

CHEMICAL APPROACHES TO PROBE ENVIRONMENTAL
STRESS IN ARCHAEA

by

Pavel Victorovich Tarlykov

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Dr. Brian Bothner

Approved for the Department of Chemistry and Biochemistry

Dr. David Singel

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TABLE OF CONTENTS

1. LITERATURE REVIEW	1
Introduction.....	1
Archaea, the Third Domain of Life	2
<i>Sulfolobus Solfataricus</i>	4
Environmental Stress	6
2. PROTEOMIC RESPONSE OF SULFOLOBUS SOLFATARICUS P2 TO HYDROGEN PEROXIDE INDUCED OXIDATIVE STRESS	9
Introduction.....	9
Introduction to 2DE and 2D-DIGE.....	9
Introduction to Cysteine Reactivity Profiling.....	10
Introduction to Phosphoproteome.....	12
Introduction to Activity-Based Protein Profiling.....	14
Sample Preparation	15
Cell Culture and Harvesting.....	15
Extraction of Proteins	16
2D DIGE Analysis	17
Gel Imaging and Analysis.....	18
In-gel Digestion and Protein Identification.....	18
Cysteine Reactivity Profiling.....	20
Experimentation.....	20
1D HEPES SDS-PAGE	20
2DE	21
Activity-Based Protein Profiling.....	22
Experimentation	22
Results.....	22
2D DIGE Analysis	22
Cysteine Reactivity Profiling.....	28
Activity-Based Protein Profiling.....	31
Discussion	33
3. PROTEOMIC RESPONSE OF SULFOLOBUS SOLFATARICUS P2 TO ARSENIC INDUCED STRESS	41
Introduction.....	41
Materials and Methods.....	43
Results.....	45
Discussion	53

TABLE OF CONTENTS – CONTINUED

4. CONCLUSIONS	59
BIBLIOGRAPHY	63

LIST OF TABLES

Table	Page
1. List of regulated proteins in 2D DIGE experiment.....	25
2. Proteins identified from phospho-stained spots.....	28
3. List of proteins with altered cysteine availability after exposure to H ₂ O ₂	31
4. List of proteins regulated in response to arsenite exposure	48
5. Changes in protein regulation in <i>S. solfataricus</i> exposed to pentavalent arsenic	51
6. Proteins with regulation in cysteine reactivity upon exposure to As (III)	51
7. Proteins identified from phospho-stained spots (As V)	51

LIST OF FIGURES

Figure	Page
1. Simplified phylogenetic tree of three domains of life	3
2. Transmission electron microscopy image of <i>S. solfataricus</i> P2	5
3. Reduction of molecular oxygen that leads to formation of various ROS	7
4. Thiol modifications of proteins.....	11
5. Chemical structures of thiol-reactive probes used in this study	12
6. Modification of a protein by phosphorylation	13
7. Schematic representation of binding of FLISP to serine hydrolases	15
8. Proteins of the <i>S. solfataricus</i> proteome regulated in response to oxidative stress	24
9. Post translational modifications of proteins in <i>S. solfataricus</i>	26
10. <i>S. solfataricus</i> phosphoproteome	27
11. Protein thiol reactivity profile	29
12. <i>S. solfataricus</i> proteome labeled with thiol-specific dye	30
13. Fluorescent scanned 1D gel of FLISP labeled proteins of <i>S. solfataricus</i>	32
14. Fluorescent scanned 2D gels of FLISP labeled proteins of <i>S. solfataricus</i>	33
15. Functional annotation of proteins regulated in response to oxidative stress	36
16. Schematic of the oxidative stress response in <i>S. solfataricus</i>	40
17. Growth of cells in the absence and presence of different concentrations of As(III) ..	46
18. 2D gels of <i>S. solfataricus</i> proteome exposed to different concentrations of arsenite.	47
19. Growth of cells in the absence and presence of different concentrations of As(III) ..	49
20. Growth of cells in the absence and presence of different concentrations of As(V) ...	50

LIST OF FIGURES – CONTINUED

Figure	Page
21. Redox-sensitive profile of <i>S. solfataricus</i> upon treatment with As(III).....	52
22. <i>S.solfataricus</i> phosphoproteome upon exposure to arsenate	53
23. General scheme of arsenite methylation	56
24. Network of regulated <i>S. solfataricus</i> proteins.....	60
25. <i>S. solfataricus</i> proteins found to be regulated in response to both As(V) and As(III) 61	
26. <i>S. solfataricus</i> proteins that are most likely involved in universal stress response	61

GLOSSARY OF ABBREVIATIONS

ABPP – Activity-Based Protein Profiling

PAGE – Polyacrylamide Gel Electrophoresis

FLISP – Fluorescently Labeled Inhibitor of Serine Proteases

DMSO – Dimethyl Sulfoxide

IAA – Iodoacetamide

2D-DIGE – Two-dimensional Differential Gel Electrophoresis

DTT – Dithiothreitol

SDS – Sodium Dodecyl Sulfate

ROS – Reactive Oxygen Species

P – Inorganic Phosphate

LC-MS/MS – Liquid Chromatography- Mass Spectrometry/Mass Spectrometry

MW – Molecular Weight

PB – Phosphate Buffer

CHAPS – 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate

ASB-14 – Amidosulfo betaine-14, 3-[N,N-Dimethyl(3-myristoylamino propyl)ammonio]propanesulfonate

IPG – Immobilized pH Gradient

TRIS-HCl - 2-Amino-2-(hydroxymethyl)-1,3-propanediol, Hydrochloride

ROI – Region of Interest

ABSTRACT

Little is known about strategies and mechanisms employed by thermophilic organisms to adapt to environmental stress. *Sulfolobus solfataricus* is a thermophile that belongs to Archaea, the third domain of life, and can be found in unusual habitats, such as the hot springs of Yellowstone National Park. This archaeon can tolerate high temperature, extreme acidity and high concentrations of heavy metals and other toxic substances. Thus, *S. solfataricus* has been chosen by many researchers as a model system for biochemical, structural, and genetic studies. In this work *S. solfataricus* has been exposed to hydrogen peroxide as a natural mild oxidant and arsenic as a common toxic metalloid. One of the aims was to quantitatively define the regulation of proteins upon treatment with hydrogen peroxide or arsenic species in different time periods and concentrations. In this sense, two-dimensional gel electrophoresis approach in conjunction with novel chemical tagging probes has been applied to detect changes on the level of regulation and chemical modification of individual proteins within the whole proteome in response to the stressors. Proteins expression levels have been monitored, redox-sensitive and phosphoproteomic profiles of the *S. solfataricus* proteome have been identified. Synthesis of the results has allowed a general scheme for how *S. solfataricus* fights H₂O₂- and As-induced stress. Lists of mapped proteins have been created and potential biomarkers for oxidative stress have been identified, which can guide further research to better understand mechanisms of proteomic response to the environmental stress in Archaea on the example of thermophilic archaeon *S. solfataricus*.

CHAPTER 1

LITERATURE REVIEW

Introduction

We live in the era of technological progress when science is blooming and new branches of knowledge are emerging. Tremendous amounts of information and cutting-edge technologies present us with a unique chance to decipher some of the biggest mysteries of life and evolution. Nowadays, it is well established that biological events arise from complex networks of molecular interactions. The desire to understand biological processes and realization of their complexity have lead to the development of global approaches to studying organelles, cells, and organisms. Genomics and proteomics are two approaches that allow cellular events to be studied from a global perspective. The knowledge of full genomes and comparative studies based on this knowledge has created the possibility to tell us about evolutionary relationship among different species and the components that are coded in the genes to carry out cellular processes such as transcription or DNA replication. In turn, proteomic analysis studies the “workhorses” of the cell. The later is a more challenging task due to the functional and structural diversity of proteins and the dynamic nature of the proteome. In this study, a proteomic approach has been used to investigate and characterize global changes in *Sulfolobus solfataricus* P2 upon exposure to a heavy metal and an oxidative stress. Standard proteomic techniques, such as two-dimensional differential gel electrophoresis (2D-DIGE), in conjunction with more novel activity-based chemical tagging were used.

Archaea, the Third Domain of Life

Until late 1980's, all organisms were classified into one of the two domains of life: prokaryotes or eukaryotes. The situation began to change in 1977, when it was first proposed using phylogenetic analyses based on sequences from small-subunit ribosomal RNAs that a certain group of organisms did not belong to either of the existing domains (Woese and Fox 1977). Further analysis revealed distinct features such as the unusual chemical composition of cell walls (Kandler and König 1998) and unique DNA dependant RNA polymerases (Zillig, Klenk et al. 1989). Eventually, this led to a major reclassification that placed many of the prokaryotic organisms that are found in extreme environments into a new domain, the Archaea. In general, distinctions among the domains of Bacteria, Eukarya, and Archaea are based on differences in genetic and molecular characteristics. Archaea are prokaryotes but on the molecular level they are neither Bacteria nor Eukarya (Woese 1990). Typical environments in which they can be found include hot springs, salt lakes, hydrothermal vents, sewage, acid mine drainage soil and many other habitats (Chaban 2006). It has been shown that in general the mechanisms of transcription, DNA repair and replication in Archaea are very similar to that of Eukarya, but with their own unique features (Qureshi 1997; Bell 1998; Bell 1998; van der Oost 1998). At the same time, the translation machinery of Archaea tends to be homologous to that of bacteria (Bell 1998; van der Oost 1998). Archaea appeared to possess a bacterial-like compact chromosomal organization with clustering of genes as polycistronic units (operons), and only a few interrupted genes (e.g. introns). The

Archaeal domain is currently divided into four phyla: Crenarchaeota, Euryarchaeota, Korarchaeota and Nanoarchaeota (Figure 1).

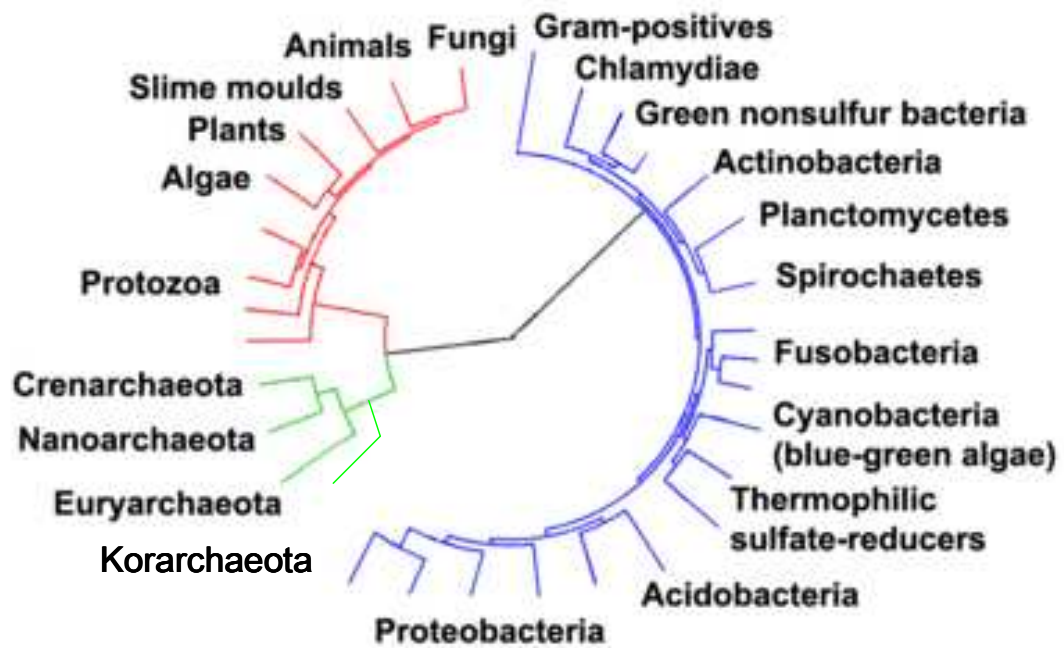


Figure 1. Simplified phylogenetic tree of three domains of life based on small-subunit ribosomal RNA sequence. Eukaryotes are colored red, Archaea green and bacteria blue. Adapted from Ciccarelli (Ciccarelli, Doerks et al. 2006).

To date, most of the species to be investigated are members of the crenarchaea and euryarchaea. The Crenarchaeota members are mostly thermophilic or hyperthermophilic organisms while the Euryarchaeota are generally methanogens. The Korarchaeota represent a distinct group based primarily on 16S rRNA sequences. Finally, the Nanoarchaeota are symbiotic hyperthermophilic organisms that have minimal genomes (Huber 2002). The Crenarchaeota, although not obligate extremophiles, thrive in environments characterized by temperatures above 75°C and pHs below 4.0. Within the Crenarchaeota, the *Sulfolobus* genus has emerged as a model organism because it

can grow aerobically and heterotrophically to high cell densities and its genome was one of the first complete archaeal genomes to be sequenced and published (She 2001).

Sulfolobus solfataricus

Sulfolobus solfataricus is an extremophile member of the Archaea, has been used by many researchers in biochemical, structural, and genetic studies to investigate how Archaea tolerate high temperatures, extreme acidity, toxic substances, and high concentrations of heavy metals (Edgcomb, Molyneaux et al. 2004; Golyshina and Timmis 2005; Remonsellez, Orell et al. 2006; Auernik, Cooper et al. 2008). Due to these efforts, *S. solfataricus* is now one of better understood archaea and is emerging as a model system for archaeal and extremophile research. Investigations into mechanisms employed by Archaea in response to extreme environmental factors promise to provide information about molecular “guardians” that are required for this adaptation.

S. solfataricus (Figure 2) usually grows at temperatures of 60°C to 85°C within a pH range from 1 to 4 and inhabits environments like solfataric hot springs where elemental sulfur is abundant. The *Sulfolobus* cell envelope is composed of a cytoplasmic membrane combined with a firmly attached S-layer of arrayed proteins on its surface (Kandler and König 1998). This type of membrane was found to be a good insulator (Hanford and Peebles 2002) and is probably responsible for the observed low permeability to ions. A large pH gradient between the inside and the outside of the cell can be established and this is exploited to generate energy via ATP synthase (She 2001).

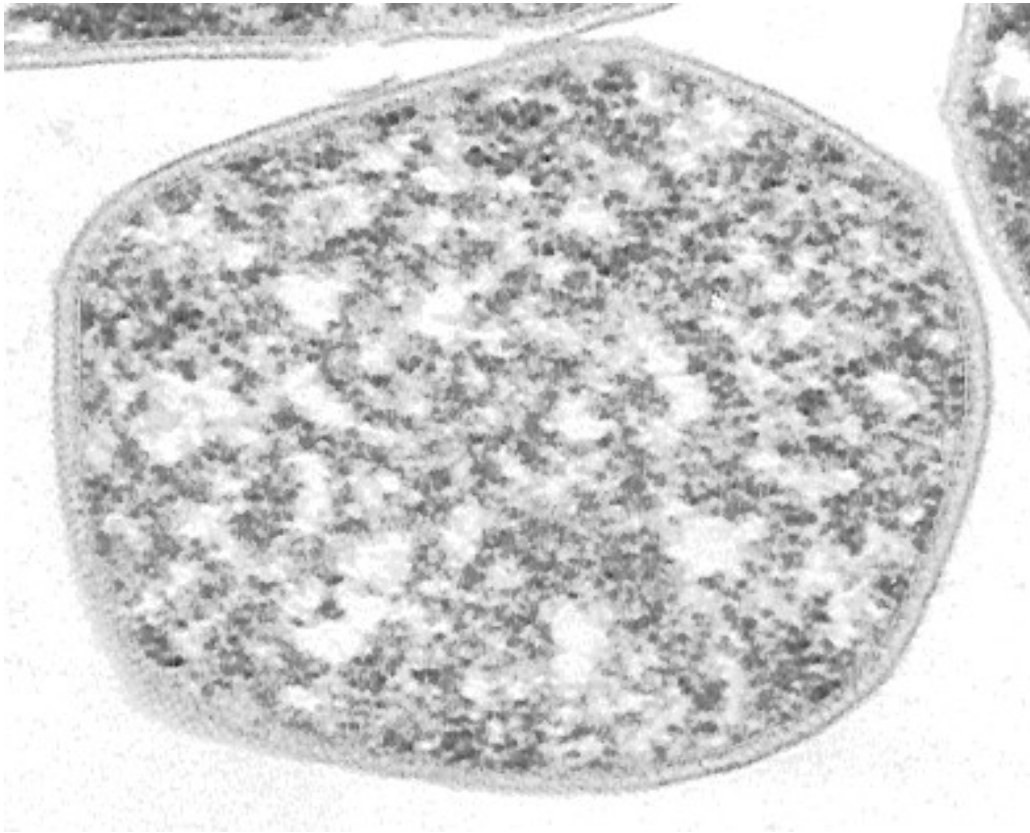


Figure 2. Transmission electron microscopy image of *S. solfataricus* P2.

S. solfataricus is an aerobic organism with a heterotrophic life style (Ciaramella 2002; Snyder 2003). The genome was sequenced in 2001 and contains 2,992,245 base pairs on a single chromosome and encodes approximately 3000 proteins and many RNAs (She 2001). Based on amino acid homology, 40% of the predicted proteins were homologous to other Archaea, 12% were exclusive to bacterial proteins and 2.3% to eukaryotic proteins. The total G+C content of the genome is 36% and with respect to the open reading frames (orfs), the usage of alternate start codons is significant (DeVendittis and Bocchini 1996). Presence of unique class of introns in the preRNA is another important characteristic of this crenarchaeon and several other thermophilic species

(Watanabe, Yokobori et al. 2002). In addition to the circular genome, other genetic elements may exist in *Sulfolobus*, including plasmids and viruses (Snyder 2003).

Environmental Stress

Specific environmental factors such as elevated temperatures, UV radiation and high concentrations of heavy metals are common agents of cellular stress (Dowling, O'Driscoll et al. 2006). The analysis of an organism's proteome allows the detection of changes on the level of individual or groups of proteins and modified proteins in response to environmental stressors. This potentially leads to the discovery of biomarkers indicative of exposure and helps to gain insights into mechanisms of toxicity and resistance to stress. Bacteria, yeast and mammalian cells have evolved strategies combat such insults and *S. Solfatarius* is not an exception. However, only a few studies have been conducted to investigate stress response pathway mechanisms in Archaea.

Oxygen, as a terminal electron acceptor, is a major player in the process of aerobic respiration. Nevertheless, along with its beneficial properties, oxygen is able to form reactive oxygen species (ROS), including superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), among others. Many characterized antioxidant proteins target the degradation of either superoxide or hydrogen peroxide. ROS can damage cells by inactivating proteins, damaging nucleic acids, and altering fatty acids of the lipids that compose cell membranes (Halliwell and Gutteridge 1990; Farr and Kogoma 1991). For example, in the presence of oxygen, ferrous ion (Fe^{2+}) readily generates aforementioned ROS that damage biomolecules, and are toxic to cells (Figure 3). The damaging effects of

oxygen are not unique to aerobic organisms and all organisms must deal with deleterious properties of ROS. For example, the contents of anaerobic cells are highly reduced, and by oxidizing such essential biomolecules as thiols and iron-sulfur proteins, oxygen can halt metabolism.

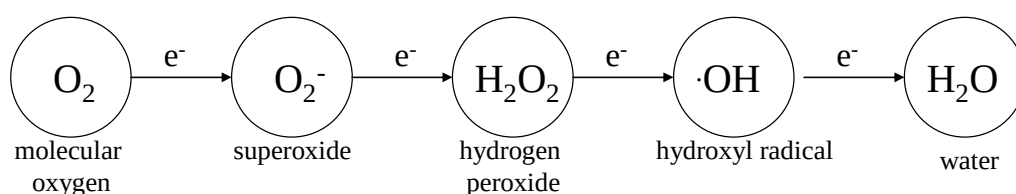


Figure 3. Reduction of molecular oxygen that leads to formation of various ROS.

What are the defense mechanisms against oxidative damage? Cellular defense mechanisms to fight oxidative damage include antioxidative enzymes and small molecules (e.g. superoxide dismutases, catalases, peroxidases, thioredoxins, peroxiredoxins and glutathione). Extensive research has been done to investigate the role of these enzymes in the detoxification of ROS within eukaryotes and bacteria (Storz and Imlay 1999; Touati 2000). In addition to this, recent work has suggested that early responses to oxidative stress involve reversible and irreversible modifications of selected protein thiols, which play important role in redox signaling (Stamler and Hausladen 1998; Stamler, Lamas et al. 2001; Missall, Lodge et al. 2004; Camerini, Polci et al. 2005; Sun, Steenbergen et al. 2006). Two common generators of ROS are H_2O_2 and heavy metals.

Hydrogen peroxide is an oxidant, stabilized by the oxygen-oxygen bond. Fluctuations in H_2O_2 levels affect the intracellular activity of key signaling components

including protein kinases and phosphatases (Rhee 2003). H_2O_2 can also be generated in the cellular response to ultraviolet radiation (Finkel 1998).

Arsenic is one of the most common toxic substances in the environment and geothermal environments are not an exception (Gihring and Banfield 2001; Langner, Jackson et al. 2001; Macur, Jackson et al. 2004). Bacterial and eukaryotic resistance to toxic substances has been well studied. Generally, in bacteria resistance is accomplished by enzymatic reduction and energy-dependent efflux of toxins through membrane protein pumps (Silver 1996). Eukaryotic cells have a tendency to resist metal toxicity by inducing the expression of heat-shock proteins (HSP), with arsenite being the biggest inducer of HSPs in several organs and systems (Del Razo, Quintanilla-Vega et al. 2001). Arsenic is not a metal, it is rather a metalloid. Nevertheless, there is significant evidence from mammalian studies that exposure to inorganic arsenic results in the generation of ROS through the conversion of arsenate to arsenite (Lynn, Shiung et al. 1998; Flora 1999; Hartley-Whitaker, Ainsworth et al. 2001). Arsenic resistance systems of Archaea on the level of proteome have not received much attention yet.

The goal of this research project was to identify key players of the *S. solfataricus* P2 proteome that are part of cellular response pathways activated by common environmental stress conditions. Hydrogen peroxide has been used as a natural mild oxidant. Another set of experiments was conducted using two different arsenic species (As (III) and As (V)) that are commonly present in the natural habitats of *S. solfataricus* at concentrations that are toxic to many other organisms.

CHAPTER 2

PROTEOMIC RESPONSE OF SULFOLOBUS SOLFATARICUS P2 TO HYDROGEN PEROXIDE INDUCED OXIDATIVE STRESS

Introduction

In order to understand proteomic response of *S. solfataricus* to oxidative stress, classical proteomics and mass spectrometric methods were applied to separate and resolve large number of *S. solfataricus* proteins and detect changes in protein expression patterns.

Introduction to Two-Dimensional Electrophoresis and 2D-DIGE

Conventional two-dimensional electrophoresis (2DE) enabled separation of complex protein samples in two dimensions, where proteins are separated by their isoelectric point in first dimension and by molecular weight in the second dimension. The proteins are distributed over a gel strip that contains a preformed pH gradient. An electric current is passed through the gel, creating a positive and negative end. The proteins move along the gel and accumulate at their isoelectric point where the overall charge on the protein is zero. Before the second dimension, that is a separation by mass, the proteins are treated with sodium dodecyl sulfate (SDS) along with urea and other reagents. This treatment denatures proteins and coats them with uniform negative charge due to binding of SDS. SDS forms negatively charged SDS-protein complexes, where the amount of negative charge bound is directly proportional to the mass of the protein. In second dimension, an applied electric potential causes the proteins to migrate toward the positive

side of the polyacrylamide gel, proportionally to their mass-to-charge ratio.

Polyacrylamide gel represents molecular sieve and is composed of crosslinked polyacrylamide chains that have absorbed a lot of solvent. As a result of this sieving process, the proteins can then be detected on the gel by a variety of stains.

Introduction of highly sensitive fluorescent cyanine dyes adapted to 2D, the well-known techniques of polyacrylamide gel electrophoresis, followed by isoelectric focusing, have been greatly improved. This approach, called 2D-DIGE, relies on pre-electrophoretic labeling of samples with one of three spectrally distinct fluorescent dyes (Unlu, Morgan et al. 1997; Tonge, Shaw et al. 2001). Up to three samples prelabeled with these dyes can be simultaneously run on the same gel, eliminating the gel-to-gel experimental variation issue. The inclusion of an internal standard pooled from all samples allows normalization across all gels. The samples can be visualized individually by scanning the gel at different wavelengths. Image analysis software packages can then be used to generate volume ratios for each spot between each channel, which allows quantification of expression differences.

Introduction to Cysteine Reactivity Profiling

The redox status of cells can be studied from global proteomic perspective by comparing the level of chemical labeling before and after exposure to the stressors. It is well-established that, because of the nucleophilicity, sulfhydryl groups on cysteine residues are very sensitive to changes in the redox level of the cell. It means that reactivity of individual protein thiols to oxidation can be part of signal transduction pathways (Leichert and Jakob 2004). Hydrogen peroxide itself is a mild oxidant and is

relatively inert to most biomolecules. However, hydrogen peroxide is able to oxidize cysteine residues in proteins to cysteine sulfenic acid or disulfide, both of which are readily reduced back to cysteine by various cellular reductants (Figure 4).

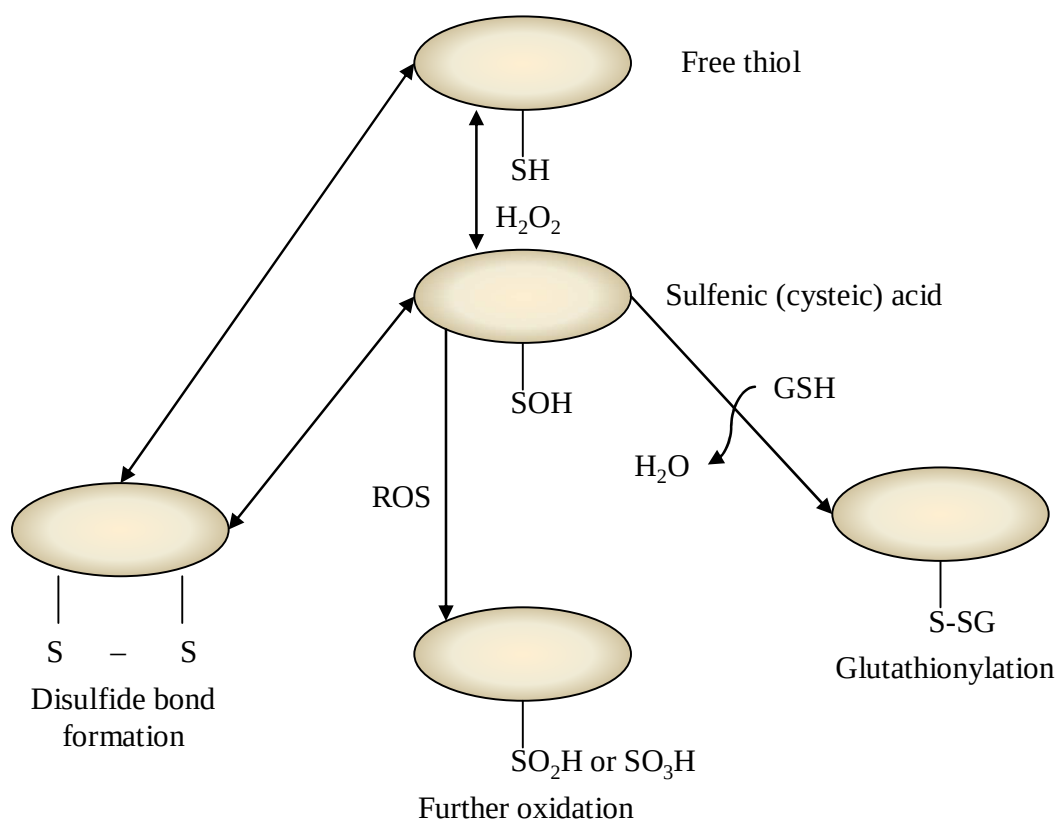


Figure 4. Thiol modifications of proteins. Formation of sulfenic acid from the reaction of H_2O_2 with protein free thiols leads to different protein modifications. In proteins without second sulfhydryl group, sulfenic acid may react with ROS to generate further oxidized sulfinic ($-\text{SO}_2\text{H}$) and sulfonic ($-\text{SO}_3\text{H}$) derivatives. Alternatively, a disulfide bond can form between the two sulfur atoms. In addition, sulfenic acid may react with glutathione or glutathione-like molecule, leading to a mixed disulfide.

There are several novel chemical probes that can specifically label free, unmodified protein thiols which can be used to attach a fluorescent tag (Figure 5). Some of them are very hydrophobic, like BODIPY 577/618 maleimide. There is likely to be considerable aggregation of bulky hydrophobic dye molecules that interferes with

isoelectric focusing of proteins making this particular dye unsuitable for 2D electrophoresis. In turn, BODIPY FL-maleimide seems to be compatible with 2DE experiments.

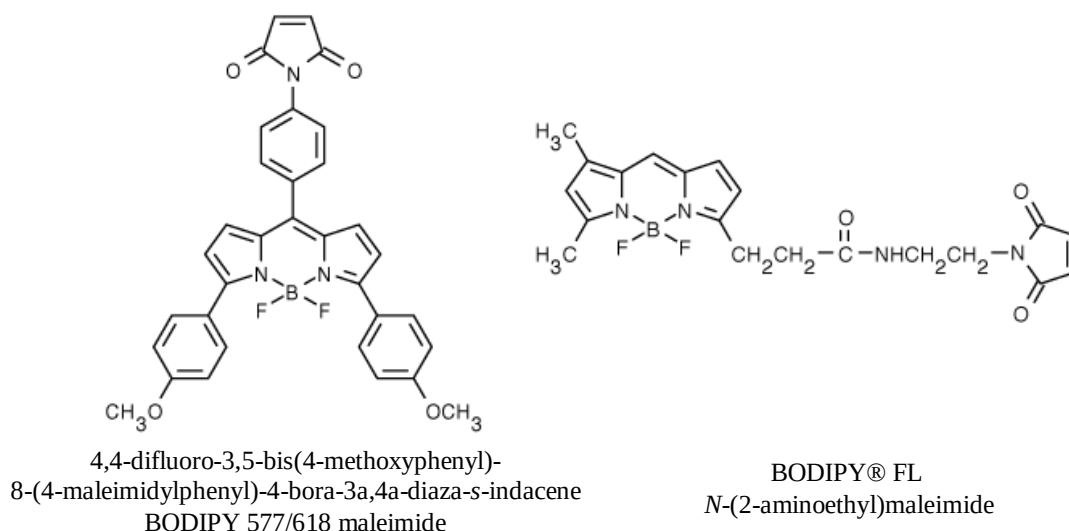


Figure 5. Chemical structures of two thiol-reactive probes used in this study.

Introduction to Phosphoproteome

Although originally believed to be a eukaryotic phenomenon, protein phosphorylation is now known to occur in all three domains of life and it is considered as a most common post-translational modification. The phosphorylation status of a cell's phosphoprotein population is controlled by the regulated activities of protein kinases and phosphatases that catalyze phosphorylation and dephosphorylation, respectively (Figure 6). Knowledge of properties of the protein phosphorylation-dephosphorylation events present in the members of the Archaea is scarce.

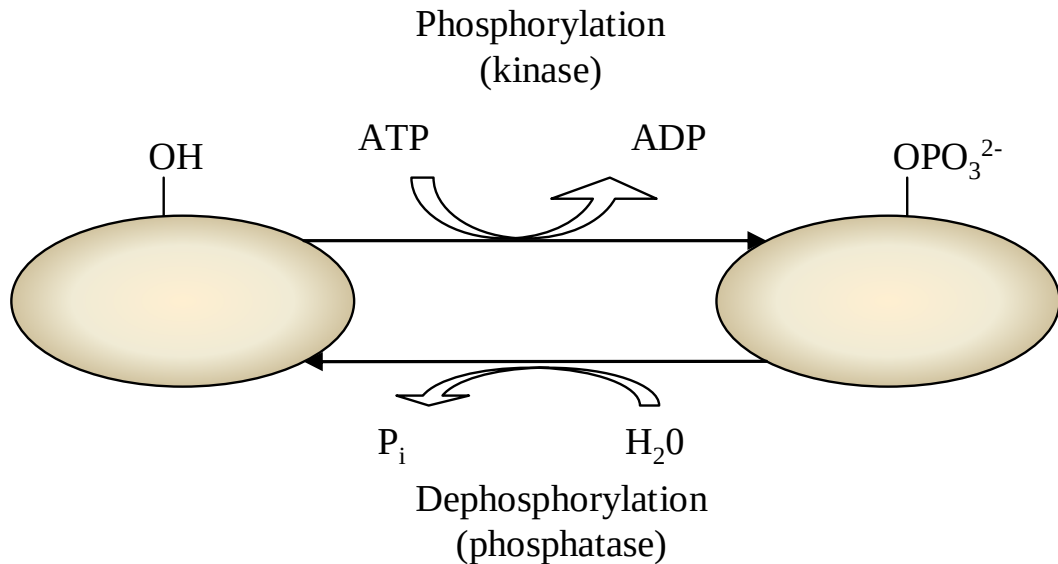


Figure 6. Modification of a protein by phosphorylation. Protein kinases catalyze the transfer of a phosphoryl group from ATP to the acceptor protein. Protein phosphatases catalyze the hydrolysis of the phosphoamino acid, releasing inorganic phosphate.

Serine, threonine and tyrosine residues are the most common targets for phosphorylation. The addition or removal of a phosphate group changes protein pI, resulting in a horizontal shift on a 2D gel. Thus, it is typical to observe a “train” of post-translationally modified protein on a gel. However, unambiguous mapping of phosphorylated proteins on a proteome-wide scale is a challenge even by mass spectrometry, partially due to a huge variety of other PTMs (e.g. glycosylation, carbonylation, cysteine oxidation). A fluorescent phosphoprotein specific stain, Pro-Q Diamond[®] (Molecular Probes) is a stain that has the ability to tag phosphorylation sites on a proteome-wide scale (Schulenberg, Aggeler et al. 2003). The mechanism of labeling is proprietary.

Introduction to Activity-Based Protein Profiling

As a result of creation of new therapeutic drugs researchers has been designing many small molecules that bind tightly within the active site of a protein. The ability of these molecules to react only with active forms of enzyme has led to invention of new family of chemical probes, called activity-based probes. ABPs have been designed for many families of enzymes and with different properties. Currently, probes exist for serine hydrolases (Liu, Patricelli et al. 1999; Kidd, Liu et al. 2001), cysteine hydrolases (Greenbaum, Arnold et al. 2002; Schmidinger, Hermetter et al. 2006), cysteine proteases (van Swieten, Maehr et al. 2004; Kato, Boatright et al. 2005; Gredmark, Schlieker et al. 2007), metalloproteases (Freije and Bischoff 2003; Chan, Chattopadhyaya et al. 2004; Saghatelian, Jessani et al. 2004), tyrosine phosphatases (Kumar, Zhou et al. 2004; Liang, Kumar et al. 2007; Zhang, Chen et al. 2007), serine phosphatases (Schmidinger, Hermetter et al. 2006), serine proteases (Sieber, Mondala et al. 2004; Fonovic, Verhelst et al. 2007), and many other families. They have been used to label active enzymes in cell lysates, whole cells, tissues, and organisms. Probes can be obtained with fluorescent or biotin tag.

In this study, one of the novel fluorescently labeled chemical probes, FAM-Phe-DAP (5(6)-carboxyfluorescein-L-phenylalanyl diphenyl alkane phosphonate ester), has been used to map serine hydrolases. A common feature of serine hydrolases, which comprise proteases, lipases and esterases, is the active site consisting of the catalytic triad Ser-His-Asp/Glu (Figure 7), that participates in the formation of a transient acyl-enzyme complex between substrate and protease (Rawlings and Barrett 1994; Wong and Schotz

2002). Hydrolases are critical to the maintenance of cellular function. They hydrolyze both external and internal nutrient sources and recognize and break down unneeded or abnormal polypeptides, that are produced as a result of environmental stress (Tomoyasu, Mogk et al. 2001). Global profiling of serine hydrolase activity in hyperthermophiles has not been undertaken yet.

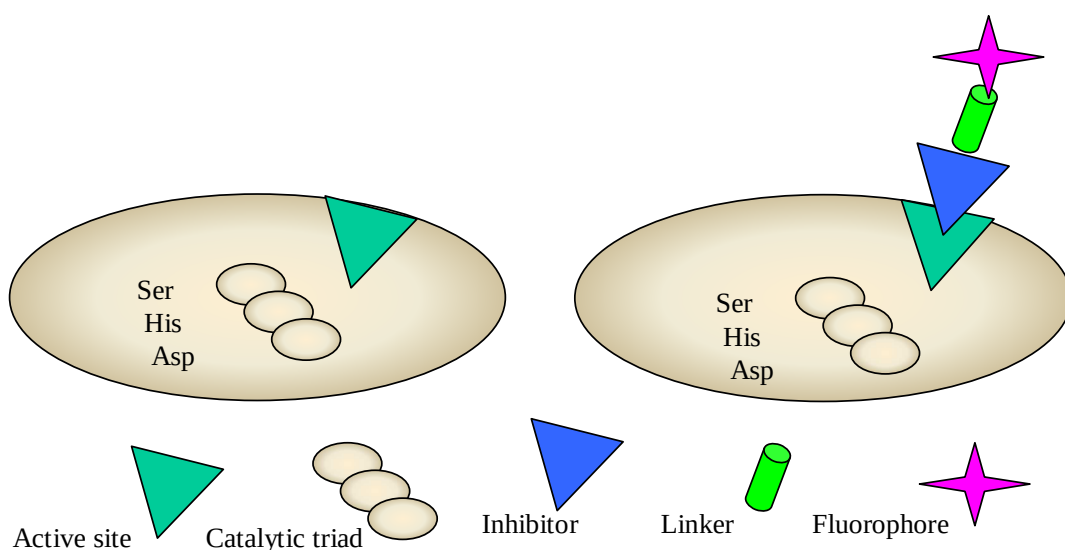


Figure 7. Schematic representation of binding of fluorescent inhibitor of serine hydrolases (FLISP) to the respective enzyme. Serine, histidine and aspartic/glutamic acid are present in the active site of serine hydrolase family. The specific fluorescent inhibitor binds covalently to the active site of respective enzyme.

Sample Preparation

Cell Culture and Harvesting

Liquid cultures of *S. solfataricus* (P2), were grown aerobically in DSMZ media 182 (22.78 mM KH_2PO_4 + 18.90 mM $(\text{NH}_4)_2\text{SO}_4$ + 0.81 mM MgSO_4 + 1.7 mM CaCl_2 + 0.2% Yeast Extract) pH adjusted to 2.8 with 6N H_2SO_4 . All cultures were grown in long neck Erlenmeyer flasks at 80°C. Hydrogen peroxide was added to stress cultures, to a

final concentration of 30 μ M. Hydrogen peroxide concentrations were determined using the molar extinction coefficient (43.6M⁻¹cm⁻¹) at 240nm.

Three liters of DSMZ media 182 (pH~2.8) was inoculated with 15 ml of a late-log phase (OD₆₅₀ 0.52) *S. solfataricus* culture and then divided evenly between three, 2-liter long neck culturing flasks. At 62.5 hrs after the start of culturing (OD₆₅₀ ~0.3), 20 ml of each 1 liter culture was removed and placed in a separate 50 ml flask as non- H₂O₂ stressed growth controls. The three separate cultures (~930 ml) were then treated with hydrogen peroxide (final concentration of 30 μ M). At 1, 2, 4, 8, 15, 30, 45, 60, 75, 90, 105, 120, 150 and 195 minutes post H₂O₂ addition, 50 ml aliquots were removed for protein isolation.

Extraction of Proteins

The cells were harvested by centrifugation at 5000 x g at 4°C for 15 minutes and washed with ice-cold PB. Cells were broken by freeze and thaw followed by sonication in urea lysis buffer (30 mM Tris-HCl pH 8.5, 7 M urea, 2 M thiourea, 4% CHAPS, 1% ASB-14, 50 mM DTT, 0.5% IPG carrier ampholytes and protease inhibitor cocktail). After the supernatant was clarified by centrifugation, soluble proteins were purified and concentrated by precipitation with 5 volumes ice-cold acetone, and resolubilized for 1h in urea lysis buffer. Protein concentration was measured with the RC/DC Protein Assay Kit (Bio-Rad). Samples were kept frozen until use.

2D-DIGE Analysis

S. solfataricus protein samples (30 min, 105 min post-H₂O₂ treated and non-treated control sample) were labeled with CyDyes according to the manufacturer's protocol. 2D-DIGE experiment was performed in triplicate. Briefly, 50 µg of each protein extract was labeled separately at 0°C in the dark for 30 min with 400 pmoles of the N-hydroxysuccinimide esters of cyanine dyes (Cy3 and Cy5 CyDyes; GE Healthcare). The internal standard, an equimolecular mixture of all the protein extracts, was labeled with Cy2. Total protein labeled with Cy2, Cy3 and Cy5 for matched control and H₂O₂ stressed samples were combined and mixed with the urea lysis buffer. 2-DE was performed as described elsewhere (Gorg, Weiss et al. 2004; Maaty, Ortmann et al. 2006), using precasted IPG strips (pH 3–11 NL, non-linear, 24 cm length; GE Healthcare) for the first dimension (IEF). 150 µg of protein (50 for each dye) was 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 µA per strip. Active rehydration was achieved by applying 50 V for 12 h. This was followed by a stepwise progression of 500 V up to 8000 V for a total of 44,000 Vhr. After IEF separation, the strips were equilibrated twice for 15 min with 50 mM Tris-HCl, pH 8.8, 6 M Urea, 30% glycerol, 2% SDS and a trace of bromophenol blue. The first equilibration solution contained 25 mM DTT, and 50 mM iodoacetamide was added in the second equilibration step instead of DTT. Second-dimension SDS-PAGE was performed in Dalt II (GE Healthcare) using 1 mm-thick, 24-cm, 13% polyacrylamide gels, and electrophoresis was carried out at a constant current (15 mA/gel for ~16h at 20°C). Electrophoresis was completed once the bromophenol blue

dye front reached the bottom of the gel. ProQ™ Diamond Phosphoprotein Gel Stain (Invitrogen), SYPRO® Ruby (Bio-Rad), and Coomassie® Brilliant Blue (Thermo Scientific) stains were used according to the manufacturer's instructions.

Gel Imaging 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 µm resolution. Images were subjected to automated difference in gel analysis using Progenesis SameSpots software version 3.1 (Nonlinear Dynamics Ltd.). The Cy3 gel images were scanned at an excitation wavelength of 532 nm with an emission wavelength of 580 nm, Cy5 gel images were scanned at an excitation wavelength of 633 with an emission wavelength of 670 nm, while the 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 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.

In-gel Digestion and Protein Identification

Selected spots were manually excised from corresponding gels. The gel slices were destained using 50% acetonitrile in 50 mM ammonium bicarbonate and vacuum dried. Samples were rehydrated with 1.5 mg/ml DTT in 25 mM ammonium bicarbonate at 56°C for 1 hour, subsequently alkylated with 10 mg/ml IAA in 25 mM ammonium bicarbonate (pH 8.5), and stored in the dark at room temperature for 1 hour. The pieces

were subsequently washed with 100 mM ammonium bicarbonate for 15 min, washed twice with 50% acetonitrile in 50 mM ammonium bicarbonate for 15 min each, vacuum dried, and rehydrated with 4 μ l of proteomics grade modified trypsin in 25 mM ammonium bicarbonate. The pieces were covered in a solution of 10 mM ammonium bicarbonate with 10% acetonitrile and incubated at 37°C for 18 hours. The solution containing peptides released during in-gel digestion were transferred to sample analysis tube prior to mass analysis. LC/MS/MS used an Agilent XCT-Ultra 6330 ion trap mass spectrometer which is fitted with an Agilent 1100 CapLC and ChipCube (Agilent, Inc.). Samples were trapped and desalted on the Zorbax 300SB-C18 Agilent HPLC-Chip enrichment column (40 nl volume) in 5% acetonitrile 0.1% formic acid delivered by an auxiliary CapLC pump at 4 μ l/min. The peptides were then reverse eluted and loaded onto the analytical capillary column (43 mm x 75 μ m ID, also packed with 5 μ m 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 200 to 2 200 at 24,300 m/z -s. MGF compound list files were used to query an in-house database using with MS and MS/MS ion mass tolerances of 1.2 and 0.5 amu respectively. Protein identification from peptide MS and MS/MS data was based on MASCOT (Matrix science, London, UK) searches of an in-house database. The in-house database contained the complete genome of *S. solfataricus* P2 translated in all reading frames with user-defined modifications. For example, a pyrrolysine codon was included in place of the

amber stop codon. MS score is a probability based implementation of the MOWSE scoring algorithm that is used by Mascot search engine (Pappin, Hojrup et al. 1993). Positive identification required two significant peptides (score >50), however most protein scores were significantly above the minimum criteria.

Cysteine Reactivity Profiling

Experimentation

S. solfataricus cells H₂O₂-treated for 0, 30 or 105 min were resuspended in phosphate buffer (PB, pH 7.4). Fluorescent sulfhydryl-modifying reagent 4,4-difluoro-3,5-bis(4-methoxyphenyl)-8-(4-maleimidylphenyl)-4-bora-3a,4a-diaza-s-indacene (BODIPY[®] 577/618 maleimide; Figure 5) was added to the cells prior to the lysis procedure.

The reagent was added to a final concentration of 100 uM. Cells were lysed by sonication (three 30 sec cycles; 50% duty cycle; power output control at 2 (Branson Sonifier). After this step, the reaction was handled differently for 1D and 2D analysis as described below.

1D HEPES SDS-PAGE: The reaction with BODIPY[®] 577/618 maleimide was carried out for two hours in the dark in the presence of denaturing agent (1% SDS). The protein supernatants were clarified by centrifugation at 20,000 × *g* for 60 min at 4°C and collected. Samples were mixed with 4x SDS sample buffer (non-reducing conditions) before separation over mini 4-20% HEPES SDS-PAGE gel. Labeled mixtures were run on SDS gel in triplicate. Fronts were run off the gels to remove unreacted dye and obtain cleaner fluorescence images. Fluorescence image was obtained on Typhoon Trio Imager

(GE Healthcare) using green laser (532 nm) with 610 nm filter at 400 V. Then the gel was stained with Coomassie[®] Brilliant Blue (CBB) and scanned in 48 bit color mode at 600 dpi resolution without color correction. Scans were stored as TIF images with no compression. Background subtraction was done using ImageJ (National Institutes of Health; version 1.39m). Lanes from triplicate experiment were averaged using Image Calculator and Region of Interest (ROI) manager. Graph of fluorescent intensity versus pixel width was plotted using Microsoft Excel 2003 (Microsoft) software.

2DE: After sonication, the reaction with 100 μ M BODIPY[®] FL maleimide (Figure 5) was carried out for two hours in the dark. The protein supernatants were clarified by centrifugation at $20,000 \times g$ for 60 min at 4°C and collected. Protein samples were purified and concentrated with ice-cold acetone precipitation. Afterwards, protein samples were resolubilized for 1h in urea lysis buffer. Protein concentration was measured with the RC/DC Protein Assay Kit (Bio-Rad). Samples were kept frozen until use. 400 μ g of protein sample were loaded into precasted IPG strips (pH 3–11 NL, non-linear, 24 cm length; GE Healthcare). 2DE procedure was performed as described earlier. Experiment was done in three technical replicas with nine gels in total. Detection of fluorescent proteins by laser scanning was performed on Typhoon Trio Imager using green laser (532 nm) with 610 nm filter at 450 V. The whole protein pattern was subsequently visualized by staining with Coomassie[®] Brilliant Blue following the manufacturer's instructions and scanned in 48 bit color mode at 600 dpi resolution without color correction.

Activity-Based Protein Profiling

Experimentation

S. solfataricus cells H₂O₂-treated for 0, 30 or 105 min were resuspended in PB (pH 7.4). The FLISP reagent, FAM-Phe-DAP, which specifically labels active forms of serine hydrolases, was reconstituted in DMSO and mixed thoroughly. Afterwards, stock solution was diluted with PB, pH 7.4. The diluted reagent was added to test samples to a final concentration of 20 μ M. Cells were lysed by sonication as described earlier. The whole cell lysates and FLISP reagent were incubated for 2 hours at 37°C and periodically vortexed during the incubation time. The protein supernatants were clarified by centrifugation at 20,000 $\times g$ for 60 min at 4°C and collected. Samples were split for 1D SDS PAGE and 2DE experiments. The gels were scanned on Typhoon Trio Imager with 488 nm excitation and 610 nm emission optics pairing. The whole protein pattern was subsequently visualized by staining with Coomassie[®] Brilliant Blue.

Results

2D-DIGE Analysis

In order to map differentially regulated proteins after H₂O₂ exposure, 3 gels were analyzed with Progenesis SameSpots software. The number of detected spots exceeded 1000. However, only 818 spots were confirmed after exclusion of the gels' artifacts. These spots were used in the analysis across all gels. Representative 2D image is displayed in Figure 8 and list of regulated proteins can be found in Table 1. There was one regulated protein spot after 30 minutes and 29 at 105 minutes (19 of which were

more abundant and 10 less abundant). Protein fold changes were ranging from -4 to +8 fold.

The largest fold change in the proteomic analysis was for the DPS-Like protein (DNA-binding Protein from nutrient Starved *E. coli* cells). The DPSL protein was found in three different gel spots that all increased in abundance at 105 min after H₂O₂ exposure. The three forms of the protein differed in pI and fold difference (Figure 8 and 9). Ferritin-like nonheme iron binding protein rubrerythrin (SSO2642) represents another example of up-regulation. Moreover, it is present in three spots on both the 30 and 105-minute 2D gels. These spots (Figures 8 and 9) had different pIs and expression levels. For example, the isoform in spot 6 increased significantly in abundance in stressed cells, whereas spots 2 and 8 decreased in abundance by 3.8-fold. Peroxiredoxin (SSO2121), also known as Bcp2, is also up regulated at the 30 and 105-minute time points in the proteome (Figures 8 and 9).

A number of proteins, including superoxide dismutase, small heat shock protein, peroxiredoxin, elongation factor-1 alpha, MarR-Like transcriptional regulator (SSO1082) and hypothetical protein SSO2588 are up-regulated.

Of the 19 regulated proteins found in this study, 5 were identified in more than one spot; DPSL (SSO2079), superoxide dismutase (SSO0316), probable peroxiredoxin (SSO2121), rubrerythrin (SSO2642), elongation factor 1-alpha (SSO0216) and thermosome alpha subunit (SSO0862).

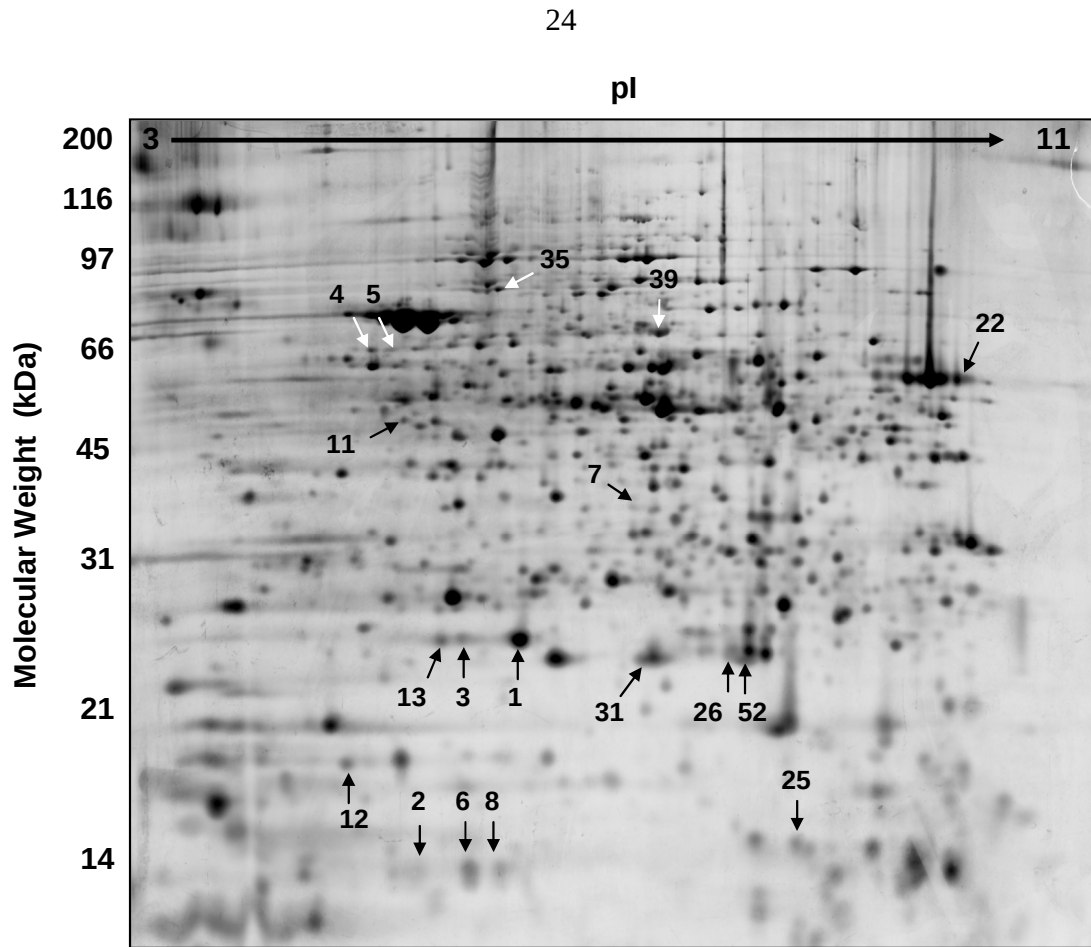


Figure 8. Regulated proteins of the *S. solfataricus* proteome after 105 minutes treatment with 30 mM H_2O_2 . 818 spots were used in the CyDye 2D-DIGE analysis. 18 protein spots changed significantly in abundance after 105 minutes exposure. Gel was stained with SYPRO Ruby.

Table 1. List of regulated proteins in 2D-DIGE experiment.

2D spot #	Gene ID	Regulated protein	Mass (Da)	pI	Fold Difference	MS SCORE
1	SSO2079	DPS-Like	21927	5.38	8.12	219
13	SSO2079	DPS-Like	21927	5.38	5.20	233
3	SSO2427	Small heat shock protein hsp20	20084	5.31	4.92	260
3	SSO2079	DPS-Like	21927	5.38	4.92	86
5	SSO0862	Thermosome alpha subunit	59695	5.35	2.86	491
5	SSO0282	Thermosome beta subunit	60387	5.56	2.86	501
4	SSO0862	Thermosome alpha subunit	59690	5.35	2.52	283
25	SSO2588	Oxidoreductase	15686	7.74	2.35	172
25	SSO1082	Transcriptional regulator, marR family	18313	9.12	2.35	104
25	SSO0276	Small nuclear ribonucleoprotein	16504	7.82	2.35	163
22	SSO0216	Translation elongation factor EF-1alpha	48573	8.93	2.03	540
26	SSO2121	Peroxiredoxin	24786	6.85	1.93	119
26	SSO0316	Superoxide dismutase [Fe] (sod)	24228	6.71	1.93	144
12	SSO3216	gamma-glutamyltranspeptidase	53136	5.67	1.89	75
31	SSO2121	Peroxiredoxin	24786	6.85	1.87	98
6	SSO2642	Rubrerythrin (rr)	16081	5.44	1.79	134
52	SSO0316	Superoxide dismutase [Fe] (sod)	24228	6.71	1.70	163
35	SSO0936	Glutamyl-tRNA(Gln) amidotransferase	71661	5.91	-1.61	161
39	SSO2044	NAD specific glutamate dehydrogenase	46091	6.5	-2.13	436
11	SSO0348	Signal recognition particle receptor protein	40390	5.29	-2.84	237
11	SSO0010	X-Pro aminopeptidase	40594	5.35	-2.84	141
8	SSO2642	Rubrerythrin (rr)	16081	5.44	-3.06	118
2	SSO2642	Rubrerythrin (rr)	16081	5.44	-3.83	277
7	SSO1045	Ribose phosphate pyrophosphokinase	32517	6.13	-4.07	161

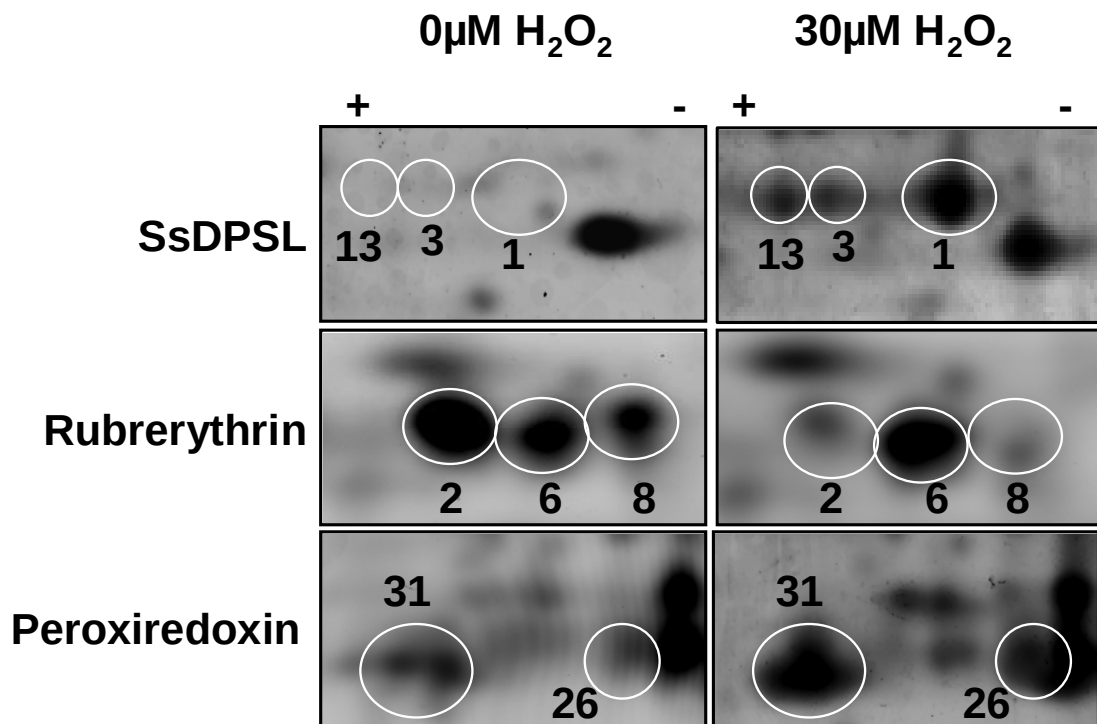


Figure 9. Post translational modification is a common feature in *S. solfataricus*. Three of the proteins that are regulated after H₂O₂ treatment are known to be important in oxidative stress and were found in multiple gel spots. Modifications can alter the pI and MW position on 2D gels. The paired panels show close ups of Sypro Ruby stained 2D gels, 105 minutes after addition of 30 µM H₂O₂. White circles indicate DPSL (SSO2079; 21639 Da; pI 5.25) spot 1,3 &13, Rubrerythrin (SSO2642; 16081 Da; pI 5.44) spot 2,6 & 8 and Peroxioredoxin (SSO2121; 24786Da; pI 6.85) spot 26 &31.

Staining of control and H₂O₂ treated SDS PAGE gels with Pro-Q Diamond[®] showed clear changes in the pattern of phosphorylation. Spots containing high levels of phosphorylation were selected for in-gel digestion. Fourteen spots were excised. Ten of the 14 spots contained more than one protein; therefore, the specific phosphorylated protein could not always be unambiguously determined. Twenty different proteins were identified from the 14 spots, 7 of which were found in more than one horizontally separated position (SSO1355, 1952, 242 and 2044 in spots P5, P6 and P7; SSO199, 1389

and 2044 in spots P9 and P10; SSO530 and 755 in spots P11 and P12; SSO in spots P13 and P14), as would be expected for differential phosphorylation (Figure 10, Table 2).

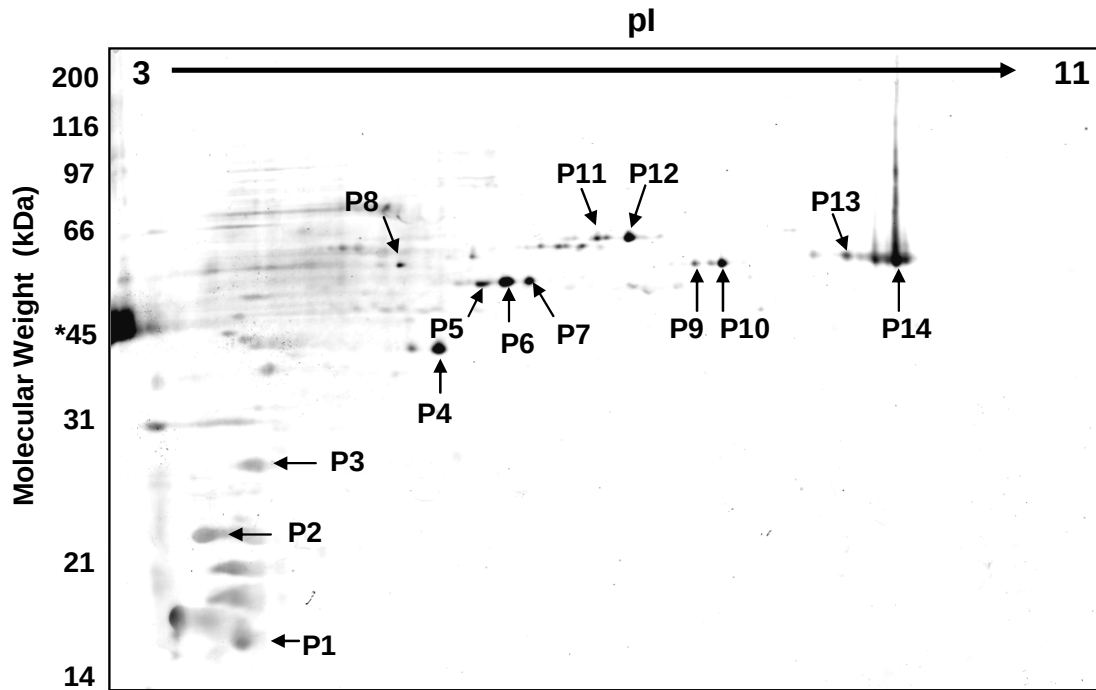


Figure 10. 2D gel of the *S. solfataricus* phosphoproteome 105 minutes after H_2O_2 treatment. Gel was stained with phosphoprotein specific stain ProQ Diamond. Each of the numbered spots was picked and the proteins were identified using in-gel proteolysis followed LC-MS/MS. Ovalbumin (*) on left) is a 45 kDa phosphoprotein standard.

Table 2. Proteins identified from phospho-stained spots.

2D spot #	Gene ID	NAME	Mass (Da)	pI	MS Score
P1	SSO0342	ribosomal protein L12	11284	4.84	178
P1	SSO0353	SSU ribosomal protein S19E	18107	10.03	155
P1	SSO0696	ribosomal protein L15	16187	10.45	135
P2	SSO0073	ribosomal protein S4	20735	9.89	258
P2	SSO1195	Phosphohistidine phosphatase	18169	5.52	100
P3	SSO0192	Disulfide oxidoreductase	25902	4.7	394
P3	SSO0746	30S ribosomal protein S3AE	23585	9.71	138
P4	SSO2483	Succinyl-CoA synthetase, beta subunit	37388	5.57	512
P5	SSO0286	hypothetical protein	42681	5.78	298
P5,P6,P7	SSO1355	Thermostable Carboxypeptidase	43326	5.93	218,419,183
P5,P6,P7	SSO1952	Thermostable carboxypeptidase	43250	5.93	143,301,150
P6, P7	SSO0242	Adenylosuccinate synthase	37417	5.83	206, 94
P7, P10	SSO2044	Glutamate dehydrogenase	46091	6.5	157,278
P8	SSO0369	Long-chain-fatty-acid-CoA ligase	51587	6.11	163
P8	SSO2635	Hypothetical protein	52481	5.6	741
P9	SSO0199	S-adenosylmethionine synthetase	45382	5.86	680
P9	SSO1389	Conserved hypothetical protein	43073	6.13	55
P11, P12	SSO0530	serine hydroxymethyltransferase	48535	6.22	261,463
P11, P12	SSO0755	S-adenosylhomocysteine hydrolase	45936	6.3	146, 189
P13, P14	SSO0216	Elongation factor 1-alpha	48573	8.93	573,104

Cysteine Reactivity Profiling

Comparison of whole proteome labeling patterns before and after treatment with H₂O₂ revealed a limited number of proteins that were highly sensitive to the redox potential (Figure 11). The curves show the average fluorescence intensity after normalization for protein concentration from three separate experiments. The percentage of reduced-thiols across the proteome decreased 30 minutes after H₂O₂ exposure (black line). By the second time point (105 minutes, red line) there was a general recovery and some protein bands were more reactive than before treatment.

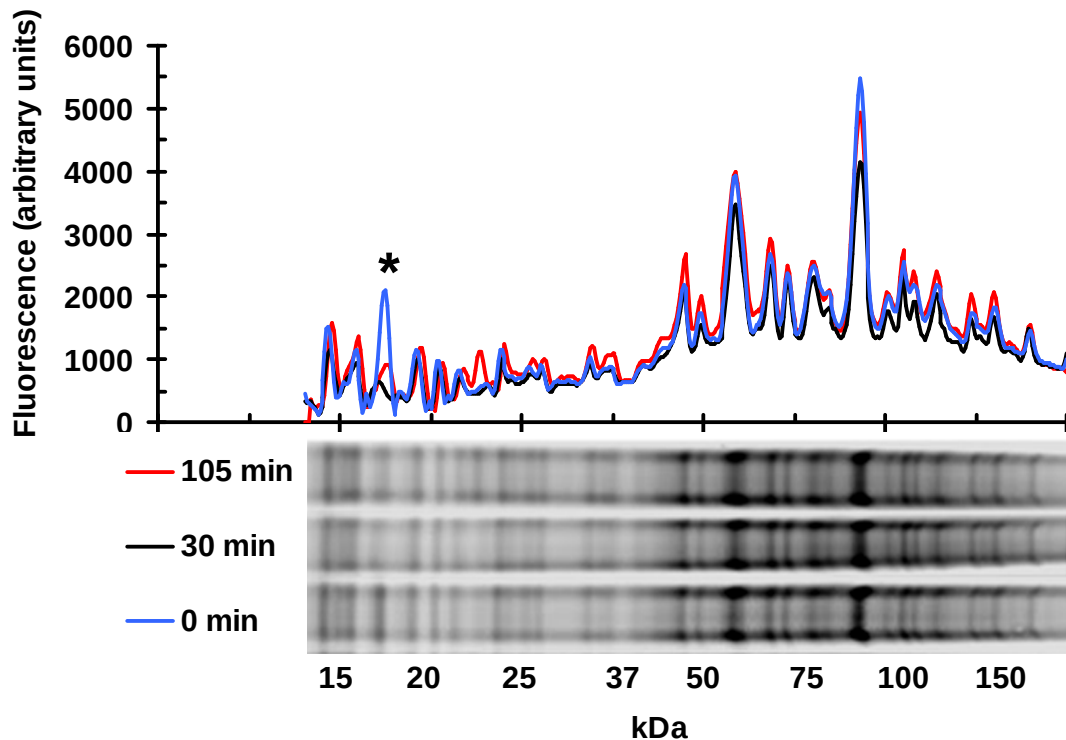


Figure 11. Protein thiol reactivity changes in H_2O_2 stressed *S. solfataricus*. Proteome-wide labeling of free cysteine thiols, with BODIPY 577/618 maleimide, shows that there is a population of redox sensitive proteins. Plot on the top shows the average fluorescent signal with respect to molecular weight. The fluorescent signal from three experiments was combined and normalized for total protein. The gel lanes at the bottom show the actual data for the 0 (blue line), 30 (black line) and 105 minute (red line) samples. * indicates protein band that were highly sensitive to changes in redox potential.

To further investigate thiol reactivity to the external stimulus, 2DE experiment was performed with the same set of samples (Figure 12). Less hydrophobic BODIPY FL maleimide was used instead BODIPY 577/618 to avoid protein precipitation during IEF. 29 regulated protein spots were detected. The biggest fold difference in the expression analysis was found for the DPSL protein, which is consistent with the results from 2D-DIGE experiment. In fact, the DPSL protein was present in three different gel spots that all increased in abundance at 105 min after H_2O_2 exposure. Interestingly, some

fluorescently detected spots that correspond to proteins with labeled sulfhydryl groups do not match in intensity with non-specifically labeled ones (Figure 12). Specific and nonspecific labeling is shown on the example of DPSL protein (Figure 12; Table 3). Three isoforms of DPSL show different levels of cysteine reactivity in response to oxidative stress.

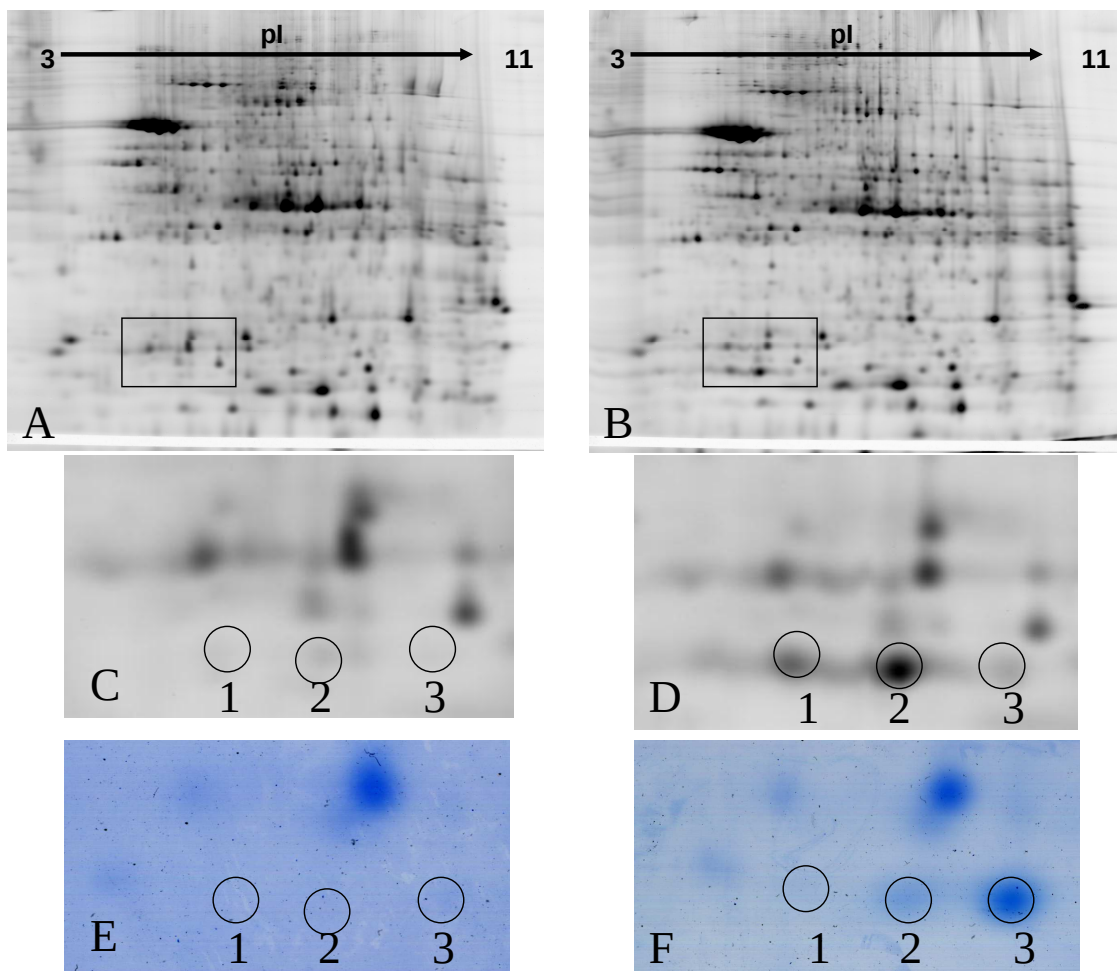


Figure 12. Proteins of the *S. solfataricus* labeled with thiol-specific BODIPY-FL dye. 2D gels on the upper panel represent changes in thiol reactivity at two time points: 30 minutes (A) and 105 (B) minutes after exposure to H_2O_2 . The paired panels (C&D, E&F) show close-ups of the same set of gels. Black circles indicate spots where different isoforms of DPSL (SSO2079; 21639 Da; pI 5.25) were found. Detection of fluorescently tagged proteins was performed using green laser (532 nm) with 610 nm filter (A, B, C, D). Coomassie Brilliant Blue was used as a non-specific protein stain (E, F).

Table 3. List of proteins with altered cysteine reactivity after 105 min of exposure to 30 μ M H_2O_2 .

Gene ID	Regulated protein	# of Cys	Score	MW	PI	Regulation
SSO1817	Thiosulfate sulfurtransferase	1	95	33691	6.41	2.022
SSO0564	ATP synthase subunit B	1	161	51114	5.3	2.161
SSO2121	Peroxiredoxin (bcp2)	1	170	24786	6.85	2.49
SSO0576	Ketol-acid reductoisomerase	1	217	36845	6.56	2.393
SSO0530	Serine hydroxymethyltransferase	1	255	48535	6.22	1.671
SSO2586	hypothetical protein	1	123	48698	6.18	1.671
SSO2079	DNA protection protein DPS	3	111	21927	5.38	9.6
SSO0738	proteasome alpha subunit	1	258	26629	5.69	-1.799
SSO0232	Glutamyl-tRNA -Gln amidotransferase	7	85	52703	6.93	-1.756
SSO0561	ATP synthase subunit E (atpE)	1	114	22676	6.69	-1.807

Activity-Based Protein Profiling

Activity-based profiling of serine hydrolases in the whole cell extract of *S. solfataricus* (Figure 13) was analyzed on 4-20% gradient 1D SDS PAGE gels and 13% non-gradient 2DE gels (Figure 14). The results from the 1D gels showed several intensified protein bands. Fluorescent signal from a low molecular weight protein (~30-35 kDa) is absent at 30 min time point; however, it is present at 0 and 105 min. Fluorescent intensities from other distinct bands (~25 kDa and ~55 kDa) have not changed significantly over time. Bands of interest were excised from the gel and identified by LC-MS/MS. Upper band and lower bands correspond to thermostable carboxypeptidase (SSO3105; 54807 Da) and proteasome alpha subunit (SSO0738; 26629 Da) respectively. MASCOT search for digested peptides could not provide trustworthy identification with two significant peptides for 30-35 kDa protein, but it was suggested that the protein of interest is lipase (SSO2493, 33583 Da).

Identification of the proteins was a challenge, mostly due to difficulties with aligning of fluorescently scanned 2D images with the total protein ones. Automation of this process may resolve this problem. Regardless of the protein identification, it is most likely that the same set of proteins showed up. The assumption is based on comparison of experimental molecular weight and pI properties of the visible spots with theoretical data. Predicted proteins are thermostable carboxypeptidase (pI 6.17; 54807 Da), lipase (pI 5.51, 33583 Da), proteasome subunit (pI 5.32; 26629 Da) and tricorn protease homolog (pI 7.25; 123380 Da). JVirGel software was used for the simulation of proteomics data to confirm the prediction of aforementioned proteins (<http://www.jvirgel.de/index.html>, Figure 14).

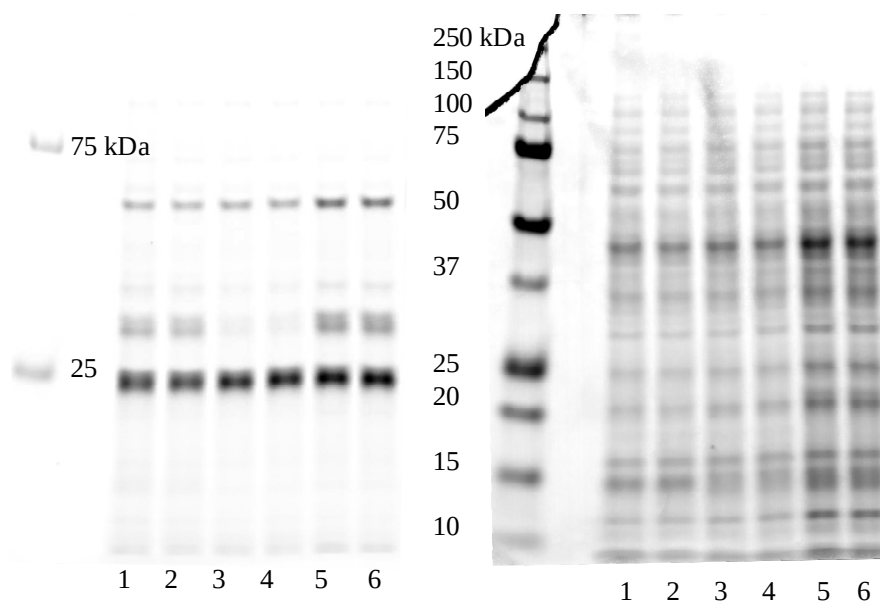


Figure 13. Fluorescent scanned 1D gel of FLISP labeled proteins of *S. solfataricus*. Samples were collected at 0 (lanes 1 and 2), 30 (lanes 3 and 4) and 105 (lanes 5 and 6) minutes after exposure to H_2O_2 and analyzed on 4-20% SDS PAGE gel. Chemical probe, FAM-Phe-DAP, was used as a fluorescent labeled inhibitor of serine hydrolases. Scanning was performed with 488 nm excitation and 610 nm emission optics pairing (left panel). The whole protein pattern was visualized by staining with Coomassie Brilliant Blue (right panel).

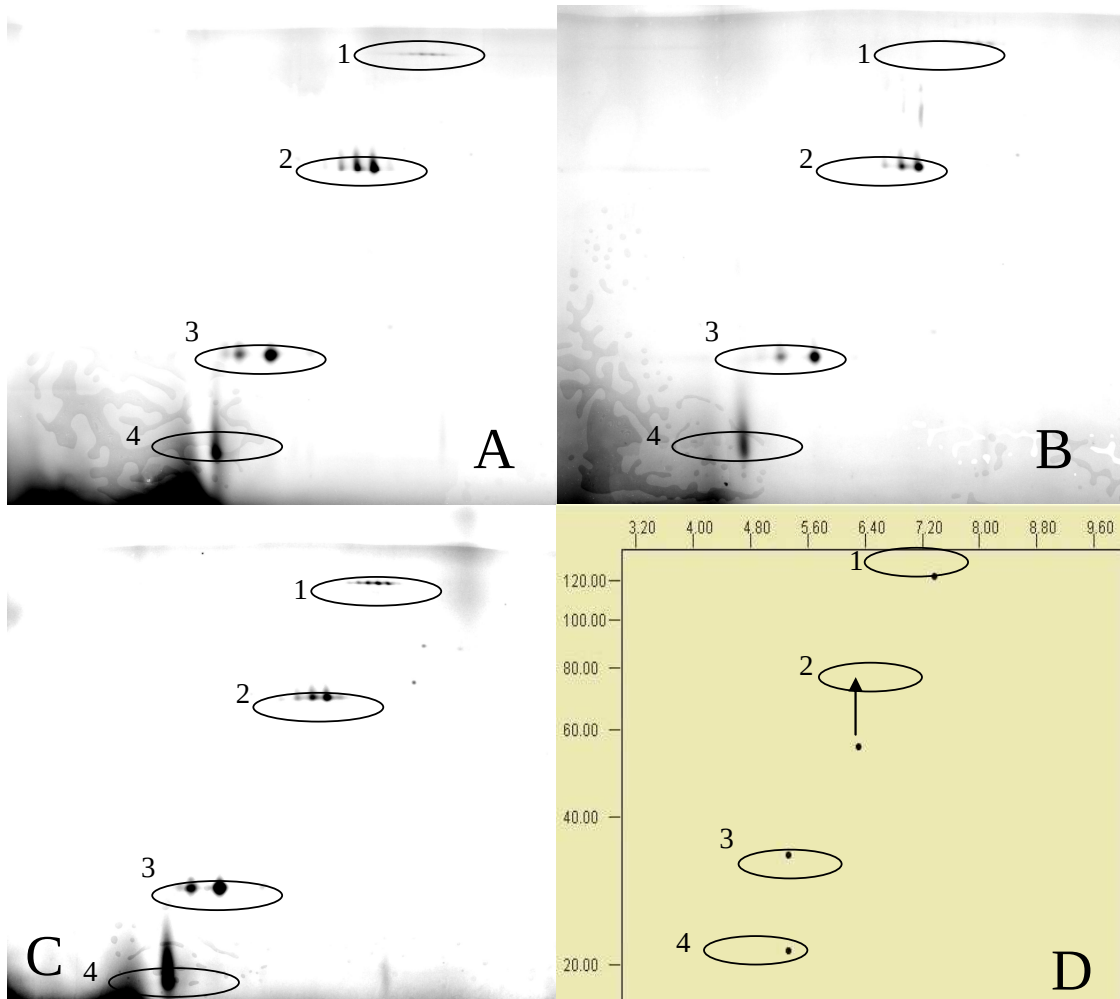


Figure 14. Fluorescent scanned 2D gels of FLISP labeled proteins of *S. solfataricus* after 0 (A), 30 (B) and 105 (C) min after treatment with H_2O_2 . Four groups of proteins were predicted to be thermostable carboxypeptidase (number 2; pI 6.17; 54807 Da), lipase (number 3; pI 5.51, 33583 Da), proteasome subunit (number 4; pI 5.32; 26629 Da) and tricorn protease homolog (number 1; pI 7.25; 123380 Da). JVirGel software (D) determined the theoretical pI and the calculated molecular weights MW of the proteins and visualizes these as a virtual 2D protein map. The lone group of proteins having a mismatch is indicated by the arrow.

Discussion

The results from H_2O_2 2D-DIGE experiment revealed 24 regulated proteins from 29 protein spots. The identified proteins are listed in Table 1 according to their fold

difference along with *Sulfolobus* gene number, molecular weight, pI, and Mascot score (MS). In this experiment, MOWSE scores ranged from 75 to 540 using only peptide scores > 32 , which indicates identity ($p < 0.05$). Several proteins were identified from multiple spots, which can occur if the protein is post-translationally modified. This observation indicates that the existence of PTMs is a common feature of *S. solfataricus* proteome. The largest increase in abundance (8.5-fold) was found for DPSL protein (SSO2079). This finding was consistent with the microarray data and the increased abundance observed by northern and western analysis (Maaty et al., unpublished data). Moreover, the three isoforms of DPSL show different levels of cysteine reactivity in response to oxidative stress. Together, these results suggest that DPSL is important component in the regulation of cellular redox potential and oxidative stress. The role of DPSL is also supported by in vitro data showing that DPSL is able to use hydrogen peroxide in the oxidation of Fe (II). One hydrogen peroxide molecule oxidizes two Fe (2+) ions, which prevents hydroxyl radical production, removing two types of reactive molecules at once. The oxidized iron, in the presence of hydrogen peroxide, becomes mineralized and is stored as a mineral core on the interior surface of the protein cage (Wiedenheft, Mosolf et al. 2005).

Regulated proteins were mostly cytosolic. However, the relationship and biological significance of the 19 up and 10 down regulated proteins and the pathways that may connect them were not immediately obvious. Functional annotation was required to solve this problem. DAVID (The **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery) is web-based software that provides a comprehensive set of functional

annotation (<http://david.abcc.ncifcrf.gov/home.jsp>). DAVID uses an algorithm to measure relationships among the annotation terms based on the degrees of their co-association genes to group the similar, redundant, and heterogeneous annotation contents from the same or different resources into annotation groups. The results of this analysis are shown in Figure 15. Functional groups include: ion and metal binding (3 proteins out of 24), nucleotide binding (4), metabolic processes (12) and oxidoreductase activities (7). In DAVID, P-Value equal or smaller than 0.05 is considered strongly enriched in the annotation categories. Based on this, the only strongly enriched cluster belongs to “oxidoreductase activity”. Oxidoreductase activities include a variety of different functions starting with protein folding, denaturation, degradation, electron transport, and oxidative stress response (Niimura, Nishiyama et al. 2000; Nishiyama, Massey et al. 2001). Seven proteins involved in oxidoreductase activities are of particular interest. This group includes DPSL protein (SSO2079), superoxide dismutase (SSO0316), rubrerythrin (SSO2642), peroxiredoxin (SSO2121), acyl-CoA dehydrogenase (SSO2877), NAD specific glutamate dehydrogenase (2044) and malate oxidoreductase (SSO2869). Together with DAVID analysis, earlier investigations have also demonstrated that superoxide dismutase, peroxiredoxin and rubrerythrin are involved in oxidoreductase activities in various species; moreover, these proteins are known to target the degradation of either superoxide or hydrogen peroxide.

Enrichment Score: 0.41			Count	P_Value
oxidoreductase activity	RT		7	4.7E-2
transition metal ion binding	RT		3	5.9E-1
cation binding	RT		3	6.2E-1
metal ion binding	RT		3	7.3E-1
ion binding	RT		3	7.5E-1
Enrichment Score: 0.28			Count	P_Value
nucleotide-binding	RT		3	2.1E-1
purine nucleotide binding	RT		4	5.3E-1
nucleotide binding	RT		4	5.7E-1
binding	RT		9	5.8E-1
purine ribonucleotide binding	RT		3	7.6E-1
ribonucleotide binding	RT		3	7.6E-1
Enrichment Score: 0.23			Count	P_Value
cellular protein metabolic process	RT		4	3.8E-1
protein metabolic process	RT		4	3.8E-1
cellular macromolecule metabolic process	RT		4	4.0E-1
cellular process	RT		10	5.5E-1
macromolecule metabolic process	RT		6	7.5E-1
cellular metabolic process	RT		8	8.5E-1
metabolic process	RT		12	8.6E-1
primary metabolic process	RT		7	8.9E-1

Figure 15. Functional annotation of proteins regulated in response to oxidative stress. “Count” column and blue bars show how many genes involved in individual term. “P-value” column is the modified Fisher Exact P-value. Usually P-Value is equal or smaller than 0.05 to be considered strongly enriched in the annotation categories. Overall “enrichment score” is based on the P-value scores of each term members.

In this study, superoxide dismutase (SOD; SSO0316) is up-regulated. It is not surprising, because the importance of SOD as an antioxidant is evident in SOD-deficient strains of *Escherichia coli*, which exhibited big growth defects when exposed to air (Carlioz and Touati 1986). In general, superoxide species are managed by either superoxide dismutases or superoxide reductases (Mccord and Fridovic.I 1969; Adams, Jenney et al. 2002). Hydrogen peroxide is a product of such superoxide detoxification.

Thus, other proteins are required to minimize intracellular H₂O₂ concentration and mitigate oxidative damage. It is most likely that identified up-regulated members of oxidoreductase cluster are key players in the “game”.

DAVID results lead to another protein with oxidoreductase activity, rubrerythrin. Role of rubrerythrin in cellular redox potential is well established (Weinberg, Jenney et al. 2004). In the referenced study, the peroxidase activities of the recombinant forms of rubrerythrin were proportional to the iron content. It was proposed that protein functions in an NADH-dependent, hydrogen peroxide: rubredoxin oxidoreductase system. Therefore, it is most likely that *S. solfataricus* uses rubrerythrin in its oxidative stress protection mechanism. Rubrerythrin was present on both the 30 and 105-minute 2D gels in multiple isoforms. These three isoforms (spots 2, 6 and 8; Figure 9) had different pIs and different levels of expression. For example, the intensity in spot 6 increased significantly in abundance in stressed cells, whereas spots 2 and 8 decreased in abundance by 3.8-fold. Identification of PTMs is always a challenge; thus, the nature of these modifications remains to be unveiled.

Peroxiredoxin (SSO2121) is up-regulated in the proteome at the 30 and 105-minute time points; in addition it is present in two isoforms (Figure 9). It plays an important role in the peroxide-detoxifying system in *S. solfataricus* (Limauro, Pedone et al. 2006). Moreover, the protein reveals high sequence identity with peroxiredoxins from other organisms, such as aerobic hyperthermophilic archaeon *Aeropyrum pernix* (61%) (Jeon and Ishikawa 2003), hyperthermophilic bacterium *Thermotoga maritima* (61%), and anaerobic hyperthermophilic archaeon *Pyrococcus horikoshii* (57%) (Kashima and

Ishikawa 2003). Peroxiredoxin along with DPSL and several other proteins shows different level of cysteine reactivity in response to oxidative stress (Table 3). As it was mentioned earlier, one of the possible modifications is oxidation of active site cysteine residues to cysteic acid (Rabilloud, Heller et al. 2002). This hypothesis is supported by mutagenesis studies that have shown that the only cysteine, Cys49, in the peroxiredoxin sequence, is involved in the catalysis (Limauro, Pedone et al. 2006).

Another important protein involved in general oxidative stress response is translation elongation factor (SSO0216), which was found to be up-regulated and also phosphorylated. Recent studies indicate that expression of this protein in rat embryonic cell line significantly increases during apoptotic events caused by H₂O₂ (Chen, Proestou et al. 2000). Intriguing connection between elongation factor and peroxiredoxin has been revealed when mouse cells transfected with both proteins have increased resistance to peroxide-induced apoptosis compared to single transfectants (Chang and Wang 2007). It will not be surprising if *S. solfataricus* possess a similar defense mechanism.

The connection of other regulated proteins to oxidative stress is not well defined yet. Although, glutaredoxin related protein (SSO0192), which appears to be phosphorylated (Table 2), was reported to be regulated in *S. solfataricus* after H₂O₂ exposure (Pedone, Limauro et al. 2006). S-adenosylmethionine synthetase and glutamate dehydrogenase showed clear changes in regulation in addition to phosphorylation (Table 1 and 2). NAD specific glutamate dehydrogenase (SSO2044) represents a down-regulated protein (Table 1). It is classified as oxidoreductase and most likely involved in oxidative stress response.

The percentage of reduced thiols across the proteome decreased 30 minutes after H₂O₂ exposure and then recovered at 105 minutes time point (Figure 7). The antioxidant machinery is not ready to counteract to high concentration of H₂O₂ at early time points; hence, sensitive cysteinyl residues are oxidized to sulfenic acid adducts that can either form disulfides with other free cysteines or be further oxidized to sulfinic or sulfenic acids (Figure 4). Once the stressor is inside the cell, it triggers the transcription and translation of the proteins, capable of detoxifying the ROS and repairing the damage (Figure 7). This is consistent with from 2D-DIGE experiments, where the overall expression profiles of the genes involved in stress response, were significantly higher, when exposure to stress reaches second time point (105 min).

There are 67 known and putative peptidases and 9 non-peptidase homologues in *S. solfataricus*. 16 of them are serine hydrolases, including serine proteases, lipases and esterases (<http://merops.sanger.ac.uk/>). FLISP reagent, FAM-Phe-DAP, labels only active catalytic sites of serine hydrolases (Figure 13, left panel) Based on that, three distinct bands were selected for further analysis and identification. Three proteins are thermostable carboxypeptidase (SSO3105; 54807 Da), proteasome alpha subunit (SSO0738; 26629 Da) and lipase (SSO2493, 33583 Da); with lipase having low probability MOWSE score. One of three identified hydrolases, thermostable carboxypeptidase, has an overlap with the phosphoproteome study. Results from the gel electrophoresis indicate that thermostable carboxypeptidase and proteasome subunit are in active conformation throughout the time course of exposure to stress and are likely used to degrade abnormally folded peptides and also proteins damaged by ROS. This gel

also reveals that the protein with approximate MW 30-35 kDa (most probably lipase) is not active at 30 min after treatment with H_2O_2 . The meaning of such behavior and the possible role of lipase in the stress response is not very clear.

The results suggest that *S. solfataricus* operates complex ROS-scavenging system in response to oxidative stress. Individual players revealed in this study and hypothesized chemical reactions that they catalyze are depicted in Figure 16. However, there are still missing pieces in the mechanism of detoxification against oxidative challenge in *S. solfataricus*. Further analysis in combination with genomics and metabolomics is needed to better understand detoxification mechanisms in Archaea.

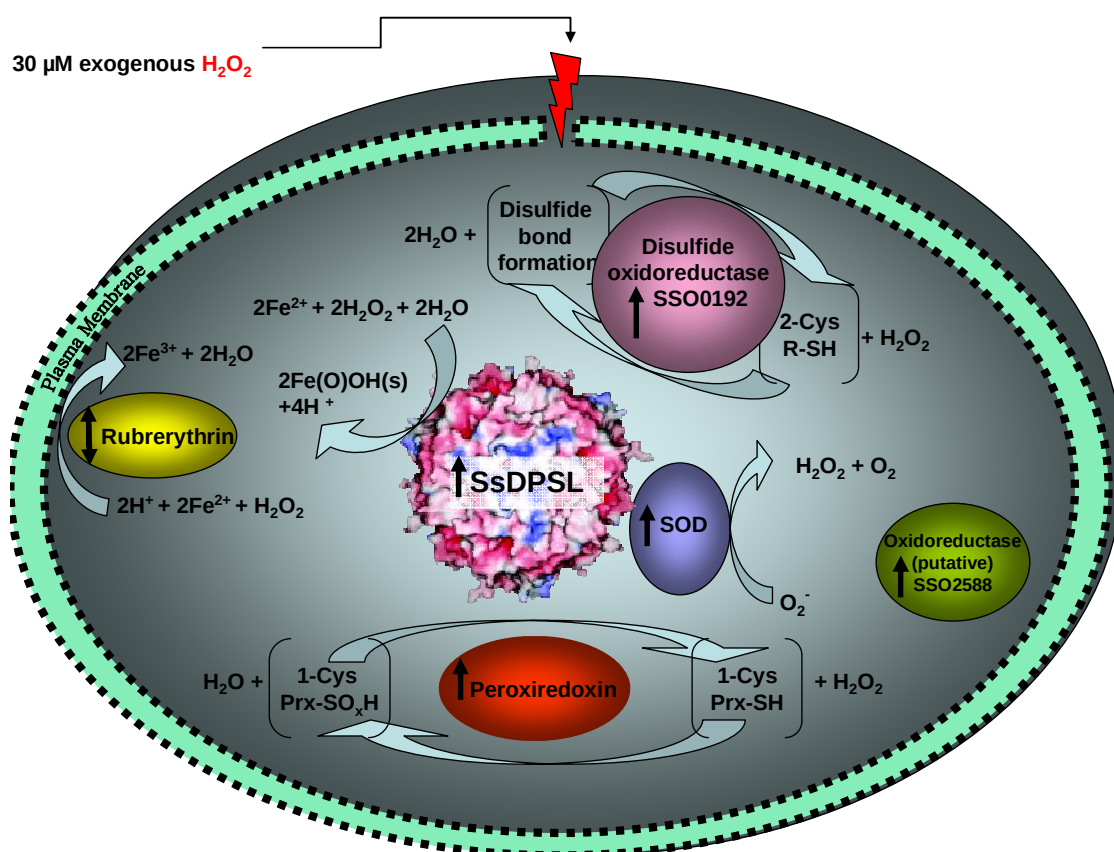


Figure 16. Schematic of the oxidative stress response in *S. solfataricus*. Black arrows indicate direction of protein regulation.

CHAPTER 3

PROTEOMIC RESPONSE OF SULFOLOBUS SOLFATARICUS P2 TO ARSENIC INDUCED STRESS

Introduction

Metals that are essential to enzyme activity, such as zinc and iron, are toxic at higher concentrations (Stohs and Bagchi 1995; Rosenzweig 2002). The toxicity of metal ions varies greatly and specific differences may be related to differences in solubility, transport, chemical reactivity, and the formation of biomolecular complexes within the cell or organism.

Arsenic is a metalloid that threatens most biological organisms. In the environment, arsenic is primarily present in its inorganic forms and mostly under two oxidation states: arsenite, As (III) and arsenate, As (V) (Connon, Koski et al. 2008). Arsenate, a phosphate analogue, can enter cells via the phosphate transport system and is toxic because it inhibits a large number of reactions, and in particular the production of ATP, by replacing phosphate (Dopson, Baker-Austin et al. 2003). Arsenate uptake would occur when phosphate levels become depleted due to cellular phosphate consumption or when arsenate is at high concentration. Noteworthy, inorganic phosphate concentration in geothermal environment is low (Langner, Jackson et al. 2001). The toxicity of arsenite is attributed to its strong affinity for sulfhydryl groups in proteins and is considerably more toxic than arsenate (Saha, Dikshit et al. 1999). Although arsenic is not a redox metal, it may cycle between oxidative states, producing radicals (Lynn, Shiung et al. 1998; Flora

1999). As a consequence, it leads to depletion of intracellular levels of antioxidants.

Recently it has been demonstrated that expression of catalase, superoxide dismutase and glutathione-S-transferase in *Zea mays* were regulated upon treatment with arsenate or arsenite (Mylona, Polidoros et al. 1998).

Arsenate tolerance in a number of plant species occurs through suppression of the phosphate/arsenate uptake system (Meharg and Macnair 1992). The suppression reduces arsenate influx to a level where the plant can detoxify it. The metabolism of arsenic in many mammalian species includes the reduction of arsenate to arsenite, with subsequent methylation yielding mono-, di- and trimethylated derivatives (Aposhian 1997).

Methylation is catalyzed by a specific methyltransferase, which uses S-adenosylmethionine as the methyl group donor and is considered to be major detoxification pathway in mammals (Zakharyan, Wu et al. 1995; Styblo, Del Razo et al. 2000). In bacteria, the *ars* operon constitutes the most ubiquitous and important scheme of arsenic tolerance (Paez-Espino, Tamames et al. 2009). The Ars system mediates the extrusion of arsenite out of the cells. There are also other microbial activities that alter the chemical characteristics of arsenic: some strains are able to oxidize arsenite or reduce arsenate as part of their respiratory processes. The biochemical mechanisms responsible for metal resistance in thermophilic Archaea remain largely uncharacterized.

Sulfolobus species can live in arsenic-rich geothermal hot springs, which have some of the highest arsenic concentrations known (Langner, Jackson et al. 2001; Hetzer, Morgan et al. 2007; Hetzer, Morgan et al. 2007). However, comparatively little attention has been devoted to these species in order to investigate the mechanism of arsenic

tolerance. In the only published study, arsenite oxidation by *Sulfolobus acidocaldarius* was 8-fold higher for cells grown in the presence of arsenite when compared with cells grown without (Sehlin and Lindstrom 1992). In this study, the toxicities of trivalent and pentavalent arsenic species at the proteome level were examined in *S. solfataricus*. Our investigation included 2DE, changes in cysteine reactivity and phosphorylation patterns under arsenic stress.

Materials and Methods

All materials and methods applied to hydrogen peroxide induced oxidative stress study were followed as previously described, with following exceptions:

For protein expression analyses, *S. solfataricus* cells were grown to late log phase (OD_{650} 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_{650} ~0.2) four separate cultures (~450 ml) were treated with 0, 60, 120 or 240 μ M arsenite ($NaAsO_2$). 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. The 2DE gels were stained with Krypton (Pierce). Detection of fluorescent proteins by laser scanning was performed on the Typhoon Trio Imager using green laser (532 nm) with 580 nm filter at 500 V. The whole protein pattern was subsequently visualized by staining with Coomassie[®] Brilliant Blue following the manufacturer's instructions and scanned in 48 bit color mode at 600 dpi resolution without color correction. Krypton-stained gels were

selected for the image and data analyses. Spots were detected and quantified with the Progenesis SameSpots software, on the basis of their relative volume.

Protein spots of interest were excised from the gels, washed, in-gel reduced and S-alkylated, followed by digestion with porcine trypsin overnight at 37°C (Maaty, Ortmann et al. 2006). Peptides eluted from the in-gel digests were analyzed on an integrated Agilent 1100 liquid chromatograph (XCT-plus) mass-selective detector. The peptides were reverse eluted and loaded onto an analytical C₁₈ capillary column (15-cm by 75-µm inside diameter, packed with 3.5-µm Zorbax 300, SB C₁₈ particles; Agilent) connected in-line to the mass spectrometer with a flow of 300 nl/min. Data-dependent acquisition of collision induced dissociation tandem mass spectrometry was utilized. Parent ion scans were run over the m/z range of 200 to 2 200 at 24 300 m/z-s. MGF compound list files were used to query an in-house database using with MS and MS/MS ion mass tolerances of 1.2 and 0.5 amu respectively. Protein identification from peptide MS and MS/MS data was based on MASCOT searches from in-house database. In all experiments, positive identification required two significant peptides (0.05<P), unless indicated otherwise.

In order to identify redox-sensitive proteins in *S. solfataricus* another set of cultures was prepared. As (III) was administered to three separate cultures (OD₆₅₀ ~0.25) in a form of sodium arsenite (NaAsO₂) to final concentrations of 0, 120 and 1000 µM. Aliquots for protein isolation were taken from the cell cultures at 8, 16, 32 and 64 hours after arsenite treatment and spun 10 min, 5 000 x g to pellet the cells. The cells, taken at 16 h time point, were resuspended in PB (pH 7.4). Fluorescent sulfhydryl-modifying

reagent BODIPY FL maleimide (Figure 5) was added to the cells prior lysis procedure. The reagent was added to a final concentration of 100 μM . Cells were lysed by sonication, followed by freeze-thaw procedure in liquid nitrogen. Proteins were extracted, separated on 13% SDS-PAGE gels and analyzed as described earlier.

In the next experiment, As (V) was added to three separate cultures ($\text{OD}_{650} \sim 0.25$) in a form of sodium arsenate (Na_2HAsO_4) to final concentrations of 0, 100 and 1000 μM . Aliquots for protein isolation were taken from the cell culture at different time points of As (V) exposure, which are 8, 16, 32 and 64 hours. The cells were pelleted by centrifugation and kept in freezer until further use. Samples from 16h time point were run on 2DE gels in triplicate, stained with ProQ Diamond phosphoprotein stain and visualized as described previously.

Results

The toxicity of different concentrations of trivalent and pentavalent arsenic species was examined in cultures of *S. solfataricus* P2 (Figure 17, 19 and 20). Cells, exposed to trivalent arsenic at 60 and 240 μM concentrations had higher OD_{650} readings than non-treated culture at 16 hours (Figure 17). At the next time point, 24 hours after inoculation, cell growth was inhibited by 60 μM and 240 μM concentrations of As (III). In another experiment, exposure to moderate (120 μM) concentration of trivalent arsenic inhibited cell growth at ~ 16 hours, while the non-treated control and 1000 μM As (III)-treated culture displayed similar growth patterns (Figure 19).

Exposure to moderate (100 μM) and high (1000 μM) concentrations of pentavalent arsenicals caused a transient increase in growth by 8 hours in both cell cultures comparing to control (Figure 20). On the other hand, standard deviation in OD_{650} measurements for this particular time point was larger than the others. 16 hour time point showed decrease in turbidity in arsenic-stressed cultures, similar to that in Figure 17, but with ~ 10 hour time-shift.

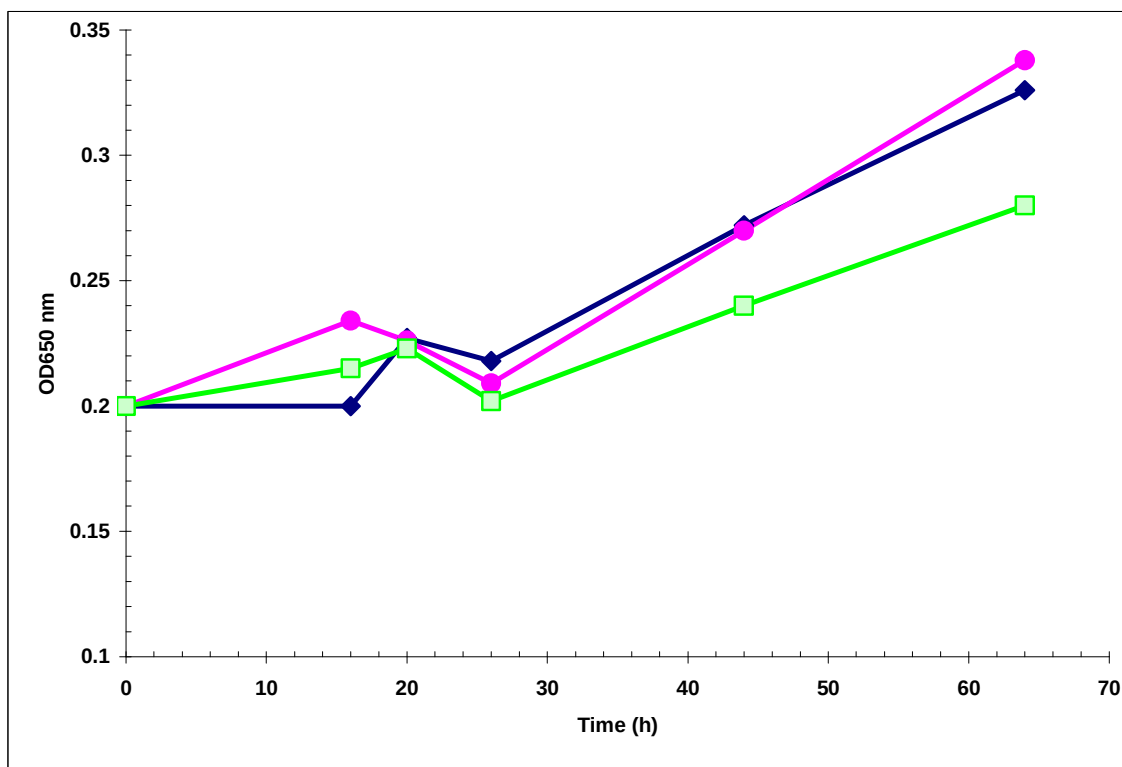


Figure 17. Growth of cells in the absence and presence of different concentrations of As (III). OD_{650} for cultures are presented without standard deviations. The blue diamonds represent the untreated control culture, while the purple circles and green squares represent the 60 and 240 μM As (III)-treated cultures respectively.

Changes in the proteome of *S. solfataricus* were monitored using 2DE on proteins isolated from the cells during As (III) treatment. Approximately 800 protein spots were reproducibly detected in each gel, stained with total protein stain (KryptonTM, Pierce).

Quantitative analysis of Krypton-stained gels revealed 24 protein spots with differences in fluorescent intensity (>1.5 -fold) (Figure 18). These spots were picked and 29 proteins were identified using in-gel proteolysis followed by LC-MS/MS (Table 4). Among them 17 were up-regulated and 12 were down-regulated in response to As (III) treatment.

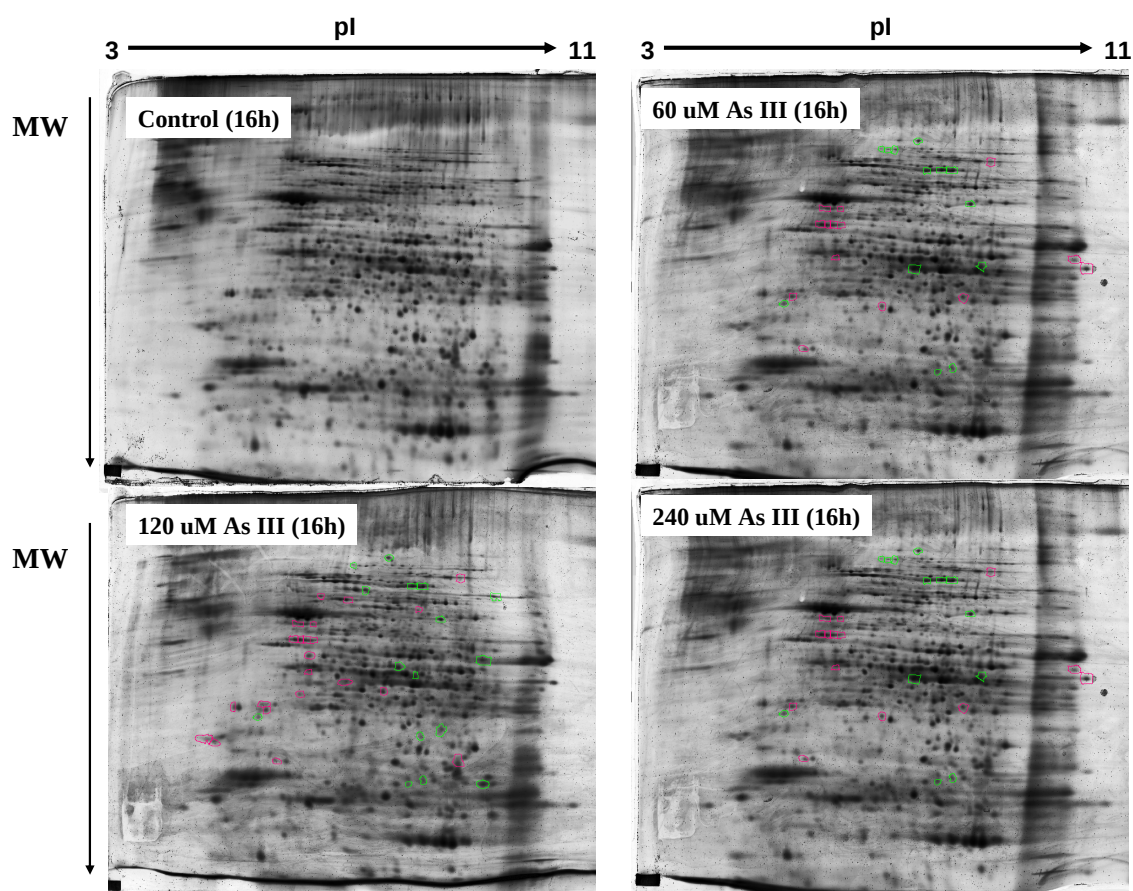


Figure 18. Representative gels of *S. solfataricus* proteome 16 h post-As (III) treatment visualized with total protein stain (Krypton). Three different concentrations of arsenite were used (60 uM, 120 uM and 240 uM). Regulated spots were picked and the proteins were identified using in-gel proteolysis followed LC-MS/MS. Red spots indicate down-regulated proteins, green spots – up-regulated.

Table 4. Concentration-dependent changes to protein regulation in cultures of *S. solfataricus* exposed to different trivalent arsenic concentrations. Fold difference were determined for 60 uM, 120 uM and 240 uM As (III)-treated samples by Progenesis SameSpots software and differences in expression values were calculated.

Gene ID	Regulated protein	60 uM As III	120 uM As III	240 uM As III
SSO1907	NAD specific glutamate dehydrogenase A2	1.5<		1.453 1.489
SSO2044	NAD specific glutamate dehydrogenase A4	1.5<		1.453 1.489
SSO0078	Tyrosyl-tRNA synthetase (tyrS)	1.5<		1.453 1.489
SSO0913	Enolase		1.532 1.5<	1.5<
SSO2182	Isocitrate dehydrogenase, probable (idh)	1.5<		1.554 1.5<
SSO3226	Conserved hypothetical protein	1.5<		1.571 1.5<
SSO0067	SSU ribosomal protein S2AB (rps2AB)	1.5<		1.571 1.5<
SSO0728	Elongation factor 2 (EF-2)		1.495	1.492 1.811
SSO3107	Dihydroxy-acid dehydratase (ilvD)		1.578	1.726 1.528
SSO2486	Threonyl-tRNA synthetase (thrS)		1.578	1.726 1.528
SSO2639	Carbon monoxide dehydrogenase	1.5<		1.492 1.811
SSO2863	Acetyl-CoA synthetase	1.5<		1.641 1.667
SSO0282	Thermosome beta subunit		1.611	1.577 1.977
SSO2098	Tricorn protease homolog		2.391	1.659 1.642
SSO2497	Acyl-CoA dehydrogenase related protein		1.927 1.5<	1.5<
SSO2450	TATA binding protein (TBP)-interacting protein		1.952 1.5<	1.5<
SSO0026	Phosphoribosylglycinamide formyltransferase 2		4.066	1.672 1.5<
SSO2241	BPS2 protein homolog (bps2)	1.5<		-2.105 1.5<
SSO0282	Thermosome beta subunit	1.5<		-2.135 -1.982
SSO1663	N-methylhydantoinase A (huyA-1)	1.5<		-2.014 -1.964
SSO1907	NAD specific glutamate dehydrogenase A2		-1.911 1.5<	1.5<
SSO0530	Serine hydroxymethyltransferase	1.5<		-2.059 -1.626
SSO0862	TF55-alpha protein		-1.45	-1.855 -1.802
SSO0755	S-adenosyl-L-homocysteine hydrolase		-1.697	-1.485 -1.698
SSO2356	Succinate dehydrogenase subunit A		-1.697	-1.485 -1.698
SSO0939	Pre mRNA splicing protein	1.5<		-1.662 -1.523
SSO2632	Hypothetical protein SSO2632		-1.515	-1.574 1.5<
SSO2815	2-oxoacid--ferredoxin oxidoreductase	1.5<		-1.535 1.5<
SSO1811	Prolidase (Xaa-Pro dipeptidase)	1.5<		-1.508 1.5<

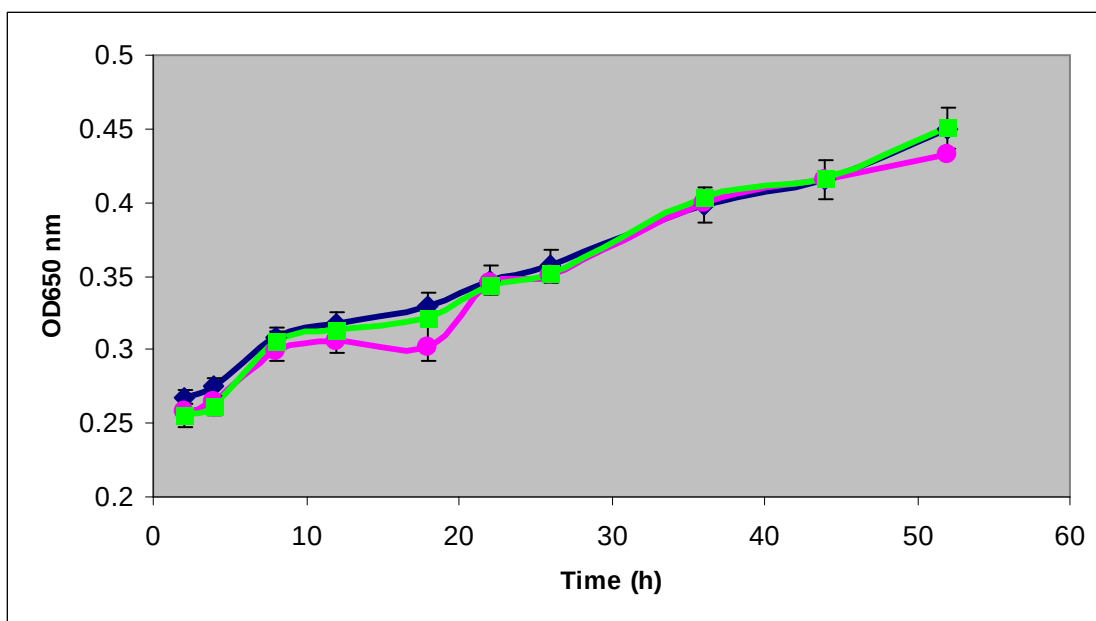


Figure 19. Growth of cells in the absence and presence of different concentrations of As (III). The average OD₆₅₀ for cultures are presented with standard deviations. The blue diamonds represent the untreated control culture, while the purple circles and green squares represent the 120 and 1000 uM As (III)-treated cultures respectively.

High affinity of arsenite for sulfhydryl groups in proteins and is well known. Thus, free thiol reactivity on the level of proteome was studied on *S. solfataricus* cells, As (III)-treated for 16 hours (Figure 21). BODIPY FL maleimide was used in 2DE experiment as a thiol-specific chemical probe. Approximately 500 protein spots were reproducibly detected in each gel. Quantitative analysis revealed a total of 45 protein spots showed more than 1.5 fold differences in expression (Figure 21). Gels selected for further analysis were stained with CBB. Only 9 proteins were identified (Table 6), mostly due to low sensitivity of the total stain, which results in difficulties with aligning and manual picking of corresponding spots from CBB-stained gel. Positive identification required two significant peptides. The biggest fold differences in thiol reactivity were found on peroxiredoxin (SSO2112) and lipase (SSO2493).

Exposure to different concentrations of arsenate resulted in 9 proteins being identified as differentially expressed (Table 5). Among them, 2 proteins were up-regulated and 7 were down-regulated in response to As (V) treatment. Pro-Q Diamond was used on the same set of gels to map and identify changes in phosphoproteome of *S. solfataricus* in response to a phosphate antagonist – arsenate (Figure 22). Most of the proteins identified from phospho-stained spots were found to be down-regulated in 1000 uM As (V)-treated cell culture (Table 5 and 7), while changes in the pattern of phosphorylation between As (V)-treated and non-treated protein samples were not significant.

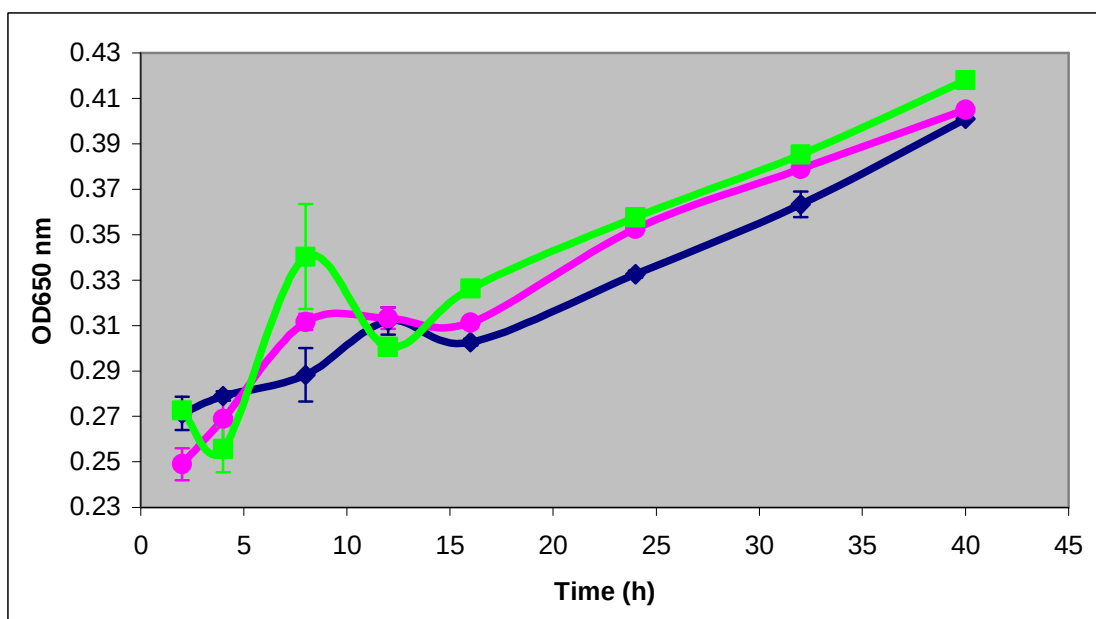


Figure 20. Growth of cells in the absence and presence of different concentrations of As (V). The average OD₆₅₀ for 3 cultures are presented with standard deviations. The blue diamonds represent the untreated control culture, while the purple circles and green squares represent the 100 and 1000 uM As (V)-treated cultures respectively.

Table 5. Concentration-dependent changes in protein regulation in cultures of *S. solfataricus* exposed to pentavalent arsenic. Fold difference were determined for 100 μ M and 1 mM As (V)-treated samples versus non-treated control by Progenesis SameSpots software and changes in expression were calculated (two right columns)

Gene ID	name	Mass	PI	MS score	100 μ M AsV	1 mM AsV
SSO0862	Thermosome alpha subunit	59695	5.35	428	1.5<	-2.601
SSO0282	Thermosome beta subunit	60387	5.56	340	1.5<	-2.601
SSO2356	Succinate dehydrogenase	62550	6.18	229	1.5<	1.864
SSO2241	BPS2 protein homolog	68510	5.56	157	1.5<	1.522
SSO0079	Bacterial-like DNA primase	45382	5.18	148	1.5<	-1.782
SSO1119	Hypothetical protein	46431	4.89	127	1.5<	-1.782
SSO0939	Pre mRNA splicing protein	46664	9.5	201	-2.307	-3.277
SSO0939	Pre mRNA splicing protein	46664	9.5	480	-2.168	-2.708
SSO0939	Pre mRNA splicing protein	46664	9.5	253	-1.988	-1.914
SSO0758	Peptidyl-prolyl isomerase	26700	6.66	99	-1.904	-2.241
SSO2493	Lipase (lipP-1)	33584	5.51	110	1.5<	-1.98

Table 6. Proteins with regulation in cysteine reactivity upon exposure to As (III). 2D gels of 120 μ M and 1 mM As (III)-treated protein samples were compared with non-treated control by Progenesis SameSpots software and fold differences were calculated (two right columns)

2D spot #	Gene ID	Regulated protein	# of Cys	score	Mass	PI	120 μ M AsIII	1mM AsIII
1	SSO0530	Serine hydroxymethyltransferase	1	168	48535	6.22	1.587	1.646
1	SSO0755	S-adenosyl-L-homocysteine hydrolase	1	163	45936	6.3	1.587	1.646
2	SSO1907	glutamate dehydrogenase	1	90	46059	6.51	1.587	1.646
3	SSO1817	Thiosulfate sulfurtransferase	1	92	33691	6.41	1.977	1.865
4	SSO0738	Proteasome subunit alpha	1	179	26629	5.69	1.535	1.692
B	SSO2121	Peroxiredoxin (bcp-2)	1	144	24786	6.85	4.146	2.247
5	SSO2061	Acetyl-CoA C-acetyltransferase	1	228	41628	5.4	1.701	1.689
6	SSO3194	Glyceraldehyde-3-phosphate dehydrogenase	1	175	56948	6.06	1.978	1.617
C	SSO2493	Lipase (lipP-1)	3	160	33583	5.51	3.645	1.757

Table 7. Proteins identified from phospho-stained spots

2D spot#	Gene ID	Protein name	Mass	PI	MS score
1	SSO0862	Thermosome alpha subunit	59695	5.35	428
1	SSO0282	Thermosome beta subunit	60387	5.56	340
2, 3	SSO2356	Succinate dehydrogenase subunit A (sdhA)	62550	6.18	229, 101
7, 8	SSO0079	Bacterial-like DNA primase (dnaG)	45382	5.18	153, 410
10	SSO1119	Hypothetical protein SSO1119	46431	4.89	127
14, 15	SSO0939	Pre mRNA splicing protein	46664	9.5	201, 480
17	SSO0758	Peptidyl-prolyl cis-trans isomerase, FKBP-type rotamase	26700	6.66	99
21	SSO0342	LSU ribosomal protein L12AB (rpl12AB)	11284	4.84	150
22, 23	SSO0216	Translation elongation factor EF-1alpha	48573	8.93	198, 223
28	SSO2493	Lipase (lipP-1)	33584	5.51	110

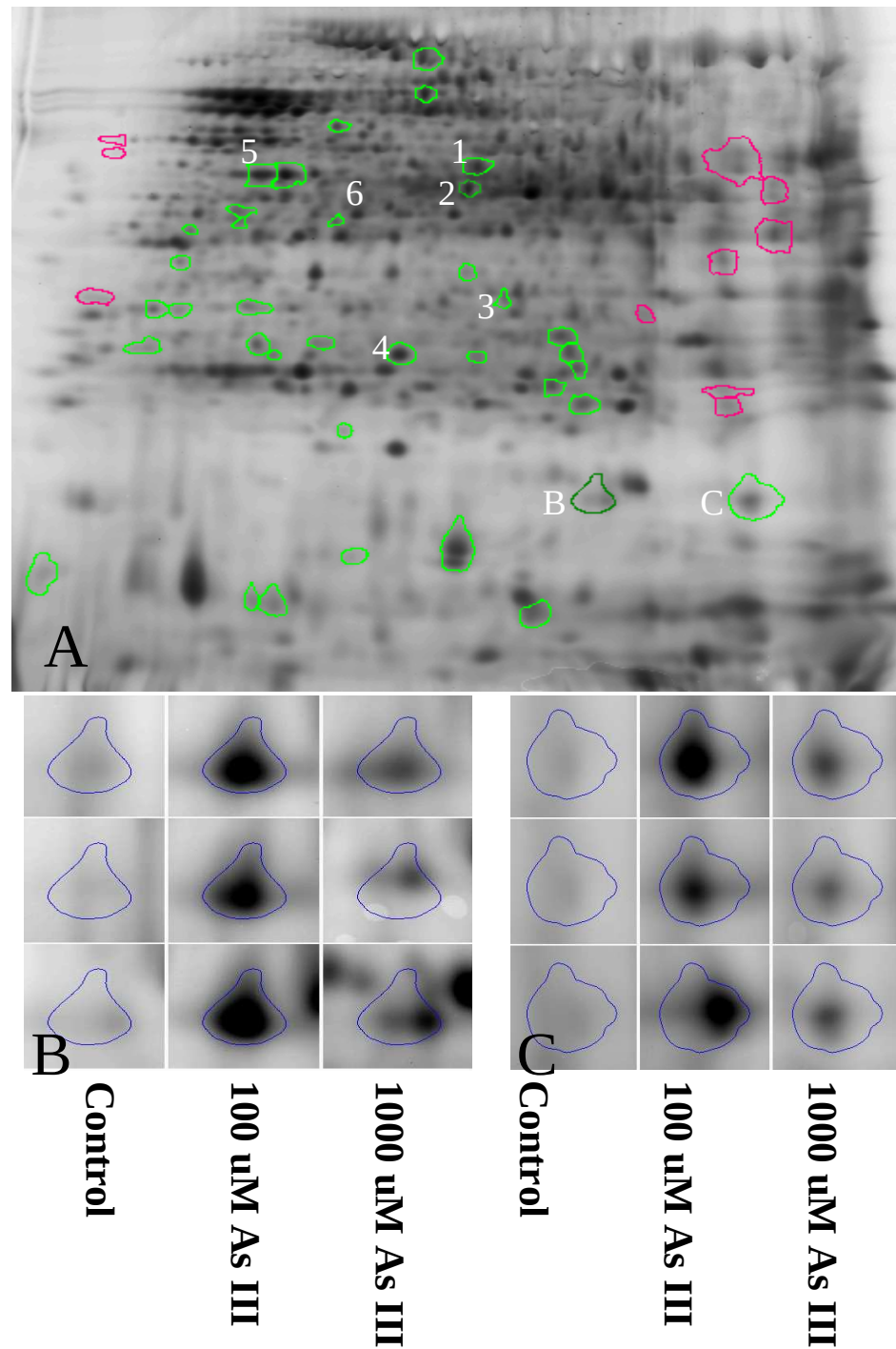


Figure 21. Redox-sensitive profile of *S. solfataricus* upon treatment with As (III). 2D gel represents 1000 uM As (III)-treated sample (A). Numbers on the gel correspond to “2D spot number” column in Table 6. Green circles indicate up-regulated and red circles down-regulated proteins. Comparison of protein thiol reactivity is shown on the example of peroxiredoxin (SSO2112; B) and lipase (SSO2493; C).

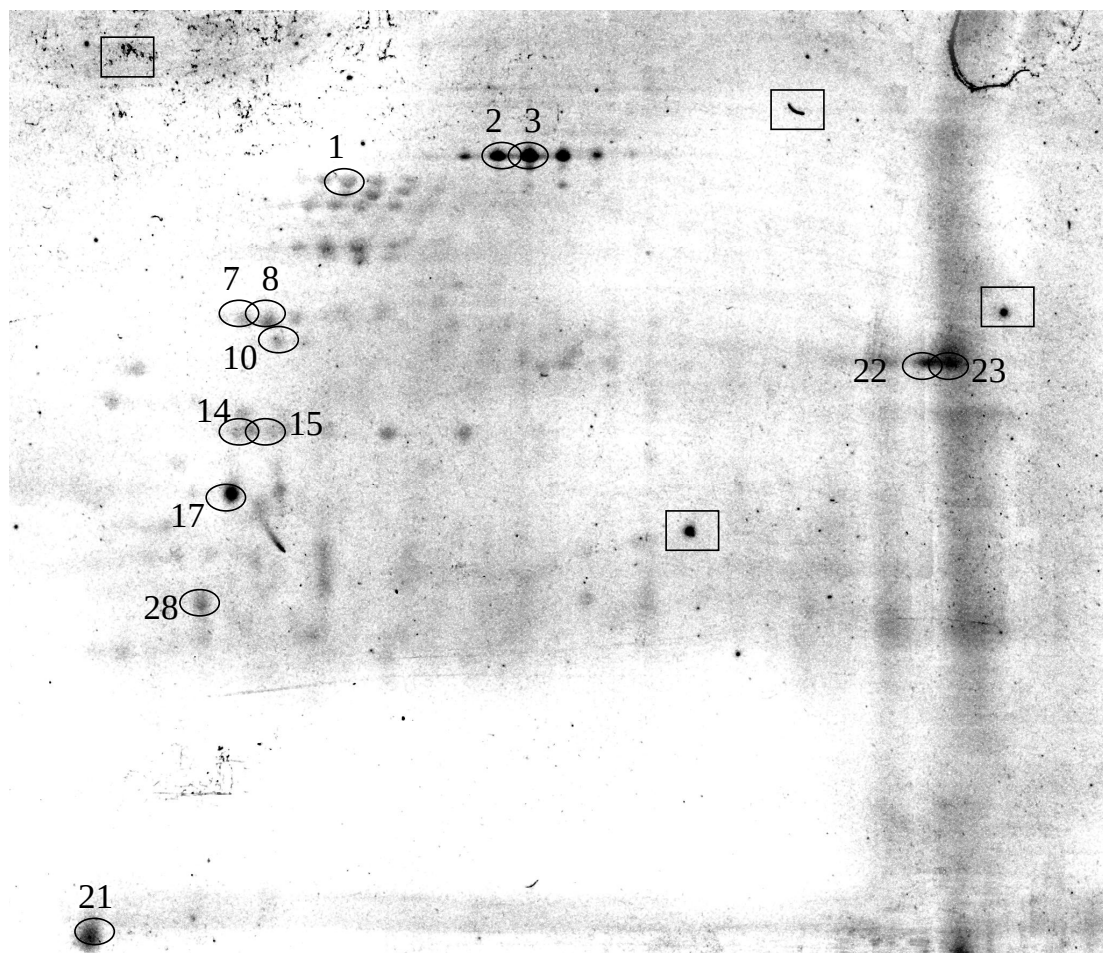


Figure 22. 2D gel of the *S. solfataricus* phosphoproteome 16 h after 1mM As (V) treatment. Gel was stained with phosphoprotein specific stain ProQ Diamond. Each of the numbered spots was picked and the proteins were identified using in-gel proteolysis followed LC-MS/MS. Squares show gel artifacts.

Discussion

The aim of this study was to investigate molecular responses in *S. solfataricus* during arsenic stress in order to understand arsenic metabolism and mechanisms of arsenic detoxification. To this end, proteins that changed in abundance in response to arsenite and arsenate exposure were determined. The list of proteins includes

dehydrogenases (NAD specific glutamate dehydrogenase, succinate dehydrogenase, isocitrate dehydrogenase, etc.), ROS scavenging systems (peroxiredoxin), protein turnover (HSPs, proteases, ribosomal proteins, translation factors, etc.) and others (see tables 4 and 5). A number of the up-regulated proteins like thermosome, peroxiredoxin and elongation factor-1 alpha found in this study are consistent with a general oxidative stress response. A similar set of proteins was found in H₂O₂ induced oxidative stress experiment.

Among the proteins related to arsenic stress management are α and β sub-units of the thermosome, the only representative of HSP60 class of molecular chaperones in *S. solfataricus*. This protein play intrinsic role in protein refolding. Thus, repair of arsenic induced damage and resistance may be enhanced by up-regulation of the chaperonin activity. Eukaryotic cells have a similar response in which the expression of various HSPs is induced by arsenite, the most potent inducer of these chaperonins in cells and organs (Del Razo, Quintanilla-Vega et al. 2001). Another protein involved in repair of arsenic damage is peroxiredoxin (SSO2121), which was discussed earlier. Briefly, it scavenges for H₂O₂ in *E. coli* (Seaver and Imlay 2001) and, most importantly, in *S. solfataricus* (Limauro, Pedone et al. 2008). In addition, peroxiredoxin interacts with translation elongation factor in mice to protect cells against apoptotic death induced by oxidative stress (Chang and Wang 2007). An increase in population of H₂O₂ may happen because of the arsenite oxidation to its pentavalent form (Masullo, Raimo et al. 1996). In fact, recent studies indicate that the expression of peroxiredoxin is induced by the presence of As (III) in humans (Lynne et al. 2000). Moreover, in the present work

peroxiredoxin (SSO2121) showed the biggest fold change in cysteine reactivity in response to As (III).

Another regulated protein is S-adenosyl-L-homocysteine hydrolase. It was found to be down-regulated upon As (III) treatment. S-adenosyl-L-homocysteine hydrolase catalyzes the hydrolysis of S-adenosyl-L-homocysteine to form adenosine and homocysteine. Since S-adenosyl-L-homocysteine is a potent inhibitor of S-adenosyl-L-methionine dependent methyltransferases, S-adenosyl-L-homocysteine hydrolase plays a critical role in the modulation of the activity of specific methyltransferases (Figure 23). It is known that the methylation of As (III) in mammalian species is catalyzed by specific S-adenosyl-L-methionine methyltransferases, where S-adenosyl-L-methionine is used as a methyl group donor (Zakharyan, Wu et al. 1995). As discussed above, methylation is considered as a major detoxification pathway in mammals; however, *S. Solfataricus* may employ different mechanisms. In support of this theory, down-regulation of S-adenosyl-L-homocysteine hydrolase results in less methylation of As (III) species. Interestingly, S-Adenosylmethionine synthetase (SSO0199), which catalyzes formation of the primary biological alkylating agent, was not found to be regulated in response to either arsenate or arsenite. This observation indirectly supports the hypothesis that methylation is not the only source of detoxification in *S. Solfataricus*. In human cells, trivalent monomethylated species were found to be the most toxic and dimethylated ones at least as toxic as arsenite (Styblo, Del Razo et al. 2000). However, pentavalent mono- and dimethylated arsenicals are less acutely toxic in human cells than arsenite. In current study, further research is

needed to figure out what organic forms of arsenic are less or more toxic for *S. solfataricus*.

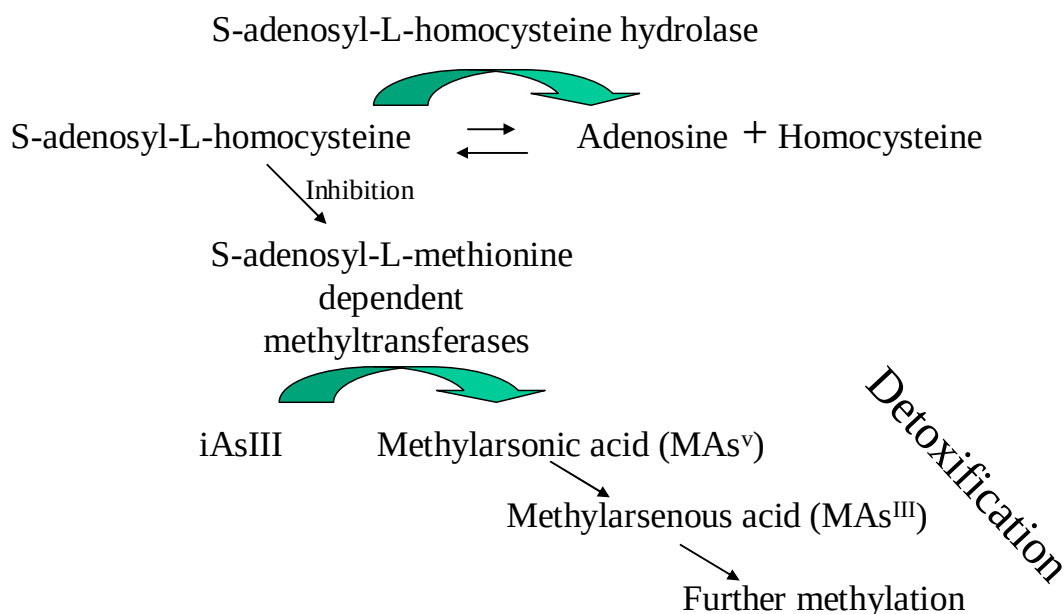


Figure 23. General scheme of arsenite methylation.

NAD specific glutamate dehydrogenase (SSO2044) which is a member of the oxidoreductase superfamily was also regulated. It participates in the glutamate metabolism pathway. Glutamate metabolism is integrated with other pathways such as glutathione metabolism, a major contributor to antioxidant protection in many cell types. However, there are no evidences of existence of glutathione synthase in *S. solfataricus*. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme which obviously has a role outside of glycolysis, particularly acting as an arsenate reductase in mammalian systems (Gregus and Nemeti 2005). In the current study, GAPDH expression was increased by the treatment of As (III) suggesting that it is somehow involved in the As detoxification mechanism in *Sulfolobus*.

Lipase (SSO2493) showed significant changes in cysteine reactivity in response to As (III) exposure. As it was mentioned, the reactivity of individual protein thiols to oxidation can be part of signal transduction pathways (Leichert and Jakob 2004). What type of signal pathway lipase is involved in is not clear yet. Interestingly, expression of other proteases, such as tricorn protease homolog (SSO2098), proteasome (SSO0738), enolase (SSO0913), was induced in response to arsenic stress. Some of them are employed by the cell to break down abnormally folded proteins, damaged by deleterious effects of environmental stress. Thus, the regulation of these proteases in *S. solfataricus* during the stress is very important.

The effect of As (V) treatment on protein phosphorylation is also intriguing. The map of phosphorylated proteins does not significantly overlap with the one, obtained in H₂O₂ study. It suggests that phosphorylation/dephosphorylation events are governed differently when the cells are exposed to phosphate-mimicking As (V). Elongation factor-1 alpha (SSO0216) and ribosomal protein L12 (SSO0342) were among the phospho-stained proteins present on both lists. The detailed characterization of specific protein phosphorylation has only been described for a few *Sulfolobus* proteins (Kim and Lee 2005; Ray, Keith et al. 2005). Thus, additional studies are required to understand the importance of protein phosphorylation in *Sulfolobus* during As (V) stress. Most likely, a mixed regulatory mechanism combining phosphorylation and the oxidation/reduction of sulfhydryl groups exists.

In the present study, comparative proteomic analyses led to identification of 40 differentially expressed proteins that include predicted and novel ones, which are

functionally involved in stress and detoxification, cell signaling, defense and development and protein biosynthesis mechanisms. Results of the findings suggest that As stress in *Sulfolobus* operates two different mechanisms: 1) generation of ROS, which could ultimately affect several pathways; 2) inhibition of methyltransferase activity, which most likely affects detoxification efficiency. Based on the proteomic investigations, As-stress responsive mechanism partially overlaps with hydrogen peroxide-induced stress response. Among the proteins identified in this study lipase, thiosulfate sulfurtransferase, DNA-primase and others (Figure 24) were regulated by both As (III) and As (V), but not by H₂O₂ treatment, suggesting that they might play very important roles in As-induced stress response.

CHAPTER 4

CONCLUSIONS

This study is the first to provide system-based evidence that complex response networks are activated in *S. solfataricus* in response to environmental stress. Components of these networks that participate in anti-oxidant and detoxifying activities were identified. In addition, changes to the phosphoproteome, and proteome redox state upon exposure to environmental stress were documented. Synthesis of the results has allowed a general scheme for how *S. solfataricus* fights H₂O₂- and As-induced stress was proposed.

The stress regulated proteins are from a range of functional classes, including oxidoreductases, hydrolases, dehydrogenases, proteins involved in primary metabolic processes and many others. Among the proteins, which are known to be involved in general stress response, are peroxiredoxin, superoxide dismutase, HSPs, elongation factor 1 and oxidoreductase. Interestingly, some of them were exclusively regulated by one stressor (H₂O₂) but not others (As III, As V). For example, DPSL protein was up-regulated only upon H₂O₂ stress and was not induced by metal (As). In order to visualize proteins that are universally regulated in response to different stressful conditions, network analysis was made, using the data from proteomics experiments described here (Figure 23). 8 out of 51 proteins were found to be regulated in response to both arsenate (As V) and arsenite (As III) stress (Figure 24), while six of them are likely to be universal stress proteins, because their expression was induced in response to both hydrogen

peroxide and arsenic (Figure 25). This suggests the existence of the common detoxification mechanisms against different toxic substances.

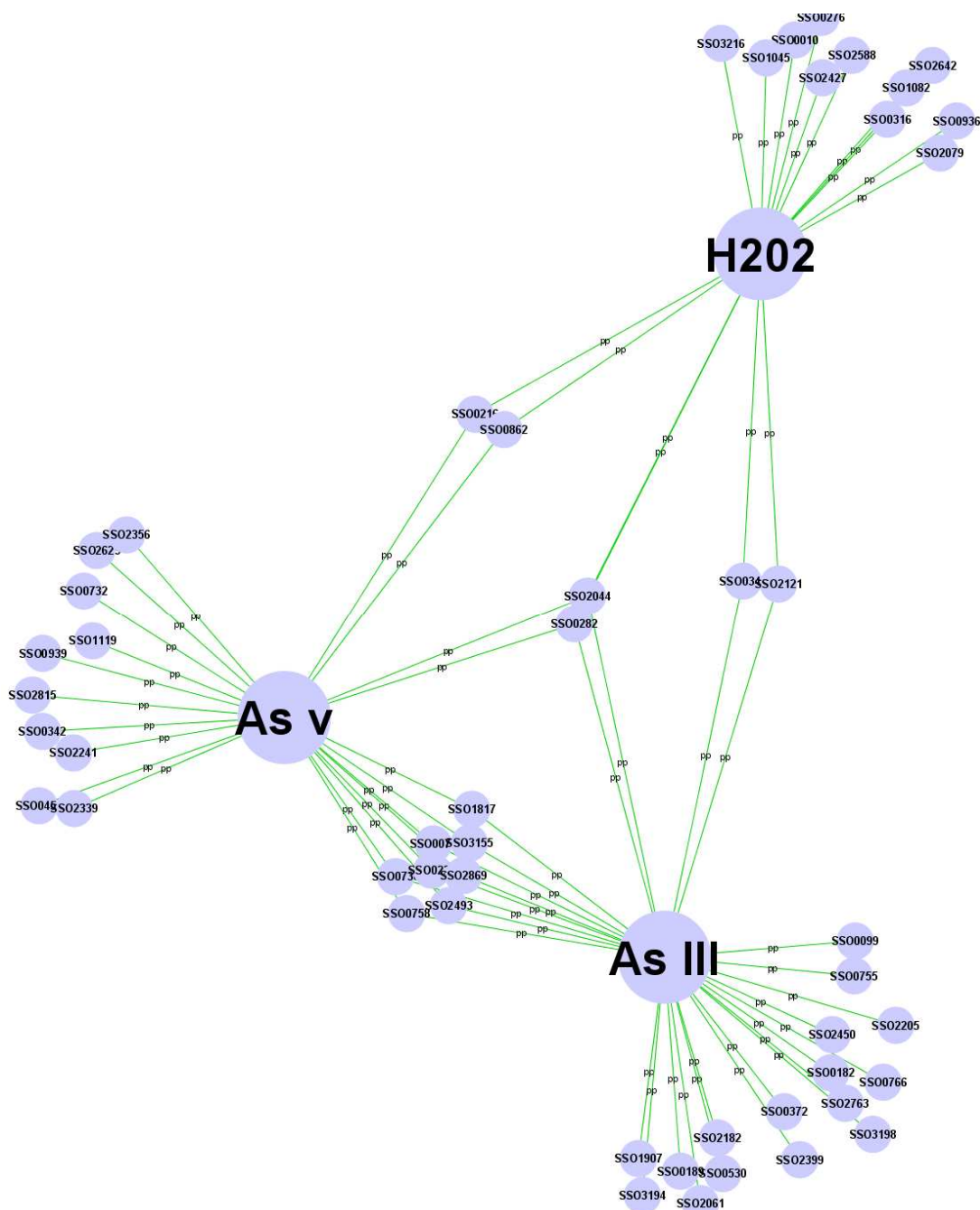


Figure 23. Network of *S. solfataricus* proteins, found to be regulated in response to hydrogen peroxide (H₂O₂), arsenate (As^V) and arsenite (As^{III}) stress.

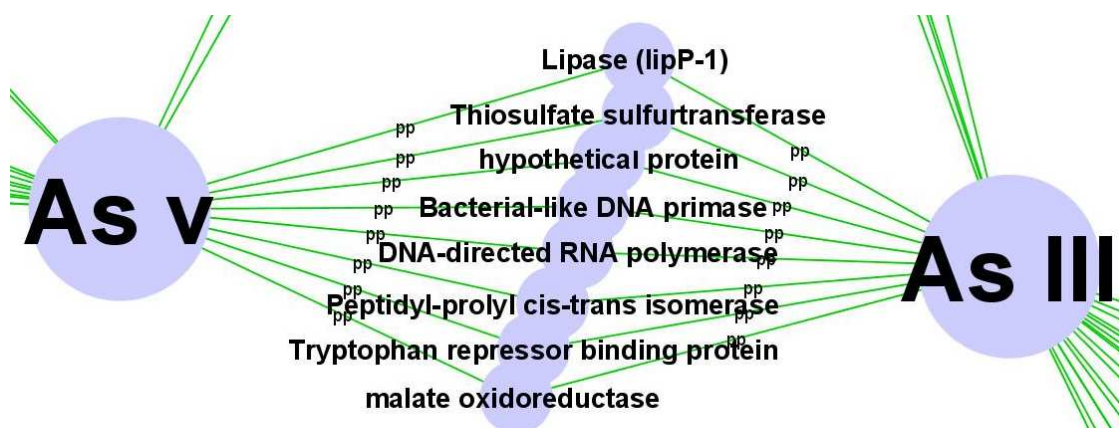


Figure 24. *S. solfataricus* proteins found to be regulated in response to both arsenate (As V) and arsenite (As III) stress. Close-up image from Figure 23.

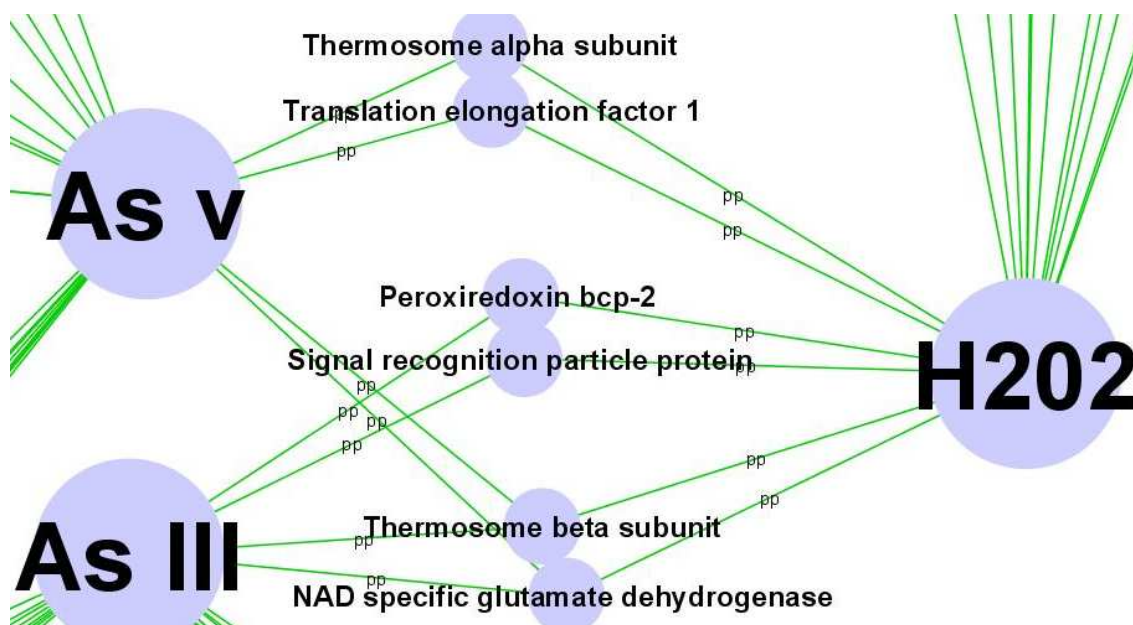


Figure 25. *S. solfataricus* proteins that are most likely involved in universal stress response. Close-up image from Figure 23.

The two-dimensional gel electrophoresis approach in conjunction with novel chemical tagging probes has been applied to investigate changes on the level of regulation and chemical modification of individual proteins within the scope of the whole proteome, in response to multiple types of environmental stress. Detection of changes in thiol reactivity, phosphorylation and enzymatic activity of proteins, separated by gel

electrophoresis, has been successfully accomplished. This “cutting-edge” methods used here can be easily adapted to any organism to elucidate a response to external stimuli, which makes this an extremely powerful technique.

The studies reported here have elucidated specific proteins and interacting pathways activated in response to stress in an archaeal organism. Future studies should continue characterizing stress responses in *S. solfataricus*. Potential stressors to be investigated are metalloids such as antimony and selenium, which are priority pollutants. The role of membrane proteins is clearly important in cellular response to environmental agents and because they are hydrophobic, the methods may not have adequately addressed this issue. Straight-forward modifications to the methods used here can be made to better investigate this important class of proteins. Studies aimed at covering the entire proteome using a wider range of stress agents will undoubtedly lead to a better understanding of the precise mechanisms and networks active in *S. solfataricus* and extremophiles in general.

BIBLIOGRAPHY

- Adams, M. W. W., F. E. Jenney, et al. (2002). "Superoxide reductase: fact or fiction?" Journal of Biological Inorganic Chemistry **7**(6): 647-652.
- Aposhian, H. V. (1997). "Enzymatic methylation of arsenic species and other new approaches to arsenic toxicity." Annual Review of Pharmacology and Toxicology **37**: 397-419.
- Auernik, K. S., C. R. Cooper, et al. (2008). "Life in hot acid: Pathway analyses in extremely thermoacidophilic archaea." Current Opinion in Biotechnology **19**(5): 445-453.
- Bell, S. D., and S. P. Jackson. (1998). "Transcription and translation in Archaea: a mosaic of eukaryal and bacterial features." Trends Microbiol **6**: 222-8.
- Bell, S. D., C. Jaxel, M. Nadal, P. F. Kosa, and S. P. Jackson. (1998). "Temperature, template topology, and factor requirements of archaeal transcription." Proc Natl Acad Sci U S A **95**: 15218-22.
- Camerini, S., M. L. Polci, et al. (2005). "Proteomics approaches to study the redox state of cysteine-containing proteins." Ann Ist Super Sanita **41**(4): 451-7.
- Carlinoz, A. and D. Touati (1986). "Isolation of Superoxide-Dismutase Mutants in Escherichia-Coli - Is Superoxide-Dismutase Necessary for Aerobic Life." Embo Journal **5**(3): 623-630.
- Chaban, B., S. Y. Ng, and K. F. Jarrell. (2006). "Archaeal habitats--from the extreme to the ordinary." Can J Microbiol **52**: 73-116.
- Chan, E. W., S. Chattopadhyaya, et al. (2004). "Developing photoactive affinity probes for proteomic profiling: hydroxamate-based probes for metalloproteases." J Am Chem Soc **126**(44): 14435-46.
- Chang, R. Y. and E. Wang (2007). "Mouse translation elongation factor eEF1A-2 interacts with Prdx-I to protect cells against apoptotic death induced by oxidative stress." Journal of Cellular Biochemistry **100**(2): 267-278.
- Chen, E., G. Proestou, et al. (2000). "Rapid up-regulation of peptide elongation factor EF-1 alpha protein levels is an immediate early event during oxidative stress-induced apoptosis." Experimental Cell Research **259**(1): 140-148.

- Ciaramella, M., F. M. Pisani, and M. Rossi. (2002). "Molecular biology of extremophiles: recent progress on the hyperthermophilic archaeon *Sulfolobus*." Antonie Van Leeuwenhoek **81**: 85-97.
- Ciccarelli, F. D., T. Doerks, et al. (2006). "Toward automatic reconstruction of a highly resolved tree of life." Science **311**(5765): 1283-1287.
- Connon, S. A., A. K. Koski, et al. (2008). "Ecophysiology and geochemistry of microbial arsenic oxidation within a high arsenic, circumneutral hot spring system of the Alvord Desert." Fems Microbiology Ecology **64**(1): 117-128.
- Del Razo, L. M., B. Quintanilla-Vega, et al. (2001). "Stress proteins induced by arsenic." Toxicology and Applied Pharmacology **177**(2): 132-148.
- DeVendittis, E. and V. Bocchini (1996). "Protein-encoding genes in the sulfothermophilic archaea *Sulfolobus* and *Pyrococcus*." Gene **176**(1-2): 27-33.
- Dopson, M., C. Baker-Austin, et al. (2003). "Growth in sulfidic mineral environments: metal resistance mechanisms in acidophilic micro-organisms." Microbiology-Sgm **149**: 1959-1970.
- Dowling, P., L. O'Driscoll, et al. (2006). "Proteomic screening of glucose-responsive and glucose non-responsive MIN-6 beta cells reveals differential expression of proteins involved in protein folding, secretion and oxidative stress." Proteomics **6**(24): 6578-87.
- Edgcomb, V. P., S. J. Molyneaux, et al. (2004). "Sulfide ameliorates metal toxicity for deep-sea hydrothermal vent archaea." Applied and Environmental Microbiology **70**(4): 2551-2555.
- Farr, S. B. and T. Kogoma (1991). "Oxidative Stress Responses in *Escherichia-Coli* and *Salmonella-Typhimurium*." Microbiological Reviews **55**(4): 561-585.
- Finkel, T. (1998). "Oxygen radicals and signaling." Current Opinion in Cell Biology **10**(2): 248-253.
- Flora, S. J. S. (1999). "Arsenic-induced oxidative stress and its reversibility following combined administration of N-acetylcysteine and meso 2,3-dimercaptosuccinic acid in rats." Clinical and Experimental Pharmacology and Physiology **26**(11): 865-869.
- Fonovic, M., S. H. Verhelst, et al. (2007). "Proteomics evaluation of chemically cleavable activity-based probes." Mol Cell Proteomics **6**(10): 1761-70.

- Freije, J. R. and R. Bischoff (2003). "Activity-based enrichment of matrix metalloproteinases using reversible inhibitors as affinity ligands." J Chromatogr A **1009**(1-2): 155-69.
- Gihring, T. M. and J. F. Banfield (2001). "Arsenite oxidation and arsenate respiration by a new *Thermus* isolate." Fems Microbiology Letters **204**(2): 335-340.
- Golyshina, O. V. and K. N. Timmis (2005). "Ferroplasma and relatives, recently discovered cell wall-lacking archaea making a living in extremely acid, heavy metal-rich environments." Environmental Microbiology **7**(9): 1277-1288.
- Gorg, A., W. Weiss, et al. (2004). "Current two-dimensional electrophoresis technology for proteomics." Proteomics **4**(12): 3665-3685.
- Gredmark, S., C. Schlieker, et al. (2007). "A functional ubiquitin-specific protease embedded in the large tegument protein (ORF64) of murine gammaherpesvirus 68 is active during the course of infection." J Virol **81**(19): 10300-9.
- Greenbaum, D. C., W. D. Arnold, et al. (2002). "Small molecule affinity fingerprinting. A tool for enzyme family subclassification, target identification, and inhibitor design." Chem Biol **9**(10): 1085-94.
- Gregus, Z. and B. Nemeti (2005). "The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase works as an arsenate reductase in human red blood cells and rat liver cytosol." Toxicological Sciences **85**(2): 859-869.
- Halliwell, B. and J. M. C. Gutteridge (1990). "Role of Free-Radicals and Catalytic Metal-Ions in Human-Disease - an Overview." Methods in Enzymology **186**: 1-85.
- Hanford, M. and T. L. Peeples (2002). "Archaeal tetraether lipids - Unique structures and applications." Applied Biochemistry and Biotechnology **97**(1): 45-62.
- Hartley-Whitaker, J., G. Ainsworth, et al. (2001). "Copper- and arsenate-induced oxidative stress in *Holcus lanatus* L. clones with differential sensitivity." Plant Cell and Environment **24**(7): 713-722.
- Hetzer, A., H. Morgan, et al. (2007). "Microbial life in a hydrothermal spring rich in arsenic compounds." Geochimica Et Cosmochimica Acta **71**(15): A401-a401.
- Hetzer, A., H. W. Morgan, et al. (2007). "Microbial life in champagne pool, a geothermal spring in Waitapu, New Zealand." Extremophiles **11**(4): 605-614.

- Huber, H., M. J. Hohn, R. Rachel, T. Fuchs, V. C. Wimmer, and K. O. Stetter. (2002). "A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont." Nature **417**: 63-7.
- Jeon, S. J. and K. Ishikawa (2003). "Characterization of novel hexadecameric thioredoxin peroxidase from *Aeropyrum pernix* K1." Journal of Biological Chemistry **278**(26): 24174-24180.
- Kandler, O. and H. König (1998). "Cell wall polymers in Archaea (Archaeobacteria)." Cellular and Molecular Life Sciences **54**(4): 305-308.
- Kashima, Y. and K. Ishikawa (2003). "Alkyl hydroperoxide reductase dependent on thioredoxin-like protein from *Pyrococcus horikoshii*." Journal of Biochemistry **134**(1): 25-29.
- Kato, D., K. M. Boatright, et al. (2005). "Activity-based probes that target diverse cysteine protease families." Nat Chem Biol **1**(1): 33-8.
- Kidd, D., Y. Liu, et al. (2001). "Profiling serine hydrolase activities in complex proteomes." Biochemistry **40**(13): 4005-15.
- Kim, S. and S. B. Lee (2005). "Identification and characterization of *Sulfolobus solfataricus* D-gluconate dehydratase: a key enzyme in the non-phosphorylated Entner-Doudoroff pathway." Biochem J **387**(Pt 1): 271-80.
- Kumar, S., B. Zhou, et al. (2004). "Activity-based probes for protein tyrosine phosphatases." Proc Natl Acad Sci U S A **101**(21): 7943-8.
- Langner, H. W., C. R. Jackson, et al. (2001). "Rapid oxidation of arsenite in a hot spring ecosystem, Yellowstone National Park." Environmental Science & Technology **35**(16): 3302-3309.
- Leichert, L. I. and U. Jakob (2004). "Protein thiol modifications visualized in vivo." Plos Biology **2**(11): 1723-1737.
- Liang, F., S. Kumar, et al. (2007). "Proteomic approaches to studying protein tyrosine phosphatases." Mol Biosyst **3**(5): 308-16.
- Limauro, D., E. Pedone, et al. (2008). "Peroxiredoxins as cellular guardians in *Sulfolobus solfataricus*: characterization of Bcp1, Bcp3 and Bcp4." Febs J **275**(9): 2067-77.
- Limauro, D., E. Pedone, et al. (2006). "Identification and characterization of 1-Cys peroxiredoxin from *Sulfolobus solfataricus* and its involvement in the response to oxidative stress." Febs J **273**(4): 721-31.

- Liu, Y., M. P. Patricelli, et al. (1999). "Activity-based protein profiling: the serine hydrolases." Proc Natl Acad Sci U S A **96**(26): 14694-9.
- Lynn, S., J. N. Shiung, et al. (1998). "Arsenite stimulates poly(ADP-ribosylation) by generation of nitric oxide." Free Radical Biology and Medicine **24**(3): 442-449.
- Maaty, W. S. A., A. C. Ortmann, et al. (2006). "Characterization of the archaeal thermophile *Sulfolobus* turreted icosahedral virus validates an evolutionary link among double-stranded DNA viruses from all domains of life." Journal of Virology **80**(15): 7625-7635.
- Macur, R. E., C. R. Jackson, et al. (2004). "Bacterial populations associated with the oxidation and reduction of arsenic in an unsaturated soil." Environmental Science & Technology **38**(1): 104-111.
- Masullo, M., G. Raimo, et al. (1996). "Purification and characterization of NADH oxidase from the archaea *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus*." Biotechnology and Applied Biochemistry **23**: 47-54.
- Mccord, J. M. and Fridovic.I (1969). "Superoxide Dismutase-an Enzymic Function for Erythrocyte." Federation Proceedings **28**(2): 346-&.
- Meharg, A. A. and M. R. Macnair (1992). "Suppression of the High-Affinity Phosphate-Uptake System - a Mechanism of Arsenate Tolerance in *Holcus-Lanatus* L." Journal of Experimental Botany **43**(249): 519-524.
- Missall, T. A., J. K. Lodge, et al. (2004). "Mechanisms of resistance to oxidative and nitrosative stress: implications for fungal survival in mammalian hosts." Eukaryot Cell **3**(4): 835-46.
- Mylona, P. V., A. N. Polidoros, et al. (1998). "Modulation of antioxidant responses by arsenic in maize." Free Radical Biology and Medicine **25**(4-5): 576-585.
- Niimura, Y., Y. Nishiyama, et al. (2000). "A hydrogen peroxide-forming NADH oxidase that functions as an alkyl hydroperoxide reductase in *Amphibacillus xylanus*." Journal of Bacteriology **182**(18): 5046-5051.
- Nishiyama, Y., V. Massey, et al. (2001). "Hydrogen peroxide-forming NADH oxidase belonging to the peroxiredoxin oxidoreductase family: Existence and physiological role in bacteria." Journal of Bacteriology **183**(8): 2431-2438.
- Paez-Espino, D., J. Tamames, et al. (2009). "Microbial responses to environmental arsenic." Biometals **22**(1): 117-130.

- Pappin, D. J. C., P. Hojrup, et al. (1993). "Rapid Identification of Proteins by Peptide-Mass Fingerprinting." Current Biology **3**(6): 327-332.
- Pedone, E., D. Limauro, et al. (2006). "Characterization of a multifunctional protein disulfide oxidoreductase from *Sulfolobus solfataricus*." Febs Journal **273**(23): 5407-5420.
- Qureshi, S. A., S. D. Bell, and S. P. Jackson. (1997). "Factor requirements for transcription in the Archaeon *Sulfolobus shibatae*." Embo J **16**: 2927-36.
- Rabilloud, T., M. Heller, et al. (2002). "Proteomics analysis of cellular response to oxidative stress - Evidence for in vivo overoxidation of peroxiredoxins at their active site." Journal of Biological Chemistry **277**(22): 19396-19401.
- Rawlings, N. D. and A. J. Barrett (1994). "Families of Serine Peptidases." Proteolytic Enzymes: Serine and Cysteine Peptidases **244**: 19-61.
- Ray, W. K., S. M. Keith, et al. (2005). "A phosphohexomutase from the archaeon *Sulfolobus solfataricus* is covalently modified by phosphorylation on serine." J Bacteriol **187**(12): 4270-5.
- Remonsellez, F., A. Orell, et al. (2006). "Copper tolerance of the thermoacidophilic archaeon *Sulfolobus metallicus*: possible role of polyphosphate metabolism." Microbiology-Sgm **152**: 59-66.
- Rhee, S. G. (2003). "Intracellular messenger function of hydrogen peroxide." Free Radical Biology and Medicine **35**: S7-S7.
- Rosenzweig, A. C. (2002). "Metallochaperones: Bind and deliver." Chemistry & Biology **9**(6): 673-677.
- Saghatelian, A., N. Jessani, et al. (2004). "Activity-based probes for the proteomic profiling of metalloproteases." Proc Natl Acad Sci U S A **101**(27): 10000-5.
- Saha, J. C., A. K. Dikshit, et al. (1999). "A review of arsenic poisoning and its effects on human health." Critical Reviews in Environmental Science and Technology **29**(3): 281-313.
- Schmidinger, H., A. Hermetter, et al. (2006). "Activity-based proteomics: enzymatic activity profiling in complex proteomes." Amino Acids **30**(4): 333-50.

- Schulenberg, B., R. Aggeler, et al. (2003). "Analysis of steady-state protein phosphorylation in mitochondria using a novel fluorescent phosphosensor dye." Journal of Biological Chemistry **278**(29): 27251-27255.
- Seaver, L. C. and J. A. Imlay (2001). "Hydrogen peroxide fluxes and compartmentalization inside growing *Escherichia coli*." Journal of Bacteriology **183**(24): 7182-7189.
- Sehlin, H. M. and E. B. Lindstrom (1992). "Oxidation and Reduction of Arsenic by *Sulfolobus-Acidocaldarius* Strain Bc." Fems Microbiology Letters **93**(1): 87-92.
- She, Q., R. K. Singh, F. Confalonieri, Y. Zivanovic, G. Allard, M. J. Awayez, C. C. Chan-Weiher, I. G. Clausen, B. A. Curtis, A. De Moors, G. Erauso, C. Fletcher, P. M. Gordon, I. Heikamp-de Jong, A. C. Jeffries, C. J. Kozera, N. Medina, X. Peng, H. P. Thi-Ngoc, P. Redder, M. E. Schenk, C. Theriault, N. Tolstrup, R. L. Charlebois, W. F. Doolittle, M. Duguet, T. Gaasterland, R. A. Garrett, M. A. Ragan, C. W. Sensen, and J. Van der Oost. (2001). "The complete genome of the crenarchaeon *Sulfolobus solfataricus* P2." Proc Natl Acad Sci U S A **98**: 7835-40.
- Sieber, S. A., T. S. Mondala, et al. (2004). "Microarray platform for profiling enzyme activities in complex proteomes." J Am Chem Soc **126**(48): 15640-1.
- Silver, S. (1996). "Bacterial resistances to toxic metal ions - A review." Gene **179**(1): 9-19.
- Snyder, J. C., K. Stedman, G. Rice, B. Wiedenheft, J. Spuhler, and M. J. Young. (2003). "Viruses of hyperthermophilic Archaea." Res Microbiol **154**: 474-82.
- Stamler, J. S. and A. Hausladen (1998). "Oxidative modifications in nitrosative stress." Nat Struct Biol **5**(4): 247-9.
- Stamler, J. S., S. Lamas, et al. (2001). "Nitrosylation. the prototypic redox-based signaling mechanism." Cell **106**(6): 675-83.
- Stohs, S. J. and D. Bagchi (1995). "Oxidative Mechanisms in the Toxicity of Metal-Ions." Free Radical Biology and Medicine **18**(2): 321-336.
- Storz, G. and J. A. Imlay (1999). "Oxidative stress." Current Opinion in Microbiology **2**(2): 188-194.
- Styblo, M., L. M. Del Razo, et al. (2000). "Comparative toxicity of trivalent and pentavalent inorganic and methylated arsenicals in rat and human cells." Archives of Toxicology **74**(6): 289-299.

- Sun, J., C. Steenbergen, et al. (2006). "S-nitrosylation: NO-related redox signaling to protect against oxidative stress." Antioxid Redox Signal **8**(9-10): 1693-705.
- Tomoyasu, T., A. Mogk, et al. (2001). "Genetic dissection of the roles of chaperones and proteases in protein folding and degradation in the Escherichia coli cytosol." Molecular Microbiology **40**(2): 397-413.
- Tonge, R., J. Shaw, et al. (2001). "Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology." Proteomics **1**(3): 377-396.
- Touati, D. (2000). "Iron and oxidative stress in bacteria." Archives of Biochemistry and Biophysics **373**(1): 1-6.
- Unlu, M., M. E. Morgan, et al. (1997). "Difference gel electrophoresis: A single gel method for detecting changes in protein extracts." Electrophoresis **18**(11): 2071-2077.
- van der Oost, J., M. Ciaramella, M. Moracci, F. M. Pisani, M. Rossi, and W. M. de Vos. (1998). "Molecular biology of hyperthermophilic Archaea." Adv Biochem Eng Biotechnol **61**: 87-115.
- van Swieten, P. F., R. Maehr, et al. (2004). "Development of an isotope-coded activity-based probe for the quantitative profiling of cysteine proteases." Bioorg Med Chem Lett **14**(12): 3131-4.
- Watanabe, Y., S. Yokobori, et al. (2002). "Introns in protein-coding genes in Archaea." Febs Letters **510**(1-2): 27-30.
- Weinberg, M. V., F. E. Jenney, et al. (2004). "Rubrerythrin from the hyperthermophilic archaeon Pyrococcus furiosus is a rubredoxin-dependent, iron-containing peroxidase." Journal of Bacteriology **186**(23): 7888-7895.
- Wiedenheft, B., J. Mosolf, et al. (2005). "An archaeal antioxidant: characterization of a Dps-like protein from Sulfolobus solfataricus." Proc Natl Acad Sci U S A **102**(30): 10551-6.
- Woese, C. R. and G. E. Fox (1977). "Phylogenetic Structure of Prokaryotic Domain - Primary Kingdoms." Proceedings of the National Academy of Sciences of the United States of America **74**(11): 5088-5090.
- Woese, C. R., O. Kandler, and M. L. Wheelis. (1990). "Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya." Proc Natl Acad Sci U S A **87**: 4576-9.

- Wong, H. and M. C. Schotz (2002). "The lipase gene family." Journal of Lipid Research **43**(7): 993-999.
- Zakharyan, R., Y. Wu, et al. (1995). "Enzymatic Methylation of Arsenic Compounds - Assay, Partial-Purification, and Properties of Arsenite Methyltransferase and Monomethylarsonic Acid Methyltransferase of Rabbit Liver." Chemical Research in Toxicology **8**(8): 1029-1038.
- Zhang, S., L. Chen, et al. (2007). "An affinity-based fluorescence polarization assay for protein tyrosine phosphatases." Methods **42**(3): 261-7.
- Zillig, W., H. P. Klenk, et al. (1989). "The Phylogenetic Relations of DNA-Dependent Rna-Polymerases of Archaeobacteria, Eukaryotes, and Eubacteria." Canadian Journal of Microbiology **35**(1): 73-80.