Sb in the different environmental compartments. While there has been a steady rate of published studies concerning Sb environmental chemistry (averaging ~10/year), the literature concerning microbe-Sb interactions is extremely limited. The characterization of microbe-Sb interactions is of interest because of the potential to harness microbes for bioremediation purposes. Oxidation of Sb (III) to Sb (V) converts the highly toxic Sb (III) to the far less toxic Sb (V). The transport and bioavailability of Sb in the environment is highly dependent on chemical speciation. At present, little is known about the mechanisms and minimum genetic requirements that control or contribute to microbial Sb redox reactions, and therefore it is necessary to identify, characterize, and understand the genes and metabolic pathways that control microbial Sb (III) oxidation.

### Microbial Antimony Uptake

The biological transformation of Sb is not well understood. Studies looking at Sb often begin with what is known about As under similar conditions or how a given organism handles As. This is because Sb and As are often found co-habiting the same environment. In *Escherichia coli*, disruption of the glycerol transporter gene *glpF* reduced Sb (III) uptake, which could be a clear demonstration of the role of GlpF in Sb(III) uptake (Gourbal et al., 2004). In *Saccharomyces cerevisiae*, a protein known as Fsp1 that is homologous to GlpF plays a similar role. In archaea, it was demonstrated that Sb is methylated via the Challenger mechanism. In this mechanism, Sb (III) is first methylated, which is then followed by the reduction of Methyl-Sb.

### Microbial Antimony Efflux

A few transporters are associated with the efflux of Sb. These are ArsB protein, the Acr3p family, and the ABC transporter superfamily (Bentley and Chasteen, 2002, Wang et al., 2004). ArsB, which mediates bacterial As (III) efflux, is also involved in Sb(III) efflux via Sb(III)/H<sup>+</sup> exchange (Meng et al., 2004). It was found that found a *arsB*-like gene in the 6ldg.6an *Halobacterium* sp. NRC-1 that confers resistance to both As (III) and Sb(III). In *S. cerevisiae*, Sb (III) resistance originates from transport of Sb(III) into a vacuole as glutathione conjugates such as Sb(GS)<sub>3</sub>. Such processes are catalyzed by the yeast cadmium factor encoded by *ycf1*, a vacuolar glutathione S-Conjugate pump. Additionally in *S. cerevisiae*, the As resistance, transport proteins Acr3-2 and Acr3-3, were also induced by Sb(III) and offers resistance to Sb(III) (Wysocki et al., 2001). This is



a clear indication that one of the mechanisms that microorganisms used to protect the cells from Sb toxicity is by extrusion, using transport proteins.

### Antimony Resistant Microorganisms

Thus far, only a small number of Sb (III)-oxidizing bacteria have been reported. This includes *Agrobacterium* (Lehr, Kashyap, & McDermott, 2007a), *Acinetobacter*, *Comamonas*, *Pseudomonas* *Stenotrophomonas*, *Variovorax* and *Brevundimonas* and *Ensifer* (Shi et al., 2013). *Stibiobacter senarmontii* is another bacteria that oxidizes Sb (Lialikova 1974). The strains *Pseudomonas* sp. S1 and *Stenotrophomonas* sp. A3 also fall in the list of antimony oxidizing bacteria (Hamamura et al., 2013).

### Antimony Oxidation

One of the major mechanisms behind heavy metal toxicity has been attributed to oxidative stress. A growing amount of data provide evidence that metals are capable of interacting with nuclear proteins and DNA causing oxidative deterioration of biological macromolecules. While each metal may have its own mechanisms of action, the generation of reactive oxygen species (ROS) by metals and the resulting effects on cell signaling appear to result from a common mechanism (Harris, 2003; Qian, Castranova, & Shi, 2003). One of the significant members of the ROS family is the superoxide anion radical ( $O_2^{\cdot-}$ ), which can be dismutated to form hydrogen peroxide ( $H_2O_2$ ) by the overall reaction and the highly reactive hydroxyl radical ( $\cdot OH$ ) in the presence of certain transition metal ions (Chen & Shi, 2002; Halliwell & Gutteridge, 1984). Highly reactive

free radicals, such as HO, initiate oxidation by abstracting a hydrogen atom from an amino acid residue and generating carbon-centered radicals. These radicals react further with O<sup>2</sup>, producing peroxy radicals. Redox-active metal ions, such as Fe (III) and Cu(II), catalyze oxidative damage of proteins in the presence of oxygen and an electron donor or by generating hydroxyl or alkoxyl radicals through Haber-Weiss and Fenton-type chemistry from hydrogen or organic peroxides, respectively (Yzed, 1993).

Three decades ago, *Stibiobacter senarmontii* was described as being capable oxidizing senarmontite [Sb<sub>2</sub>O<sub>3</sub>] to cervantite [Sb<sub>2</sub>O<sub>4</sub>] and to stibiconite [Sb<sub>3</sub>O<sub>6</sub>(OH)]. *Chlorella vulgaris* bio-concentrates Sb when grown in medium containing Sb(III), and when transferred to Sb-free media, a 60:40 Sb(III):Sb(V) mixture was observed (Maeta et al., 1997) This implies that it oxidizes Sb(III) to Sb(V). Some hetrotrophic Sb(III) oxidizing bacteria as *A. tumefaciens* have also been characterized (Lehr et al., 2007; Fan et al., 2008).

### Arsenic in the Environment

As is a heavy metal that occurs naturally in the earth's crust. In many areas around the world, As-rich groundwater is used for drinking water and for irrigation purposes. Solid-phase As is broadly distributed in nature and can be mobilized in groundwater through a conglomeration of natural processes and/or anthropogenic activities (Smedley & Kinniburgh, 2002; Welch, Westjohn, R., & B., 2000) However, at the global scale, natural processes are mainly responsible for the prominent As concentrations. Anthropogenic sources of As, such as leaching from mine tailings, can be

very important on the local scale. As exists naturally in four valence states: + V, + III, 0, and -III. The most common forms of As are + V, also denoted as As (V) or arsenate, and +III, As (III) or arsenite. Arsenite is much more toxic than arsenate. The current literature states that arsenite is anywhere from 10 to 100 times more toxic than arsenate. Not only does toxicity vary with As's valence state but also the solubility, bioavailability, and mobility. Although both As(II) and As(V) are soluble, As(III) has a higher solubility than As(V) and therefore has increased bioavailability and mobility as compared to As(V) (Ruta et al., 2011).



Figure 1.1. Documented problems with As in groundwater and the environment. Countries mostly affected by As contamination are China, Argentina, Ghana and Bangladesh. Areas that are shaded in red indicate where As contamination causes challenges with ground water contamination

### Microbial Arsenic Uptake

Detoxification of heavy metal ions by reduction has been extensively studied and As(V) reduction to As(III) is well-documented (Ruta et al., 2011; Soda, Yamamura, Zhou, Ike, & Fujita, 2006). It is believed that inorganic arsenate ( $\text{AsO}_4^{2-}$ ), which is a molecular analogue of phosphate ( $\text{HPO}_4^{2-}$ ), can compete for phosphate anion transporters and replace phosphate in some biochemical reactions (Hughes, 2002).

In Eukaryotes, aquaglyceroporins are the major cellular entrance (MCE) for As (III) and Sb (III). Sanders et al showed that inactivation of *Escherichia coli glpF* gene, encoding for the glycerol facilitator, led to Sb (III) resistance phenotype (Sanders, Rensing, Kuroda, Mitra, & Rosen, 1997). Later, based on the genetic data and direct transport measurements of radioactive As(III) it was demonstrated that the *S. cerevisiae* glycerol facilitator Fps1 mediates uptake of As(III) and Sb(III) (Wysocki et al., 2001). Both glycerol facilitators GlpF and Fps1 belong to the family of major intrinsic proteins (MIP) that encompasses the membrane channel proteins, which are selective for either water only (aquaporins) or water and other uncharged solutes, like glycerol and urea (aquaglyceroporins) (Bienert, Schüssler, & Jahn, 2008).

In the yeast *Saccharomyces cerevisiae*, there are two high-affinity, Pho84 and Pho89, and three low-affinity, Pho87, Pho90 and Pho91, phosphate transporters that have been identified and it was strongly suggested that they As(V) uptake is mediated by phosphate transport, Figure 1.3, (Bun-ya, Harashima, & Oshima, 1992; Persson et al., 2003).

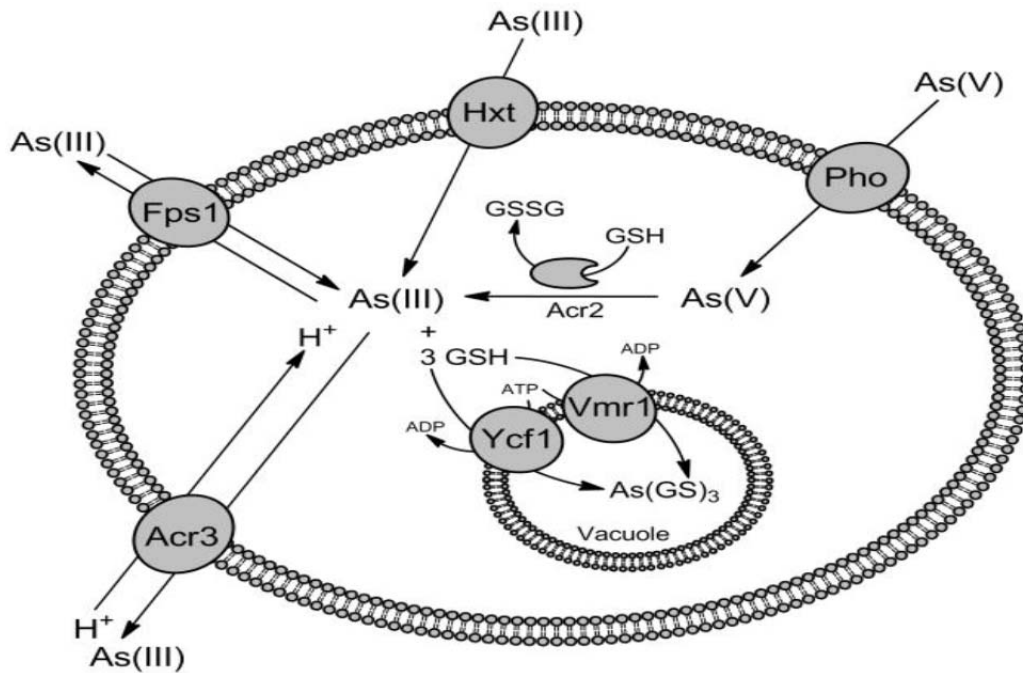


Figure 1.2. The pathway for Arsenic uptake and detoxification of As(V). Pathways for arsenic uptake and detoxification in the yeast *S.cerevisiae*. In yeast cells uptake of As (III) is facilitated mainly via the aquaglyceroporin Fps1 but in the absence of glucose As (III) also enters the cell through the hexose permeases Hxt (Hxt1-Hxt17, Gal2). As (V) is taken up by the phosphate transporters (Pho), such as Pho84 and Pho87, followed by reduction to As (III) by the arsenate reductase Acr2 in the cytoplasm. Next, As (III) is transported out of the cell by the As (III)/H<sup>+</sup> antiporter Acr3 against the concentration gradient or by the aquaglyceroporin Fps1, especially during As (V) exposure, when the internal concentration of As(III) is higher than the outside. In addition, As (III) is conjugated with glutathione (GSH) and sequestered into the vacuole by the ABC transporters Ycf1 and Vmr1.(Maciaszczyk-Dziubinska, Wawrzycka, & Wysocki, 2012)

### Microbial Arsenic Efflux

Bacterial resistance to arsenic and antimony has been described in both gram-positive (Dabbs & Sole, 1988) and gram-negative bacteria (Hedges & Baumberg, 1973). Resistant *E. coli* and *Staphylococcus aureus* are resistant to arsenic but their cells can only accumulate arsenate to only a small extent (Silver et al., 1981a).

### Arsenic Resistant Microorganisms

Some microbes can reduce As (V) to As (III) during their anaerobic respiration or as a means of arsenic detoxification. Very few bacteria have been reported to have the ability to oxidize As (III) to As (V) and to reduce AS (V). *Herminiimonas arsenicoxydans*, *Thermus sp*, bacteria, is one of those (Silver et al., 1981b). *Staphylococcus aureus* and *Escherichia coli* have the ability to reduce cellular concentrations of As and rapidly effluxing them via a plasmid-encoded arsenical pump (Silver et al., 1981b). Among different microorganisms used in bioremediation, fungi are considered as most effective species for metal removal from metal contaminated sites because they are able to survive in higher concentration of metals. *Aspergillus niger* has the ability to detoxify environmental copper and zinc. The arsenic tolerant fungal strains (ASC1 and ASB3) is able to remove a significant amount of arsenic from different arsenic enriched media in laboratory condition (Mukherjee, Das, Samal, & Santra, 2013).

### Arsenic Oxidation

Some microbes are not only resistant to As but, they have the ability to metabolize it via methylation, reduction and oxidation reactions. Methyl reactions convert As (V) to As (III) into compounds such as monomethyl arsonate (MMA(V)), methylarsonite (MMA (III) and trimethyl oxide as well as a number of volatile arsines. Arsenite (As III) oxidation is carried out by a heterodimer, arsenite oxidase (Aox). Homologs of Aox are found in the genomes of *Crenarchaeota Aeropyrum* premix and *Sulfolobus tokodaai*. They were also found in some green bacteria, *Chloroflexus*

*aurantiacus*, *Chlorobium limicola*, *Nitrobacter hamburgensis* and *Burkholderia* species. Because Aox was found in these green bacteria, it has been suggested that As (III) may be linked to photosynthesis (Stolz, Basu, & Oremland, 2010). It has been suggested that dissimilatory arsenic reduction, a microbial process requiring As (V) as an electron acceptor, became possible after the evolution of oxygen photosynthesis (Lehr, Kashyap, & McDermott, 2007b).

#### *Sulfolobus Solfataricus*, A Model Organism

The order Sulfolobales is a genus of microorganisms belonging in the family Sulfolobaceae. It belongs to the phylum of Crenarcheota of the hyperthermophilic Archaea. This domain contain the genera *Sulfolobus*, *Acidianus*, *Metallosphaera*, *Stygiolobus*, and *Sulphurisphaera*. *Sulfolobus spp.* Was formerly isolated in the USA at Yellowstone National Park and was first described by Brock et al (Brock, Brock, Belly, & Weiss, 1972).

Members of this group are hyperthermophilic obligate aerobes. They require a low Ph to grow and survive by oxidizing molecular sulfur to sulfuric acid (Brock et al., 1972). They typically colonize hot springs which are rich in heavy metals but little is known about the interactions between these elements. *S. solfataricus*, however, is strictly aerobic and grows either heterotrophically or uses cellular respiration with oxygen acting as the final electron acceptor for autotrophic growth. It has a number of transport systems for the uptake of different organic compounds and energy sources such as sugars (e.g. glucose and galactose) and amino acids (e.g. alanine, aspartate, and glutamate) (Grogant,

1989). The first sequenced genome of *Sulfolobus*, *S. solfataricus* P2, was published in 2001 (She et al., 2001). It contains 2,992,245 bp on a single circular chromosome

(Duggin & Bell, 2006), and encodes approximately 3000 proteins. About one third of the genes have no known homologs in other sequenced genomes. *Sulfolobus* is of high interest for industry and biotechnology because of its broad physiological versatility – e.g. it exhibits the ability to grow on phenol (Izzo, Notomista, Picardi, Pennacchio, & Di Donato, 2005) – and because of the unique properties of its thermostable proteins. Some *Sulfolobus* strains have been found to survive in environments which are enriched with heavy metals including As, Mercury (Hg) and Sb. With this vast versatility and a small genome, *Sulfolobus* has become a model organism for a variety of scientific studies including their reaction to heavy metals.

### Aims and Objectives

Metal (oid) s of Sb and As are found in the environment. Human exposure to these chemicals can lead to acute and chronic intoxication, a number of diseases and even cause death. In microorganisms,

If we are to understand the mechanisms and better address the problems associated with Sb (III) toxicity, then it will be necessary to elucidate the mechanisms controlling cellular Sb levels. This study aimed to elucidate aspects of a regulatory



system that controls Sb (III) levels in *Sulfolobus solfataricus* as a model for archaeal organisms. For this study, the following questions were asked:

- What effect does Sb (III) and As (III) have, on the proteome of *S.solfataricus*?
- Does Sb reduce or oxidize proteins?
- Can *S.solfataricus* adapt to Sb (III) stress?

CHAPTER 2

PROTEOMICS AND IN VIVO LABELING OF PROTEINTHIOLS  
IN SULFOLOBUS SOLFATARICUS DURING  
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Chapter 2: Proteomics and in vivo labeling of protein thiols in *Sulfolobus solfataricus* during exposure to antimony

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Contributions: Assisted and collaborated with As (III) stressing, provided valuable insight

Co-Author: Benjamin D Reeves

Contributions: Provided probes used for the whole experiment, provided valuable insight

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Contribution: Provided assistance on in vivo labeling results

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Contribution: Provided valuable insight

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Contribution: Provided probes used for the whole experiment, provided valuable insight

Contribution of Authors and Co-Authors-Continued

Co-Author: Mark J. Young

Contribution: Provided valuable insight, co- supervised

Co-Author: Brian Bothner

Contributions: Provided valuable insight; wrote manuscript; procured funding.

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## CHAPTER 2

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Abstract

Antimony (Sb) has a long history in both the chemical and social literature. As a metalloid it is often found in the environment with Arsenic (As). Extended exposure in humans causes heart disease, lung disease, diarrhea, severe vomiting and ulcers. In plants, it inhibits early crop growth. A large body of data on bacterial response and mechanisms for detoxification also exists. In contrast, knowledge about how archaeal species respond to Sb is much less extensive. The model crenarchaeal organism *Sulfolobus solfataricus*, can survive in environments with high antimony concentrations, and the genetic and biochemical mechanisms responsible for antimony tolerance have yet to be reported. As a first step in bringing to light the biological response of *S. solfataricus* to antimony, a set of proteomic and chemical tagging experiments were undertaken. Two-dimensional differential gel electrophoresis (2D-DIGE) showed a limited response from intracellular and membrane proteins with respect to their abundance. In contrast,

chemical targeting of cysteine residues revealed that extensive oxidation had occurred to both cytosolic and membrane proteins upon exposure to antimony. To remove any possible experimental artifacts that could alter the oxidation state of protein thiols, a method for labeling cytoplasmic proteins in live *S. solfataricus* cells was developed. This method used the recently described Z-dye probes for quantitative comparisons. Together, our results suggest that Sb response is primarily focused on a general stress factors likely stemming from oxidative damage to proteins. No evidence for a specific transport or bioconversion was present. Cysteine residues in membrane proteins displayed the most significant oxidative changes. The demonstration that chemical biology approaches can be applied to prokaryotic cells, even those growing at extremes of temperature and Ph, should have broad appeal for microbiologists well beyond those investigating archaea.

### Introduction

Antimony and arsenic are intimately linked through their elemental nature and chemical properties. In the environment, Antimony (Sb) is often associated with arsenic (As) because they have similar geochemical properties and toxicology. In addition to the natural distribution in soil and water, antimony is released by a number of human activities. Sources of anthropogenic release of antimony into the environment include batteries, paints, pigments, ceramics, fire retardants, and glass. As with other metalloids, the physiological and toxicological behaviors of antimony depend on its oxidation state, binding partners, potential ligands and solubility. It can exist in a variety of oxidation states (-III, 0, III, V) but it primarily is present as Sb (III) and Sb (V) in environmental,

biological and geochemical samples(Hamamura et al., 2013). Antimony potassium tartrate (APT) is the predominant trivalent form. Generally, Sb (III) compounds have a 10-fold higher toxicity than Sb (V) compounds. Due to the toxic effects, Sb is listed as a priority pollutant of interest by the USEPA (the maximum contaminant level in drinking water is 6  $\mu\text{g L}^{-1}$ ).

In microorganisms, metals have been reported to impair cell membranes, alter enzyme specificity, protein expression, disrupt cellular functions, and damage DNA. Current estimates indicating that over half of all proteins are metalloproteins, containing metal ions either as a structural component or as a catalytic co-factor(Cvetkovic et al., 2010). Microorganism have evolved numerous mechanisms that ensure efficient metal homeostasis, such as sequestering of metals by metallothioneins(Camakaris, Voskoboinik, & Mercer, 1999) and trafficking of metal ions by metallochaperones(Rosenzweig & Biology, 2002). A small number of Sb (III)-oxidizing bacteria have been reported. This includes *Agrobacterium tumefaciens*(Lehr et al., 2007a), *Acinetobacter*, *Comamonas*, *Pseudomonas Stenotrophomonas*, *Variovorax*, and *Brevundimonas* and *Ensifer* (Shi et al., 2013). Other bacteria said to be Sb (III)-oxidizing are *Stibiobacter senarmontii* isolated 30 years ago (Lialikova 1974) and the recently described *Pseudomonas* sp. S1 and *Stenotrophomonas* sp. A3. (Hamamura et al., 2013). Some of the Sb (III)-oxidizing strains have been shown to also oxidize As (III) as well(Cai, Rensing, Li, & Wang, 2009; Xiong et al., 2011), the ability to oxidize both of these Group 15 elements is not universal (Li et al., 2013A, B). The geographically disparate origin of these Sb (III)-oxidizing bacteria indicates that microbial Sb (III) oxidation may be widely distributed across bacterial

lineages and mediate detoxification of Sb where it exists in high concentrations in the environment.

*Sulfolobus* species are hyperthermo-acidophiles growing optimally at 70–85°C and Ph 2–3 that are found worldwide in geothermic environments such as solfataric fields and hot springs. While these natural environments can be rich in heavy metals, little is known about the interaction between heavy metals and resident archaea. Some studies have been carried out to elucidate the mechanisms that *Sulfolobus sp.* Utilizes to resist the presence of copper, mercury and antimony. (Schelert, Rudrappa, Johnson, & Blum, 2013), (Auernik, Maezato, Blum, & Kelly, 2008). For example, P-type CPX-ATPases are responsible for the export of copper (Deigweiher, Drell, Prutsch, Scheidig, & Lübben, 2004) through biological membranes. Gene disruption was used to demonstrate that Mercury resistance mediated by mercuric reductase (MerA) in *S. solfataricus*(Schelert et al., 2004). Thus far, there are no studies on the prospective genetic and biochemical mechanisms that enable *S. solfataricus* to survive in high concentrations of antimony. Understanding these mechanisms could be particularly useful.

In this study, we have analyzed the physiological response of *S.solfataricus* to varying concentrations and lengths of exposure to Sb. Cells were stressed with Sb (III) at levels inducing low levels of toxicity. Proteomics analysis using 1-Dimensional (1D) gels 2-Dimensional (2D) Differential Gel Electrophoresis (DIGE) was used to investigate changes in protein abundance and post-translational modifications. As part of the analysis, a novel method for characterizing the redox status of protein thiols *in vivo* labeling was developed. Zwitterionic fluorescent chemical probes reported on the



oxidized to reduced balance of cysteines in both cytoplasmic and membrane proteins. Results show that Sb (III) the immediate cellular response at the protein level is specific, with the relatively few proteins showing changes in abundance. Proteins that did show regulation tended to be associated with the cellular membrane. In addition there was an overall oxidation of cysteine residues across the proteome.

### Materials and Methods

*S. Solfataricus* ATCC strain was grown in DMZ medium 182 (22.78 Mm  $\text{KH}_2\text{PO}_4$  + 18.90 Mm  $(\text{NH}_4)_2 \text{SO}_4$  + 0.81 Mm  $\text{MgSO}_4$  + 1.7 Mm  $\text{CaCl}_2$  + 0.2% Yeast Extract) Ph adjusted to 3.0 with  $\text{H}_2\text{SO}_4$ . All cultures were grown in long neck Erlenmeyer flasks at 80°C. Growth was monitored by taking the optical density of the cultures at a wavelength of 650 nm.

### Growth Curves and Protein Analysis of Antimony Stressed Cells

The cells were grown in triplicates and were allowed to reach the optical density (OD) of ~0.2. Cells were stressed with 0, 50, 240 and 1000  $\mu\text{M}$  Sb (III) ( $\text{C}_8\text{H}_4\text{K}_2\text{O}_{12}\text{Sb}_2 \cdot 3\text{H}_2\text{O}$ , Sigma Aldrich, US). Growth curves of the effects of Sb (III) on *S. solfataricus* (P2) constructed using cultures of 75ml in a 150ml long neck Erlenmeyer flask. Two sets of triplicate cell cultures were treated with 50  $\mu\text{M}$ , and 240 $\mu\text{M}$  concentrations of Sb (III). A third set of the triplicates was not treated and it acted as the control. Samples from each flask were taken to test  $\text{OD}_{650}$  at 6,16,24,48 and 72 hour time points.

For protein expression analyses, *S. solfataricus* cells were grown to late log phase (OD<sub>650</sub> 0.52) in DSMZ media and then divided evenly between four, 1-liter long neck culturing flasks. At 56 hrs after the start of culturing (OD<sub>650</sub> ~0.2) (~450 mls) were treated with 0, 50, 240 or 1000  $\mu$ M antimony. At 8 and 24 hours after addition, 50 ml aliquots were taken for protein isolation.

### 2-Dimensional – Differential Gel Electrophoresis

The cells were pelleted after 8 hours and 24 hours respectively and washed with 0.5 ml PBS buffer. Cells were lysed with a combination of freeze–thaw and lysis buffer (30 mM Tris-HCl pH 8.5 (4 °C), 7 M urea, 2M thiourea, 4% CHAPS, 50 mM DTT, 0.5% IPG carrier ampholytes and a cocktail of protease inhibitors). The supernatant was clarified by centrifugation. Proteins were purified and concentrated by precipitation with acetone, and resuspended for 1 h in lysis buffer without DTT. Protein concentration was measured using a colorimetric Bradford Protein Assay dye (Bio-rad). 50  $\mu$ g of each protein samples was labeled with custom made Z-CyDyes using a minimal labeling protocol (Maaty, Steffens, Heinemann, Ortmann, Reeves, Biswas, & Edward, 2012). The labeling reactions were performed at the same time on all 3 biological replicates, using dye at a final concentration of 400 Pico moles of the N-hydroxysuccinimide esters dyes (Z-Cy3 and Z-Cy5) dissolved in 99.8% DMF (Sigma). The internal standard (Z-Cy2) was an equimolecular mixture of all samples. Labeling reactions were quenched by the addition of 1 ml of a 10-mM L-lysine solution (Sigma) and left on ice for 10 min. Z-Cy2, Z-Cy3 and Z-Cy5 labeled samples were combined appropriately and mixed with

rehydration buffer (7M urea, 2 M thiourea, 4% CHAPS) containing 50 Mm DTT and 0.5% IPG2-DE was performed as detailed by Shevchenko et al. Precasted IPG strips (Ph 3–11 NL, nonlinear, 24 cm length; GE Healthcare) in the first dimension (IEF) were used. Ar, 24 cm length; GE Healthcare) in the first dimension (IEF). Labeled samples were combined and made up to 450 Ml rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG buffer Ph 3–11 NL, 40 Mm DTT, and a trace of bromophenol blue) and loaded onto IPG strips. 150 µg proteins were loaded on each IPG strip and IEF was carried out with the IPGPhor II (GE Healthcare). Focusing was carried out at 20 °C, with a maximum of 50 Ma/strip. Rehydration was achieved by applying 50 V for 12 h. A total of 44,000 Vhr were used to complete focusing. Prior to the second-dimensional electrophoresis, the strips were equilibrated twice for 15 min with 50 Mm TrisHCl, Ph 8.8, 6 M Urea, 30% glycerol, 2% SDS and a trace of bromophenol blue. The first equilibration solution contained 65 Mm DTT, and 153 Mm iodoacetamide was added in the second equilibration step instead of DTT (Hatzimanikatis, Choe, & Lee, 1999). The strips were sealed on the top of the gels using a sealing solution (0.75% agarose in SDS-Tris HCl buffer). The second-dimension SDS-PAGE was performed in a Dalt II (GE Healthcare), using 1 mm-thick, 24-cm, 13% polyacrylamide gels, and electrophoresis was carried out at a constant current (45 min at 2 W/gel, then at 1 W/gel for ~16 h at 25 °C). Gels were stained with Coomassie Brilliant Blue stains for spot picking.

### In Vivo Labeling of Intact Cells

To measure the oxidation state of protein thiols *in vivo* and to examine if the presence of antimony causes oxidation in *S. Solfataricus*, cells were grown as outlined in 2-D DIGE section. Untreated cells and cells treated with 50  $\mu\text{M}$  and 240  $\mu\text{M}$  Sb (III) were allowed to grow until they reach the OD of  $\sim 0.2$ . The cells were gently pelleted after 8 and 24 hours by centrifugation at 800 rpm for 10 minutes at 37°C. They were washed with 0.5 ml PBS. 100  $\mu\text{l}$  of PBS was resuspended in the pellets. Labeling was performed on all 3 biological replicates using custom made Z-CyX maleimide (ZB-M LC-01-56) at a final concentration of 200. As per figure 4, the labeling reactions were performed for 20 min at 60°C for 10 minutes. For the cyanine dye, the reactions were quenched with 10 mM Lysine and 1M DTT (Sigma) for Z-Dye linked to maleimide. Succeeding that, the cells were centrifuged at 800 rpm and the supernatant was retained. This was followed by analysis on 14% 1 D SDS PAGE in a The Mini-PROTEAN Tetra system (Biorad).

### Cytosolic and Membrane Associated Protein Preparation

Subsequent to *in vivo* labeling, cells were lysed by freeze-thaw followed by sonication for 30 seconds. Cytosolic proteins were collected by centrifuging at 15000 rpm for 15 minutes. The pellet was retained and membrane associated proteins were extracted with 100  $\mu\text{l}$  0.4 % Triton-X. The samples were spun at 15000 rpm for 15 minutes. The resulting pellet was resuspended in 4X SDS loading buffer (100 mM Tris-

HCl Ph 6, 277Mm SDS, 40% glycerol) to recover integral membrane proteins. After centrifugation for 15 minutes at 15K rpm, the supernatant was retained.

### Image Acquisition and Analysis

After electrophoresis, gels were scanned using the Typhoon Trio Imager according to the manufacturer's protocol (GE Healthcare). Scans were acquired at 100  $\mu\text{m}$  resolution. Images were subjected to automated difference in gel analysis using Progenesis SameSpots software version 3.1 (Nonlinear Dynamics Ltd.). The Z-Cy3 gel images were scanned at an excitation wavelength of 532 nm with an emission wavelength of 580 nm, Z-Cy5 gel images were scanned at an excitation wavelength of 633 with an emission wavelength of 670 nm, while the Z-Cy2 gel images were scanned at an excitation wavelength of 488 nm with an emission wavelength of 520 nm. Gel spots were co-detected as DIGE image pairs, which were linked to the corresponding in-gel Z-Cy2 standard. After scanning, the gels were stored in 1% acetic acid at 4°C until spot excision. Spots were identified and volumes were quantified using Progenesis SameSpots software.

### Quantitative Determination of Free Thiols

To determine total protein thiol content, Ellman's reagent (ThermoScientific) was used. A standard curve was constructed using Cysteine Hydrochloride Monohydrate. Absorbance at 412 nm was measured.

### In-gel Digestion

Gel processing and in-gel digestion was performed in a keratin-free laminar flow cabinet. Gel slices were cut from the coomassie-stained and washed SDS-PAGE gel on a clean glass plate and were further diced in 1 mm<sup>3</sup> cubes using a scalpel and were transferred to a 1.5 ml reaction tube for further processing. The gel plugs were in-gel reduced and S-alkylated, followed by digestion with porcine trypsin (Promega) overnight at 37°. The solution containing peptides released during in-gel digestion were transferred to sample analysis tube prior to mass spec analysis.

### Protein Identification

For LC MS/MS, we used 6520 Accurate-Mass Q-TOF LC/MS (Agilent). Injected samples were first trapped and desalted on the Zorbax 300SB-C18 Agilent HPLC-Chip enrichment column (40 NL volume) for 3 min with 0.1% formic acid delivered by the auxiliary pump at 4 µl/min. The peptides were then reverse eluted and loaded onto the analytical capillary column (43 mm × 75 µm ID, also packed with 5 µm Zorbax 300SB-C18 particles) connected in-line to the mass spectrometer with a flow of 600 NL/min. Peptides were eluted with a 5 to 90% acetonitrile gradient over 16 min. Data-dependent acquisition of collision induced dissociation tandem mass spectrometry (MS/MS) was utilized. Parent ion scans were run over the *m/z* range of 400–2200 at 24,300 *m/z*-*s*. MGF compound list files were used to query an in-house database using Biotools software version 2.2 (Bruker Daltonic) with 0.5 Da MS/MS ion mass tolerance. Protein identification was accomplished by database search using MASCOT (Matrix science,

London, UK). Protein identifications were considered to be positive when the protein score was  $>50$  ( $p < 0.05$ ).

## Results

*S. solfataricus* naturally inhabits thermal features that can contain high levels of metals including antimony. The objective of this study was to characterize the response to Sb (III) in this archaeal hyperthermophile at the protein level. The cells were grown in batch cultures and allowed to reach an Optical Density (OD) of  $\sim 0.2$ , at which point antimony was added at three concentrations (50, 240 and 1000  $\mu\text{M}$ ). Growth curves were made to assess toxicity. The cultures exposed to Sb (III) at 240 and 1000  $\mu\text{M}$  grew more slowly, whereas 50  $\mu\text{M}$  did not change the growth rate, Figure 2.1. Our interest was to investigate cellular mechanisms behind tolerance to the metalloid. Therefore, the two lower concentrations were selected for further investigation because at 240  $\mu\text{M}$  the cells were clearly responding to the stress, but it was not completely inhibiting cell division nor causing overt killing.

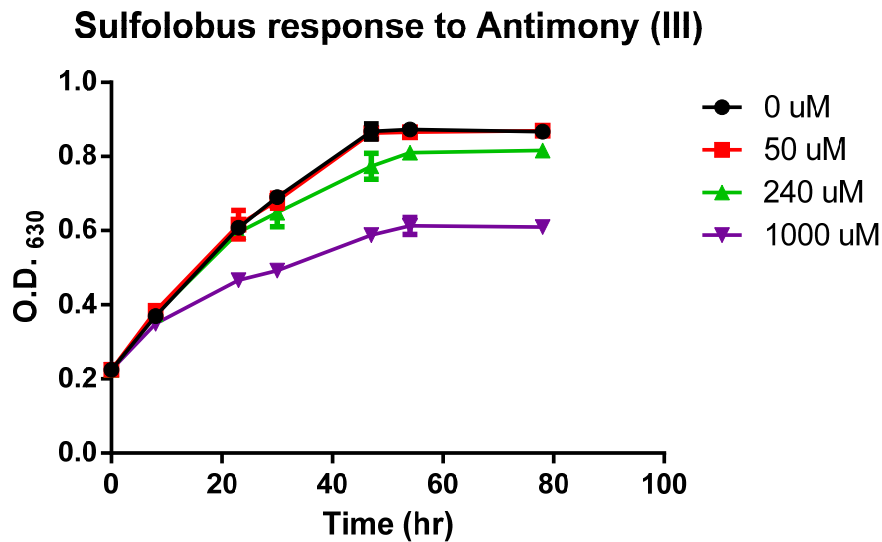


Figure 2.1: Growth of *S.solfataricus* grown in the presence of Sb (III)

#### Protein Abundance

To determine if Sb (III) exposure lead to changes in protein abundance, cells stressed with 50 and 240 Um were collected at 8 and 24 hours. Total soluble protein from three replicate experiments was minimal labeled with Zcye dyes in preparation for standard 2D-DIGE analysis (Maaty, Steffens, Heinemann, Ortmann, Reeves, Biswas, Dratz, et al., 2012). Proteins were focused on wide-range (Ph 3-11) isoelectric focusing strips and then separated using 13% SDS-PAGE. A representative 2D gel is shown in Figure 2.2. Image analysis showed that roughly 900 spots were visible on each gel. After filtering to remove irregularities, 807 spots were used in the analysis across all gels. Differences in protein spot intensities were evaluated using a 1.5 fold change and 0.05 p value. Using these criteria, only 9 protein spots changed significantly between control and Sb (III).



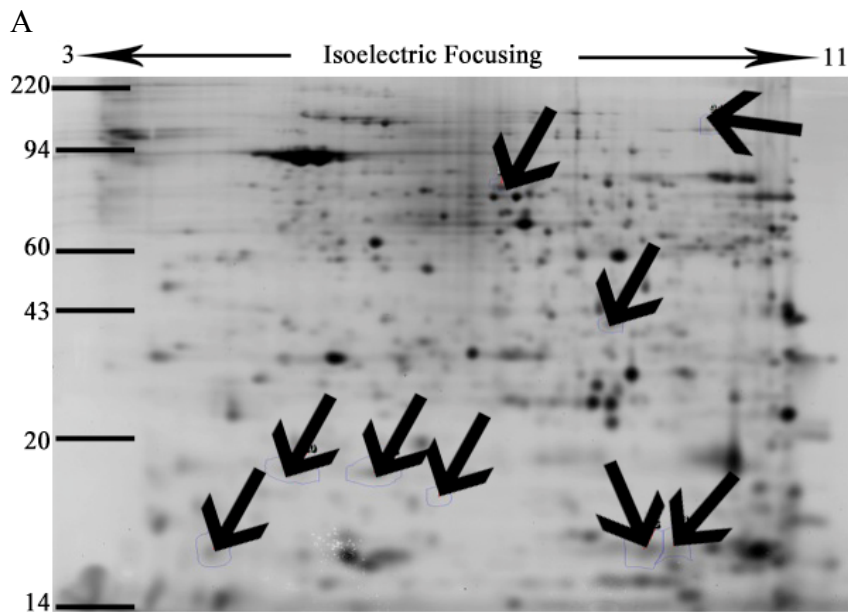


Figure 2.2. *S. solfataricus* total proteome after Sb (III) stress

To determine if the quality of the gels impacted our ability to detect changes in protein abundance, a volcano plot was constructed, figure 2.2. This shows that the average coefficient of variance for spot volume was 3.7%, indicating that the reproducibility between biological replicates and across the gels where cells were treated with 50  $\mu$ M Sb (III) (A) and cells treated with 240  $\mu$ M Sb (III) (B) was very good.

Therefore, the low number of regulated protein spots was not a technical artifact. It was concluded that exposure to levels of Sb (III) that slow *S. solfataricus* growth do not lead to large scale changes in the abundance of cytosolic proteins, suggesting that a targeted response was elicited.

B

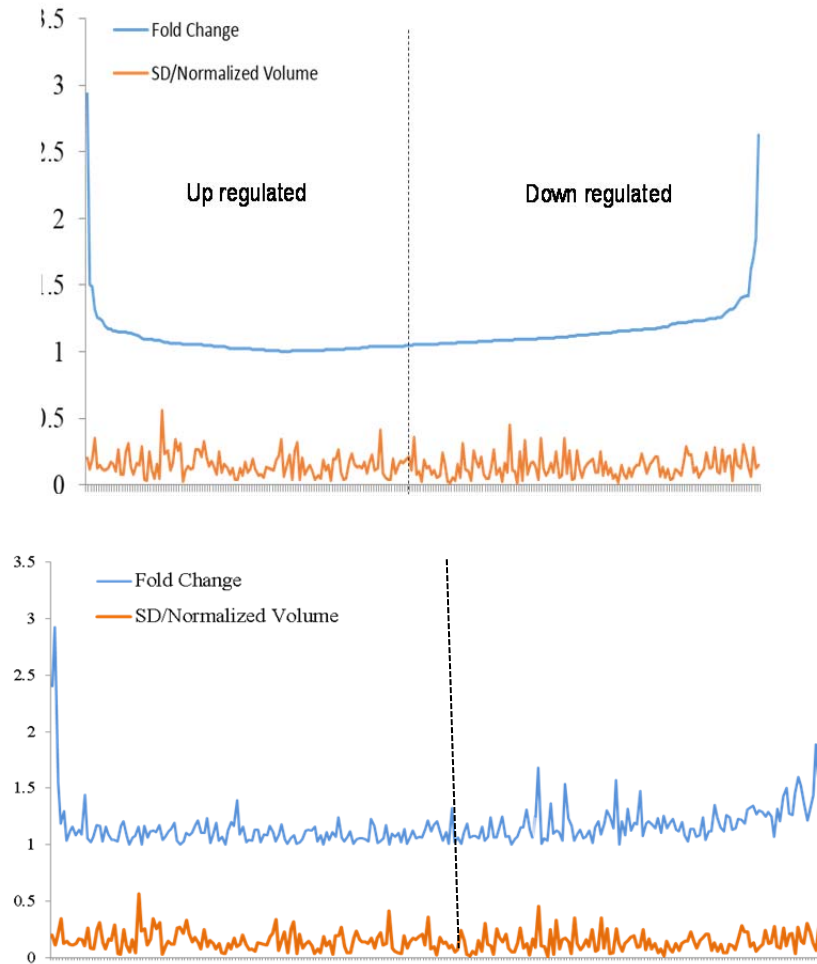


Figure 2.3. Volcano plot of 2D gel spots from cells stressed with Sb (III) 50  $\mu$ M Sb (III) (A) and 240  $\mu$ M Sb (III) (B).

To determine the identity of the proteins that did show altered abundance, gel spots were subjected to in-gel proteolysis and LCMS/MS analysis. Peptides were queried against an expanded in-house database for *SsP2*, which has approximately 5000 open reading frames (ORFs), using MASCOT (Maaty et al., 2009). The validity of all assigned MS/MS spectra used for identification of regulated proteins was confirmed by manual

inspection. From the nine regulated spots, five proteins were identified. A list of proteins, identifications scores, molecular weights, Pi, and annotation is shown in table 2.1. Identified proteins were carbon monoxide dehydrogenase, Sm-like ribonucleoprotein core, inorganic pyrophosphatase, prefoldin subunit alpha, and isochorismatase related protein. The toxicity of antimony may be due to a range of interactions at the cellular level.

Table 2. 1. Identified regulated proteins after Sb(III) stress

<b>Protein</b>	<b>MW [kDa]</b>	<b>Pi</b>	<b>SSO Number</b>	<b>Gene Identifier</b>	<b>Fold Change</b>	<b>Peptides</b>
Carbon monoxide dehydrogenase	19.1	5.7	SSO2433	<a href="#">gi 13815736</a>	2.5	1
Sm-like ribonucleoprotein core	16.5	8.9	SSO0276	<a href="#">gi 13813416</a>	2.1	4
Inorganic pyrophosphatase	19.7	4.8	SSO2390	<a href="#">gi 1453865</a>	4	7
Prefoldin subunit alpha	16.3	5.1	SSO0349	<a href="#">gi 13813493</a>	1.5	4
Isochorismatase related protein	21.2	9.3	SSO1184	<a href="#">gi 13814379</a>	1.5	2

Toxicity may result from the binding of Sb (III) to sulfhydryl groups in proteins leading to an inhibition of activity or disruption of structure (Swaran J S Flora, 2009) . Heavy metal excess may also stimulate the formation of free radicals and reactive oxygen species, which may result in oxidative stress (S J S Flora, Mittal, & Mehta, 2008) .

To explore the possible interaction between Sb (III) and membrane proteins, we designed a protocol to monitor changes in the content of reduced cysteines.

*In vivo* Labeling of Intact Cells

Thiols play a crucial role in maintaining the appropriate oxidation-reduction state of cells (Trachootham, Lu, Ogasawara, Nilsa, & Huang, 2008). The largest thiol pool *S. solfataricus* is the cysteine residues present in proteins (Heinemann et al., 2014). The specific form of the cysteine (oxidized or reduced) is critical to protein and enzyme function and is sensitive to oxidative damage. It has been shown that cysteine residues in a subset of proteins undergo oxidative modification in response to changes in the intracellular environment caused by metals (Go & Jones, 2013). To determine if thiol containing proteins were involved with the response of *S. solfataricus* to Sb (III) and if there was a global change in the oxidation state of cysteines, thiol specific chemical tagging experiments were conducted. To avoid potential artifacts introduced by cooling the cultures down and then lysing cells, a method for live cell labeling of reduced protein thiols at high temperature (70 C) was developed, Scheme 2.1. Custom made Z-Cy fluorophores which have a cystic acid side chain bearing a terminal maleimide functional group were used (Epstein, 2012; Maaty, Steffens, Heinemann, Ortmann, Reeves, Biswas, Dratz, et al., 2012). Maleimide reacts specifically with sulfhydryl groups at the Ph ~6.8. Zcy dyes have a zwitterionic structure that improves solubility in aqueous conditions, making the dye-protein conjugate less likely to precipitate during isoelectric focusing. Using this *in vivo* labeling method, we obtained information on the oxidation state of thiols from cytosolic, membrane, and membrane associated proteins in one experiment.

Proteins were labeled at 70 C. Cytosolic, membrane and membrane associated proteins were isolated. All fractions were run on 1D SDS PAGE, figure 2.4. Buffer

containing SDS was used to extract integral membrane proteins. Proteins extracts from cells labeled with Sb (III) were labeled *in vivo* with maleimide probes, after 8 and 24 hours. Figure 5 depicts membrane proteins labeled *in vivo*, after 8 hours. Unstressed cells (0  $\mu\text{M}$ ) were run as control. Image-J Software was used to generate values from protein bands. Intensity values were normalized by dividing the total concentration of the sample by the intensity of each band to normalize for any differences in loading.

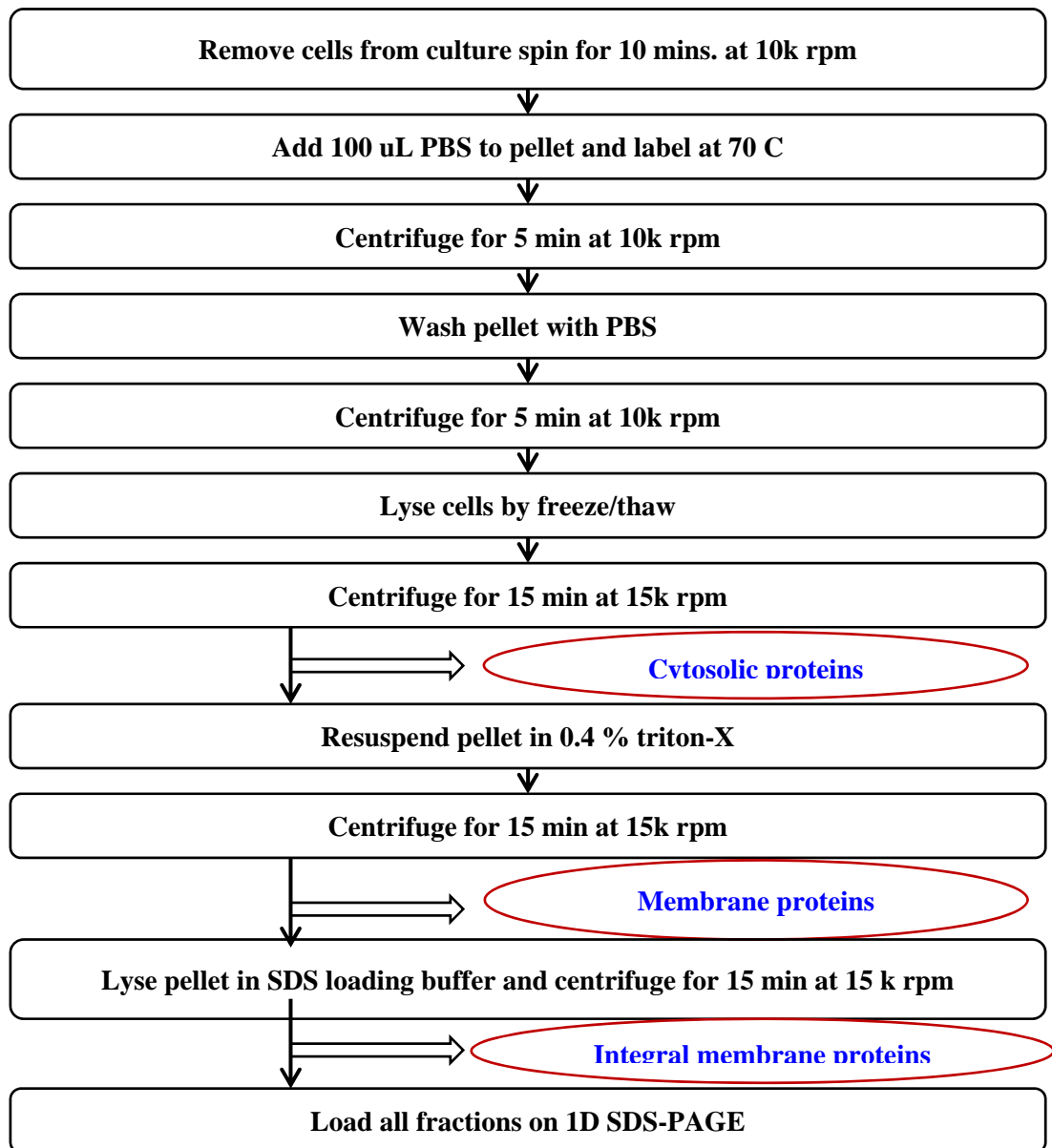
A total of 17 protein bands were excised from both gels representing samples that were treated with 50 and 240  $\mu\text{M}$  antimony, at 8 and 24 hours, for in-gel digestion. From the 17 bands, 13 proteins were identified, Table 2.2.

Some of the significantly regulated proteins were TF55-alpha protein, alpha mannosidase and elongation factor 1- alpha. TF55- alpha assists in the correct folding and assembling of other proteins (Gutsche, Essen, & Baumeister, 1999). Heat shock proteins (Hsps) are a highly conserved, ubiquitously expressed family of stress response proteins which are expressed at low levels under normal physiological conditions. Hsps can function as molecular chaperones, facilitating protein folding, preventing protein aggregation, or targeting improperly folded proteins to specific degradative pathways. A general characteristic of all chaperones and co-chaperones is their ability to reduce aggregation by “holding” misfolded polypeptides in intermediate stages of folding in order to promote refolding (Freeman & Morimoto, 1996). Hsps can intervene following oxidative stress at several levels. Firstly, some Hsps, mainly members of the Hsp70 family and its co-chaperones, play a crucial role in protein sorting and quality control by selecting and directing unusual proteins to the proteasome or lysosomes for degradation

(Mayer & Bukau, 2005). This protein was also down regulated in cells that were stressed with As.

Class II  $\alpha$ -mannosidase is another protein that was downregulated. This protein belongs to a large protein complex inside all Eukaryotes and archaea, as well as in bacteria. This class of proteins, are exohydrolases that belong to glycoside hydrolase family and hydrolyze mannosidic linkages via the retaining mechanism (Petersen, 2010). In *S. solfataricus*, there is evidence that the likely role of class II  $\alpha$ -mannosidase (ManA) is in the processing of an extracellular polymeric substance known as extracellular polymeric substances (EPS) and may also be involved with processing of N-glycans attached to S-layer proteins (Koerdt et al., 2012). The proteasomal system is a very complex comprises of a 10S core and degrades cells. It also and several-fold regulated “degrading machinery” of cells, comprising of the 20S “core” proteasome and various regulator proteins. It chores closely together with the ubiquitinating system (Jung & Grune, 2008a) . Proteasomes is the major proteolytic machinery for the removal of oxidized and misfolded proteins in the cell (Breusing & Grune, 2008; Goldberg, 2003; Jung & Grune, 2008b). In humans, proteomes play a role in the prevention of cytotoxicity induced by oxidized proteins. Proteasomal dysfunction can lead to decreased degradation of misfolded proteins, thus resulting in accumulation of oxidized proteins and subsequent protein aggregation. Protein aggregates can then feedback to further inhibit proteasome activities, form additional cellular stress, and lead to cytotoxicity and human pathologies(Ciechanover & Brundin, 2003; Dahlmann, 2007).

ManA has previously been characterized with respect to both physical properties and substrate specificity (Brouns et al., 2006). The class ManA is fully dependent on a divalent metal ion that participates in the binding of the mannosyl in site -1, preceding the scissile bond of the substrate (Shah, Kuntz, & Rose, 2008).



Scheme 2.1.. A workflow of in vivo labeling of cells and the isolation of cytosolic and membrane proteins.

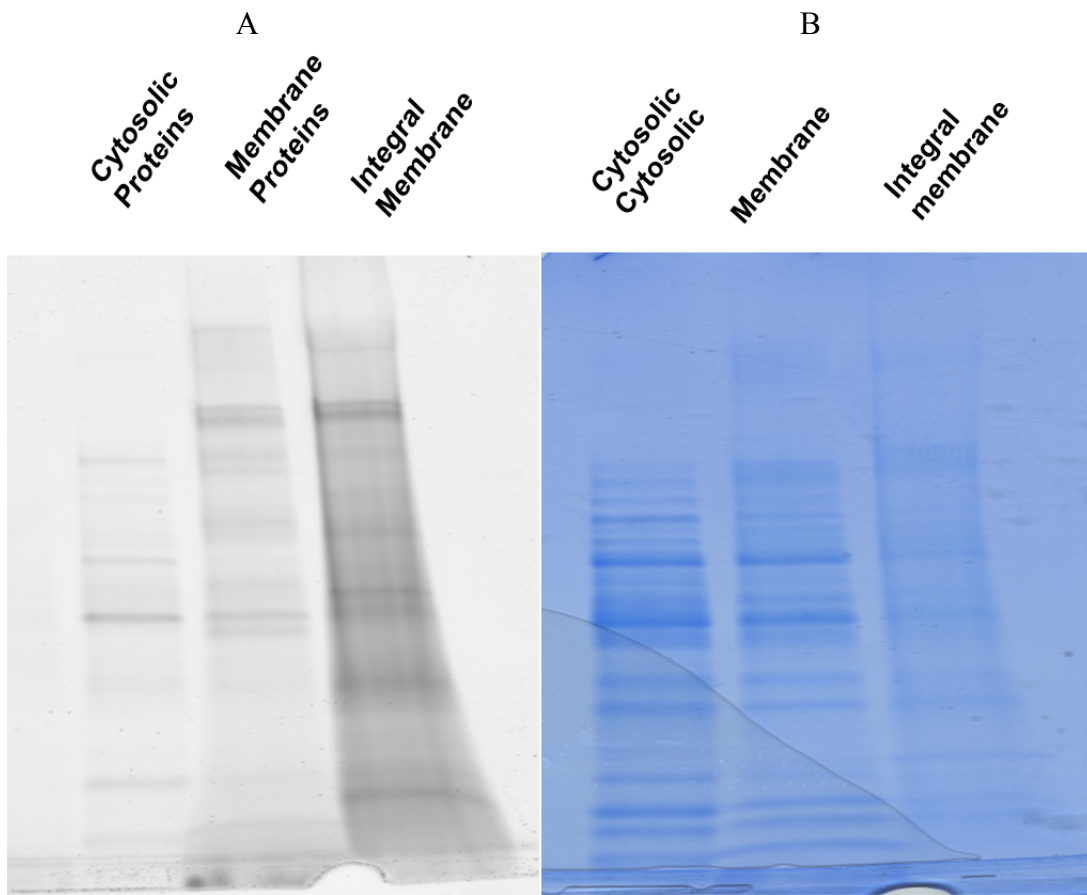
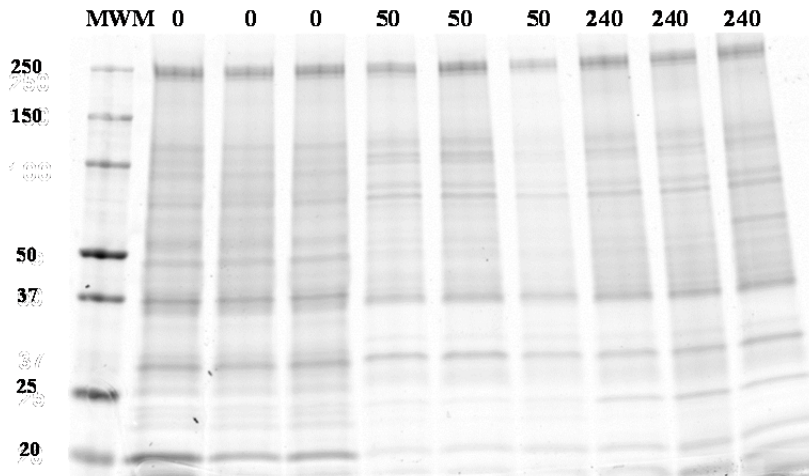


Figure 2.4 SDS PAGE of *S. solfataricus* thiols labeled with Z-Cy Maleimide probes (A) and a representative protein gel stained in coomassie protein stain (B).



A



B

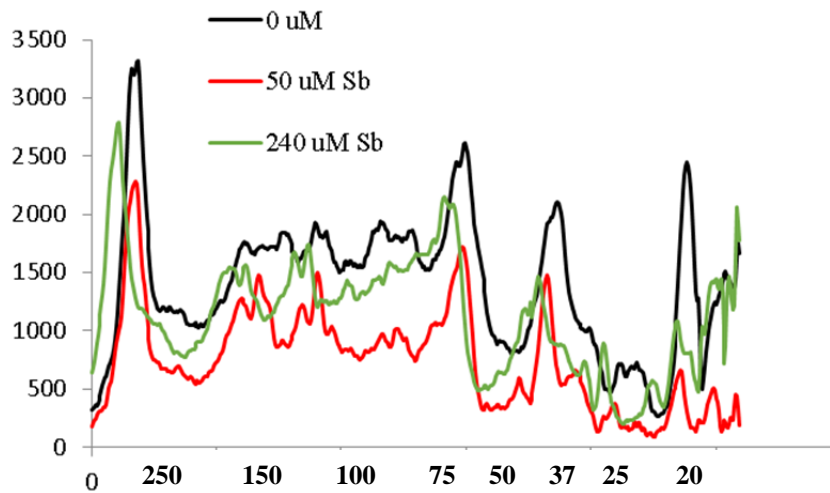
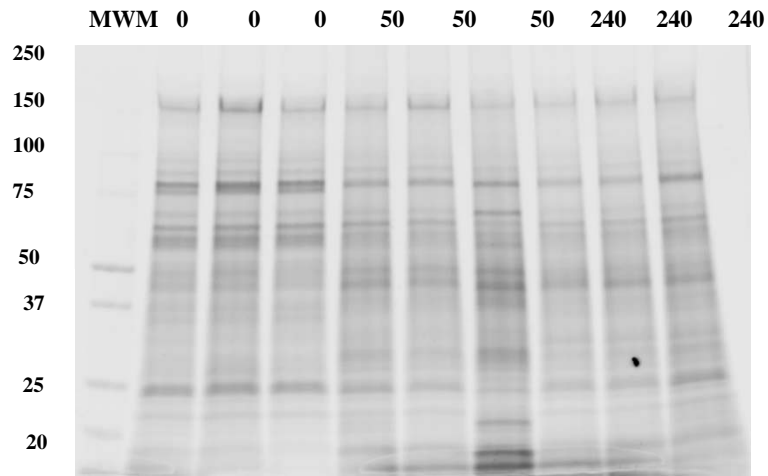


Figure 2.5 SDS PAGE gel of membrane proteins (A) labeled with Zcy5 maleimide probes *in vivo*. Cells were collected and labeled after 8 hours. Image J was used to measure the protein intensities (B). The fluorescence intensity has been normalized based protein content in each lane.

A



B

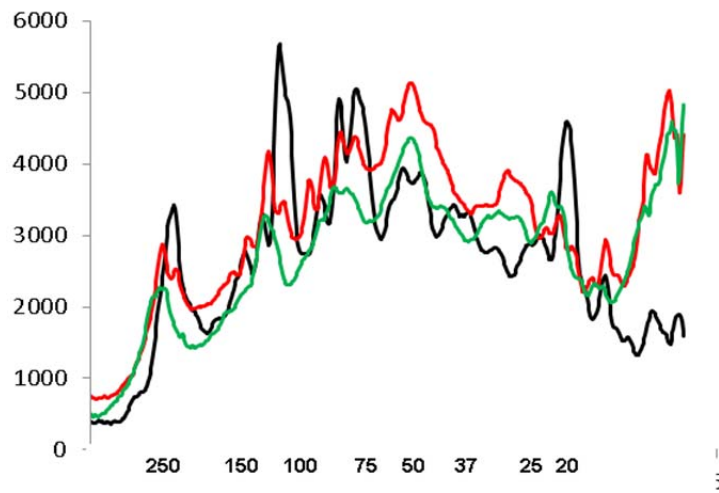


Figure 2.6. SDS PAGE gel of membrane related proteins. (A) Labeled with ZCy maleimide probes *in vivo*. Cells were collected and labeled after 8 hours. Image J was used to measure the protein intensities (B). The fluorescence intensity has been normalized based protein content in each lane.

Table 2.2. Identified differentially expressed protein bands after treating with 50 and 240  $\mu$ M Sb (III). The proteins were labeled with a thiol reactive probe.

<b>Protein name</b>	<b>SSO number</b>	<b>Mass (Da)</b>	<b>Gene Identifier</b>	<b>Peptides</b>
3-hydroxyacyl-CoA dehydrogenase/enoyl CoA hydratase	SSO2514	72993	<a href="#">gi 13815814</a>	2
Alpha-mannosidase	SSO3006	111092	<a href="#">gi 13816395</a>	2
Acetyl-CoA synthetase (acetate-CoA ligase)	SSO2863	75752	<a href="#">gi 13816221</a>	2
Elongation factor 1-alpha (elongation factor tu)	SSO2429	48459	<a href="#">gi 13813351</a>	2
Proteasome alpha subunit (N-terminus)	SSO0738	26572	<a href="#">gi 6015738</a>	2
DNA-directed RNA polymerase, subunit B $\sim$ (rpoB2)	SSO0751	54002	<a href="#">gi 6015738</a>	2
TF55-alpha protein		59633	<a href="#">gi 5930008</a>	2
Elongation factor 1-alpha (elongation factor tu) (EF-tu) (tuF-1)	SSO2429	48459	<a href="#">gi 13813351</a>	2
Citrate synthase	SSO2589	42784	<a href="#">gi 1575780</a>	2
Adenylate kinase	SSO0694	21313	<a href="#">lcl 824</a>	2

### Quantitative Determination of Free Thiols

The thiol functional group of the amino acid cysteine can experience a broad array of oxidative modifications and performs a number of physiological functions. In addition to forming covalent cross-links that stabilize protein structure and functioning as a powerful nucleophile in many enzyme active sites, cysteine a central player in redox signaling, functioning as reversible a regulatory molecular switch. The data from *in vivo* labeling indicated that the concentration of reduced thiols decreased after exposure to Sb (III), see figures 2.4 and 2.5. As an alternative to our labeling method, we used the standard method of Ellman *et al* (Courtney & Francisco, 1961). As shown in figure 2.6, it

was discovered that with the increasing concentration of antimony, the concentration of free thiols decreases, suggesting that the presence of antimony, oxidizes the proteins in *Sulfolobus*.

Members of archaea, the third domain of life, have devised mechanisms to withstand some of the most severe environments known to support life (Hirooka, Kato, Matsu-ura, Hemmi, & Nishino, 2000). *Sulfolobus species*, are usually thermoacidophilic, therefore, they can be found in severe environments. *S. solfataricus* can endure extreme temperatures, high acidity, and highly enriched metal environments.

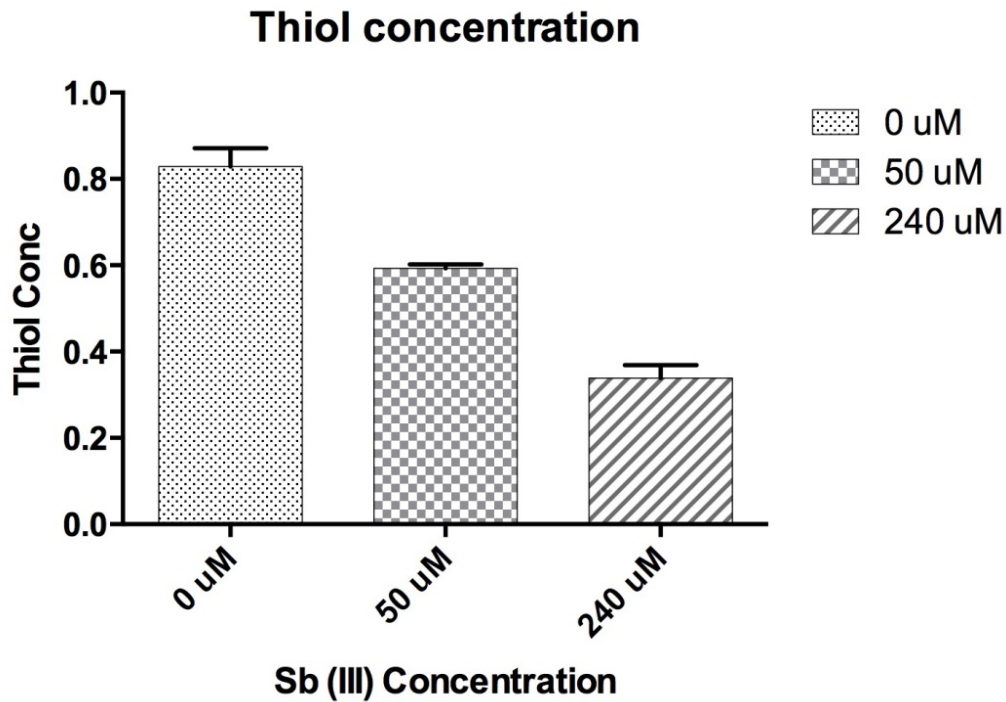


Figure 2.7. Bar graph comparing the quantity of free thiols after stressing with Sb (III) after 24 hours.

## Discussion

Members of archaea, the third domain of life, have devised mechanisms to withstand some of the most severe environments known to support life (Hirooka et al., 2000). *Sulfolobus* species, are usually thermoacidophilic, therefore, they can be found in severe environments. *S. solfataricus* can endure extreme temperatures, high acidity, and highly enriched metal environments. Metals play an important role in the life process of microorganisms. However, at high levels, both essential and nonessential metals can damage cell membranes, alter enzyme specificity, disrupt cellular functions, and damage the structure of DNA. In this study, the aim was to determine the effects of heavy metal stress on the proteome of the *S. solfataricus*.

## 2D-DIGE

We determined the effects of antimony by evaluating changes on the proteome using 2-DE. In this study, only 9 proteins showed altered abundance. Out of the nine proteins, 5 proteins could be identified by LC MS/MS. Carbon monoxide dehydrogenase, is one of the proteins that was up regulated, which has been identified to be a REDOX stress protein (Salzano et al., 2007). Carbon monoxide dehydrogenase/acetyl-CoA synthase (CODH/ACS) complex forms part of a five-subunit complex consisting of  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ , and  $\epsilon$  subunits. This complex catalyzes the reversible oxidation of CO to CO<sub>2</sub>, transfer of the methyl group of acetyl-CoA to tetrahydromethanopterin (H<sub>4</sub>MPT), and acetyl-CoA synthesis from CO, CoA, and methyl-H<sub>4</sub>MPT. The  $\beta$  subunit contains cluster A, which is a NiFeS cluster, which is the

active site of acetyl-CoA cleavage and assembly. Interactions between the CODH and the ACS subunits are required to cleavage or produce the C-C bond of acetyl-CoA. These interactions include intra-molecular electron transfer reactions between the CODH and ACS subunits. Carbon monoxide dehydrogenase is also associated with Rubrerythrin, which is a protein involved with cellular redox potential (Maaty et al., 2009). This protein was also found to be up-regulated when cells of *S. solfataricus* were stressed with Sb (III) (manuscript in preparation).

Prefoldin is another protein that was upregulated. It is a molecular chaperone found in eukarya and archaea domains and helps in the folding of newly synthesized polypeptide chains such as actin and tubulin (Siegers et al., 1999). Prefoldin also binds to proteins that have been synthesized in ribosomes and transports them to chaperonine Tric/CCT in collaboration with HSP70 and HSP40 (Geissler, Siegers, & Schiebel, 1998, Hartl & Hayer-Hartl, 2002). During stressful conditions, molecular chaperones therefore bear the responsibility of maintaining homeostasis in cells through stimulation of proper folding of proteins (Feder & Hofmann, 1999). It can be assumed that when *Sulfolobus* cells are stressed with antimony, prefoldin becomes regulated thus minimizing damage to the cells. In eukaryotes, class II  $\alpha$ -mannosidase is found in the Golgi apparatus, the cytosol, and the lysosomes, where the enzyme part takes in processing the N-glycosylated proteins and in catabolism of glycans originating from, for example, unfolded or endocytosed proteins.

Other proteins which showed significant upregulations are Sm-like ribonucleoprotein core, inorganic pyrophosphatase and isochorismatase related protein.

In the initial stages of the experiment, it was anticipated that more than 9 proteins will show significant changes in abundances.

*In vivo* Labeling of Intact Cells with  
Thiol Reactive Maleimide Probe

Since we observed such a low number of regulated proteins from the cytosol, using Z-Cy Dye minimal labels for lysines, we decided to determine if there could be any membrane proteins that could be differentially expressed. We were also interested in examining the redox state of proteins *in vivo*. We therefore came up with a protocol that labels both the cytosolic and membrane proteins *in vivo*, figure 4. We used Z-Cye dyes at a final concentration of 200 pico mol. From this experiment, 13 proteins were identified from both the cytosolic and membrane fractions,

Proteins that were identified are listed in Table 2.2. The  $\alpha$ -mannosidases are exohydrolases that belong to glycoside hydrolase family and they hydrolyze mannosidic linkages via the retaining mechanism(Howard, 1998). In eukaryotes, class II  $\alpha$ -mannosidase exist in the Golgi apparatus, the cytosol, and the lysosomes, where it participates in processing the N-glycosylated proteins as well as in catabolism of glycans which originate fromunfolded or endocytosed proteins(Herscovics, 1999). However, the function of class II  $\alpha$ -mannosidase in bacteria and archaea is more unclear, but presumably, the enzyme serves similar functions, which is supported by the discovery of protein glycosylation in prokaryotes (Messner & Sleytr, 1991) was also recently reported that the likely role of class II  $\alpha$ -mannosidase in *S. solfataricus*, is in the processing of an

extracellular polymeric substance known as EPS and maybe also in the processing of N-glycans of proteins located in the S-layer (Koerdt et al., 2012).

Temperature Factor 55 (TF55), is another protein that was differentially expressed and characterized from *Sulfolobus shibatae* (Jonathan D Trent, Nimmesgern, Wall, Hartl, & Horwich, 1991). Binding and refolding of denatured proteins have been demonstrated for TF55. In *S. shibatae*, TF55 is the major heat-shock protein observed after relocating the organism from its optimal temperature of 75°C to 88°C and, is probably liable for the acquisition of thermo tolerance (J D Trent, Gabrielsen, Jensen, Neuhard, & Olsen, 1994). Heat shock proteins (Hsps) are a highly conserved, ubiquitously expressed family of stress response proteins which are expressed at low levels under normal physiological conditions. Hsps can function as molecular chaperones, facilitating protein folding, preventing protein aggregation, or targeting improperly folded proteins to specific degradative pathways. A general characteristic of all chaperones and co-chaperones is their ability to reduce aggregation by “holding” misfolded polypeptides in intermediate stages of folding in order to promote refolding (Freeman & Morimoto, 1996). Hsps can intervene following oxidative stress at several levels. Firstly, some Hsps, mainly members of the Hsp70 family and its co-chaperones, play a crucial role in protein sorting and quality control by selecting and directing unusual proteins to the proteasome or lysosomes for degradation (Mayer & Bukau, 2005).



### Conclusion

Research has been carried out on the effects of metals on microorganisms and mostly, prokaryotes. (An & Kim, 2009; Gates, Harrop, Pridham, & Smethurst, 1997). There is evidence that antimony causes oxidative stress in those organisms however, data on the effects of antimony stress on archaea is unavailable. For the first time, this research was conducted to elucidate any differences that are brought about on the proteome of *Sulfolobus*. Our findings revealed that only a few cytosolic proteins were regulated. We also discovered that antimony does cause oxidation to some proteins. It was found that, following our *in vivo* labeling and membrane protein isolation, membrane proteins were not identified by Mass Spec. The likely explanation for this could well be that our protocol, we did not enrich our membranes. The only protein that is linked to the membranes is  $\alpha$ -mannosidase. It was reported that its likely role in *S. solfataricus*, is in the processing of an extracellular polymeric substance known as EPS and maybe also in the processing of N-glycans of proteins located in the S-layer. We also observed that some high molecular weight proteins, DNA-directed RNA polymerase- subunit B and were regulated, isolated and identified in fractions where we expected to have membrane proteins. This could be a result of contamination during the membrane proteins extraction. This could also be the result because we are not enriching out membrane proteins. Therefore, in the future, it is anticipated that we add on a step where we enrich the membrane proteins. From this data, we also learned that the mechanism of Antimony resistance by *S. solfataricus* may differ, as compared to arsenic.

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## CHAPTER 3

SULFOLOBUS SOLFATARICUS  
RESPONSE TO ARSENIC STRESS

This experiment was carried out as collaboration with Dr. Walid Maaty in the Bothner Lab. We aimed to compare the changes in the proteome when *S. solfataricus* is treated with Sb (III) to the As (III) proteome. Because Sb and As belong to group 15 of the periodic table and are often found co-habiting the same environment, it was anticipated that data and information that we obtain from As (III) research will give us insight on the toxicity levels and how microorganism responds to Sb (III). It has been demonstrated that microorganisms oxidize As (III) as a resistance mechanism towards arsenite in bacteria. It was also reported that the archaebacterium *Sulfolobus acidocaldarius* does oxidize arsenite (Sehlin & Lindstrom, 1992).

For our investigations, we included 2D DIGE changes in cysteine reactivity. We also aimed to understand a regulatory system that controls As levels in our model archaeal organism, *Sulfolobus solfataricus*.

For protein expression analyses, *S. solfataricus* cells were grown to late log phase (OD<sub>650</sub> 0.32) in DSMZ media and then divided evenly between four, 1-liter long neck culturing flasks. At OD of ~0.2, cells were stressed with 240, 1000 and 5000 uM Arsenite. At 8, 16, 24, 32, and 64 hours after addition, 50 ml aliquots were taken for protein isolation. 2DE procedure was performed on 13% SDS-PAGE gels as described earlier. All experiments were performed in triplicate, unless noted otherwise. Growth curves of the effects of Arsenite on *S. solfataricus* (P2) constructed using cultures of 75ml

in a 150ml long neck Erlenmeyer flask. These cultures were used two were treated with 240, 1000 and 5000 uM concentrations of As (III) the third acted as the control. Two samples from each flask were taken to test OD<sub>650</sub> at 8,16,24,48 and 72 hour time points, figure 3.1. High concentrations of As (III), 100 and 5000 uM, slowed down growth of *S. solfataricus*. At 16 hours, the OD of the cells stressed with 5000 uM was ~ 0.4 while those stressed with 240 uM As (III) was above 0.5.

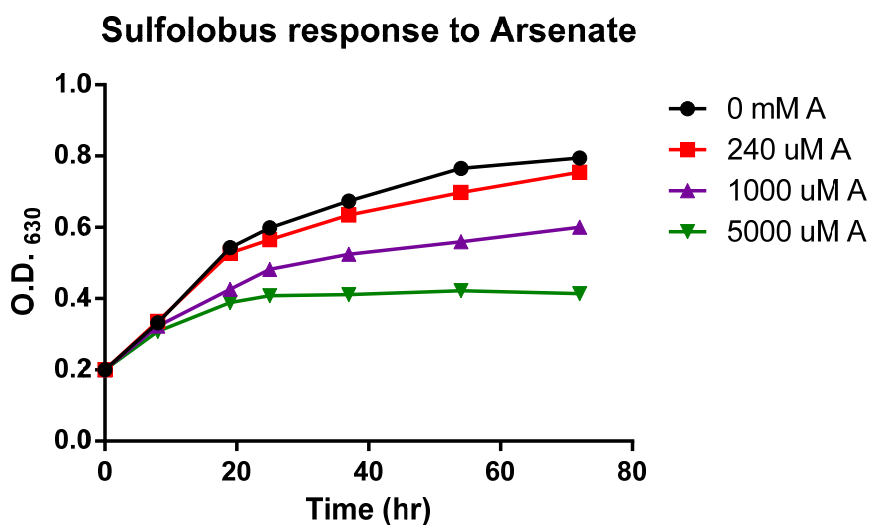


Figure 3.1: Growth curve of *S. solfataricus* grown in the presence of 50, 240 and 1000 uM Sb. Optical densities were measured between 0 and 78 hours at 650 nm.

### Protein Abundance

To determine changes in protein abundance, in the presence of As (III), cells were stressed with 240, 1000 and 5000 uM As (III). At 8, 16, 24, 32, and 64 hours after addition, 50 ml aliquots were taken for protein isolation.

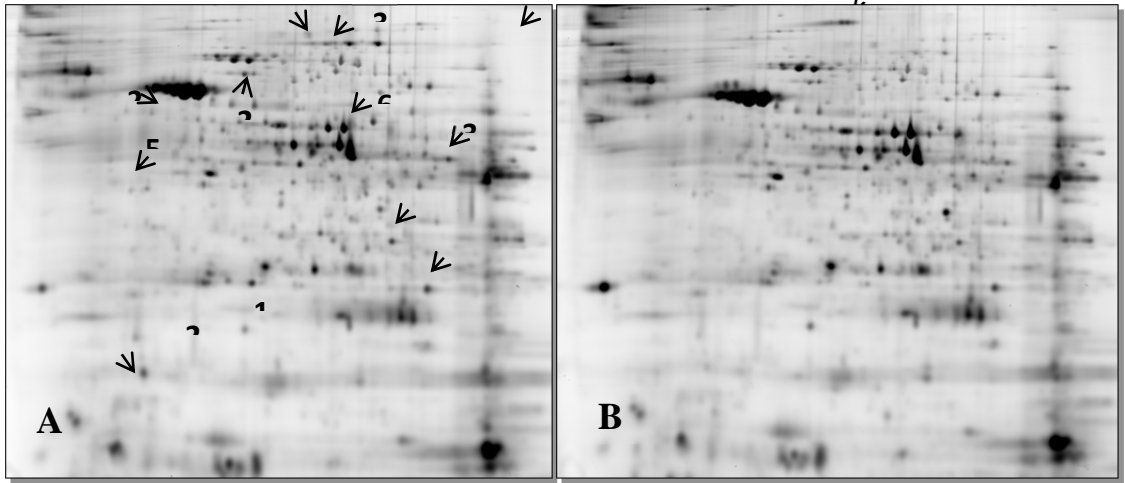


Figure 3.2: Comparison of *Sulfolobus* total proteome before and after As (III) stress. A) Control proteome before stress. B) Proteome after 8 hr stress with 240uM arsenite As(III). Approximately 700 common spots were used in the 2D-DIGE analysis. 29 protein spots changed significantly in abundance 8hr post arsenite treatment.

Figure 3.2 is a 2D-DIGE of protein extracts from cells that were stressed with 240 uM As (III), B, and cells that were not stressed, A. Approximately 700 common spots were used in the 2D-DIGE analysis. 29 protein spots showed some significant changes in abundance 8hr post As (III) treatment. Spots that changed in abundance are indicated with arrows. From the 29 regulated spots, we were able to identify 24 proteins using in-gel proteolysis followed by LC-MS/MS

Table 3.1. Identified differentially expressed protein bands after treating with 240 uM As (III).

<b>Protein Name</b>	<b>Mass kDA</b>	<b>Fold Difference</b>	<b>Peptides</b>
Geranylgeranyl hydrogenase	48.739	2.1	4
Aspartyl-tRNA synthetase	44.923	2.2	8
Geranylgeranyl hydrogenase	48.739	2.2	3
Aspartyl-tRNA synthetase	48.907	2.6	9
S-adenosylhomocysteine hydrolase	46.664	2.6	3
Pre mRNA splicing protein	39.353	2.6	2
Elongation Factor alpha 1	48.433	2.8	6
Alcohol Dehydrogenase	36.907	2.8	5
Translation initiation factor	66.839	2.8	2
Aspartate aminotransferase	45.543	3.4	3
Serine-pyruvate aminotransferase	41.951	2	4
DNA binding protein	10.967	2	3
Translation factor EF- 1 alpha	33.62	2	2
Acyl-Coa dehydrogenase related protein	33.634	2.1	8
Thiosulfatesulfuttransferase	30.175	2.1	4
exosome complex exonuclease	51.678	2.1	3
tiD Protein, putative	53.079	2.3	3
Gamma-glutamyltranspeptidase	26.573	2.8	3
Hypothetical Protein	53.079	2.4	2
isolucyl-trna synthetase	31.896	3.6	6
Tricorn protease homolog	123.038	3.6	2
Leucyl-tRNA synthetase	109.035	4.4	8
Ribonuclease PH	27.56	5.4	5
Leucyl-tRNA synthetase	109.035	5.8	5

## CHAPTER 4

## DISCUSSION AND CONCLUSIONS

For our studies, we used a model organism, *S. solfataricus*, which is found in environments with high temperature, low pH and high heavy metal concentrations. We studied the effects that Sb has on its proteome, and subsequently, genes. We also compared its effects on *S. solfataricus*, to those of As. Observing the growth patterns in figure 2.1 and figure 3.1, we found that when stressed with 240  $\mu$ M As (III), cells showed a significant slowing of growth after 24 hours with the OD of  $\sim$ 0.65. Cells stressed with Sb (III) however, had an OD reading of  $\sim$ 0.5 at the same time point. This could be an indication that Sb (III) is more toxic than As (III). This phenomenon was further observed when we carried out our proteomics studies. We initially ran 2D- DIGE for cells stressed with Sb (III). From this experiment, 29 proteins were significantly differentially expressed. With Sb (III) stressed cells however, only 9 proteins were differentially expressed ON 2D DIGE. From the 9 proteins that were regulated post stressing, the cells with only 5 proteins were identified by MS/MS. Even though antimony and arsenic are always found co-habiting, from our study, we can suggest that they do not necessarily have the same mode of toxicity. More proteins were significantly regulated when cells were treated with As (III) than when they were treated with Sb (III), table 2.1 and table 3.1.

We also developed a novel method in which cells were labeled *in vivo*. This method is able to label both the cytosolic and membrane proteins. We used our custom



made ZCye maleimide probes which are designed to label free thiols. This method is able to label live cells *in vivo*. In the process, cytosolic and membrane related proteins are labeled too. This method can be applied on all cell types. That includes archaea, eukaryotes and prokaryotes.

For our studies, one of the proteins that showed significant differential expressions are shown in table 2.2. Among the 11 regulated proteins, Heat shock protein TFF alpha protein was one of the down regulated when stressed with Sb.

Having observed significant decrease of fluorescence from isolated membranes stressed with Sb (III), we concluded that membrane proteins are the primary target of metal induced damage. We expected to identify some membrane proteins from our excised protein gels but, we were not successful. Only cytosolic proteins were identified. A possible explanation could be that there is contamination by cytosolic proteins, during the process of membrane protein isolation. Additionally, we ran out *in vivo* labeled fractions on 1 D gels. One of the disadvantages of running a one D gel is that one protein band can have more than 1 protein. We can possibly have a cytosolic and membrane protein in one band. Often, cytosolic proteins are more abundant. A possible solution for these challenges, would be to enrich our antimony treated membrane proteins. We can also run the membrane proteins on 2D SDS PAGE, where the possibility of identifying more than 1 protein is a spot, is less than in a protein band.

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