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A proteome analysis of *Corynebacterium glutamicum* after exposure to heavy metals (mercury, cadmium, cobalt, silver and lead)

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DEDICATION

To my lovely country Palestine

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Abbreviations:

2D	two-dimensional
AA	acrylamide
ADP	adenosine diphosphate
APS	ammoniumpersulfate
ATP	adenosine triphosphate
Bis	bisacrylamide
%C	percentage of crosslinker in acrylamide solution
CBB	Coomassie Brilliant Blue
Da	Dalton
DNA	desoxyribonucleic acid
DTT	dithiothreitol
GSH	glutathione
ESI	electropspray ionization
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide.
h	hour
HSP	heat shock protein
IEF	isoelectric focusing
IPG	immobilized pH gradient
IPG-DALT	two-dimensional electrophoresis with immobilized pH gradient
LC	liquid chromatography
Lys	lysine
MALDI	matrix assisted laser desorption ionization
Mr	relative molecular mass
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NAD	nicotinamide adenine dinucleotide
NBDs	nucleotide binding domains
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pl	isoelectric point
PMF	peptide mass fingerprint

RNA	ribonucleic acid
ROS	reactive oxygen species
SOD	superoxide dismutase
SDS	sodium dodecylsulfate
%T	percentage of acrylamide in total
TCA	citric acid cycle
TEMED	N,N,N',N'-tetramethylethylendiamine
TMDs	two transmembrane domains
TOF	time of flight
Tris	tris(hydroxymethyl)-aminomethane
Trx	thioredoxin reductase
V	volt
v/v	volume per volume
W	watt
w/v	weight per volume

1 Introduction

Over the last three decades there has been increasing global concern over the impacts on public health attributed to environmental pollution, in particular, the global burden of disease. The World Health Organization (WHO) estimates that about one quarter of the diseases mankind is faced with today occur due to prolonged exposure by environmental pollution. Most of these environment-related diseases are, however, not easily detected and may be acquired during childhood and manifested later in adulthood. Heavy metals are metallic elements that are present in both natural and contaminated environments. In natural environments, they occur at low concentrations. However, at high concentrations as is the case in contaminated environments, they result into public health impacts. The elements of concern include lead, mercury, cadmium, arsenic, chromium, zinc, nickel and copper. Heavy metals may be released into the environment from metal smelting and refining industries, scrap metal, plastic and rubber industries, various consumer products, and from burning of waste containing these elements. On release to the air, the elements travel for large distances and are deposited onto the soil, vegetation and water depending on their density. Once deposited, these metals are not degraded and persist in the environment for many years, poisoning humans through inhalation, ingestion and skin absorption. Acute exposure leads to nausea, anorexia, vomiting, gastrointestinal abnormalities and dermatitis.

From approximately five million well-known chemicals today, only 80,000 are used. However in the European market there are 30,000 chemicals, the most of them were not tested before neither on the toxic effect to health nor to environment. There are two main problems in the environmental analysis:

(i) Although in principle we are able to detect most different chemical substances in low concentrations with high accuracy, normally unknown or unexpected substances can not be detected. For example the analysis of many thousand compounds, which can be found in wastewater, is technically extremely difficult, if not impossible (Hoch and Seifert, 2002; Böhme and Hufe, 2004).

(ii) Chemical compounds, which can be detected by environmental analysis, their hazard effects could not be estimated, because for most pollutants either there are no, or only insufficient data exist about their toxic effect (Böhme and Hufe, 2004).

Although we can estimate the hazardous potential or toxicity of compounds with the help of biological tests due to their observed effect on organisms such as fish, water fleas, algae or

bacteria, by methods that permit us to obtain knowledge on the kind of toxicity, no accurate identification of the causal environmental chemicals can be acquired. In the case of waste water, an important question is posed as to which suitable method must be used, if it is not clear, which pollutants are present in waste water (Böhme and Hufe, 2004).

Therefore, the scientists are trying to use alternative methods by using biomarkers in order to recognize the pollutants and to detect their toxic effects (Oberemm, 2001; Oberemm et al., 2005). The principle of this detection procedure is based on the fact that the cells of living organisms on molecular level are flexible and prone to environmental changes. The exposure of cells to pollutants causes changes in some genes. Similar to physical or biological effects, the environmental chemicals cause induction of certain (stress) genes, depending on the pollutants; the stress response is either universal or highly specific. In this way it is possible to consider the expression of certain proteins or the activity of enzymes in animals, plants or microorganisms, which are involved in detoxification processes, as indicators for the exposure of environmental chemicals.

For example, if we detect the exposure of fish to Cytochrom P4501A in contaminated waters, we can estimate the pollutant spectrum (chlorinated aromatics) and on the other hand we can say that there is a toxic effect on the fish (Böhme and Hufe, 2004). Another well-known example is the overexpression of vitellogenin in male fish; it indicates that the fish was exposed to oestrogen active substances (Hoch and Seifert, 2002).

It is clear that the application of individual biomarkers supplies very little information about the identification and toxic effects of environmental chemicals (Oberemm, 2001; Oberemm et al., 2005). It is not possible to know which gene is activated or inactivated after the exposure to certain pollutants and/or a certain pollutant class.

Rapid advance in the field of molecular biology and protein chemistry enables the scientists now to use a new generation of alternative tests and detection methods. These methods give hope to establish fast and reliable detective procedures with the help of a wide spectrum of biomarkers. By using these biomarkers not only many different environmental chemicals can be identified, but also their toxic mechanisms can be characterized and better understood.

Even a relatively simple microorganism possesses thousands of different genes, which can be at least in theory differently regulated as reaction to exposure to environmental chemicals. Consequently an enormous potential exists to detect by global analysis of the differential gene expression the exposure to pollutants as well as to recognize their specific effects. The response of a cellular organism after the treatment or exposure to pollutants can be analyzed,

e.g., on transcriptom level, which means by the measurement of the change in gene expression using mRNA or DNA microarrays.

Alternatively to microarrays, the change in gene expression change can be also be analysed on protein level. This proteome analysis is usually carried out with the help of two-dimensional gel electrophoresis (2DE) and mass-spectrometry (MS) to identify differentially expressed proteins. By this way the post-translational modification of proteins can be determined, which is not possible with DNA microarrays.

1.1 Investigation of stress protein expression by proteome analysis

The proteome is the entire complement of proteins expressed by a genome, cell, tissue or organism. More specifically, it is the set of expressed proteins at a given time under defined conditions (Wilkins et al., 1996). Proteome analysis is dedicated to the survey of all proteins in a sample under a certain condition (Pandey and Mann, 2000). In consequence of the nature of the proteome, a method for proteome analysis needs to be capable of visualizing several thousand proteins in order to provide a most complete overview. Two-dimensional electrophoresis seems to be highly potential for proteome analysis. Classical 2D electrophoresis was established by O'Farrel who used carrier ampholytes to separate proteins in the 1st dimension according to their isoelectric point (*pI*), and SDS PAGE in the 2nd dimension for separation according to the molecular weight (Mr) (O'Farrell, 1975). However, the application of carrier ampholytes limits the potential of the method, because the pH gradient was not stable and, thus, protein separation was time dependent. In particular drifting of the alkaline carrier ampholytes towards the cathodic side and simultaneously the formation of a plateau in the middle of the pH gradient were observed. To overcome these problems of carrier ampholytes generated pH gradients, the concept of immobilised pH gradients was introduced in 1982 (Bjellqvist et al., 1982), and (IPG Dalt) developed by Görg et al (1988, 2000, 2004) has become the standard procedure in proteome analysis.

In current times, proteomics includes much more methods than the classical proteome analysis by 2D electrophoresis. Nearly the complete range of methods in protein biochemistry, as well as specialized applications downstream or upstream of 2D electrophoresis like protein fractionation or LC- MS/MS, joined the term proteomics (Simpson, 2004). High-performance liquid chromatography with tandem mass spectrometric (LC/MS/MS) has become the technique of choice. It can either be applied to the analysis of the actual 2D spots to improve the resolution of the separation or be used for the direct analysis of the protein mixture. This

approach, which is predominantly used as a qualitative tool, has significantly improved both the selectivity and sensitivity for complex biomolecules. Current methods in 2D electrophoresis as well as special analytical techniques applied in this thesis are briefly explained in the following sections.

Physiologically we can divide the proteome into two main groups, the first one includes proteins, which are components and responsible for growth and proliferation, and the second group is involved in the adjustment for nutrient starvation, or stress in general, and establishes an adaptive network, enabling the cell to protect itself from unsuitable environmental conditions (Auffray et al., 1992). The main fields for application of proteome analysis are traditionally placed in areas of medicine and pharmacy. By proteome analysis the identification and characterisation of differentially expressed proteins in a cell, tissue or an organism can be performed. It is possible that these proteins can be considered as biomarkers for specific diseases, or as target molecules for the development of new drugs.

This approach is applied in the field of toxicology and environmental analysis in order to find stress proteins (proteomics signatures), which can be considered as response (high specific biomarkers) due to the exposure of special environmental chemicals (Kilstrup et al., 1997). Proteomics signatures are subset proteins, which are induced after the treatment of the cell with stress factors (e.g. with environmental chemicals). They are present in treated or stressed cell but missing in untreated cells (control), or occur in small quantity (VanBogelen et al., 1999).

1.2 Aim of this study

Heavy metals are well-known toxic environmental compounds, but mechanisms of their toxicity are largely unknown. The analysis of the *C. glutamicum* proteome from cells grown in control and heavy metals enriched media was performed for comparison by two dimensional gel electrophoresis (2DE) followed by MALDI-TOF mass spectrometry. One aim of the study was the analysis of the heavy metals response of *C. glutamicum* with advanced proteomic tools to identify stress proteins involved in the heavy metals response, as some stress proteins could be considered as biomarkers for special heavy metals. These biomarkers can be used as an early warning system for heavy metals and protect the whole ecosystem from heavy metals. Identification of these proteins and their mapping into specific cellular processes may help to gain deeper insight into the toxic mechanisms of heavy metals.

Analysis of the stress response of *C. glutamicum* to heavy metals was another aim of the study to identify stress proteins, which are involved in the adaptive and protective mechanism of *C. glutamicum.* This enables to gain a global understanding of the way in which *C. glutamicum* counteract the heavy-metal stress exposure.

2 Theoretical Background

2.1 Stress proteins and biomarkers

The cellular stress response can be defined as a reaction to the threat of macromolecular damage. It comprises an evolutionarily highly conserved mechanism that protects cells from a sudden environmental change or frequent fluctuations in environmental factors.

Our current knowledge of the stress proteome, i.e. all the proteins involved in realizing the cellular stress response through induction, post-translational modification, or protein–protein/ DNA interaction, is still fragmentary and needs further elucidation.

The cellular stress response has been associated most clearly with protective effects during conditions that perturb both protein and DNA integrity. Many types of environmental stress have been shown to cause deleterious changes in protein conformation, including osmotic stress, thermal stress (Hochachka and Somero, 2002), heavy metal stress (Farrer and Pecoraro, 2002), ionizing radiation (Kempner, 1993). Moreover, the cellular stress response may play roles as yet poorly known for the stabilization of other macromolecules, such as lipid structures (membranes) and RNA. Thus, it is feasible to define the cellular stress response as a reaction to the threat of macromolecular damage (independent of the means by which such damage occurs). Cells respond to all types of stress by activating four basic mechanisms, all of which are aimed at stabilizing macromolecular structure and function during adverse, abnormal or pathological conditions, and at conserving metabolic energy for homeostatic adaptations. These four mechanisms and their transient activation can be regarded as cornerstones of the cellular stress response. They consist of: cell cycle checkpoint control leading to growth arrest – cell cycle checkpoints induced during stress in eukaryotic cells include the G1/S checkpoint (Bartek and Lukas, 2001), the G2/M checkpoint (Bulavin et al., 2002) and translational control mechanisms (Brostrom and Brostrom, 1998). Induction of molecular chaperones (HSPs) and protein stabilizers – molecular chaperones are commonly activated either by induction (Feder and Hofmann, 1999) or by post-translational modification, e.g. phosphorylation of HSP28 *via* the p38 MAP kinase signaling pathway (Kato et al., 2001).

activation of mechanisms for nucleic acid and chromatin stabilization and repair – for instance, eukaryotic pathways involved in DNA repair and chromatin stabilization include the p53 pathway (Harkin and Hall, 2000) and the NFkappaB pathway (Vermeulen et al., 2002). Removal of macromolecular debris generated by stress – this aspect of the cellular stress response is exemplified by the ubiquitin/ proteasome pathway (Fuchs et al., 1998).

Bio-monitoring which is a sub-branch of environmental monitoring is important because biological responses may be elicited at chemical concentrations below analytical detection limits or after chemical exposure has ceased (Rand et al., 1995). Also the term biomarker is referring to the use of physiological, biochemical, and histological changes as indicators of xenobiotic's effects, at the organismal or suborganismal level (Hugget et al., 1992). Therefore, biomarkers, based on biomonitoring program, have important information and biomonitoring programmer should ideally be able to demonstrate the effect of pollutants to individuals and preferably through to a population, community and ecosystem level. Thus, they can be used as an early warning system and protect the whole ecosystem from toxic chemicals which are released to it in different ways (Depledg, 1993). In practical terms, biomarkers are the endpoints of ecotoxicological tests that register an effect on living organisms (Lam, 2003).

2.2 Stress factor heavy metals

"Heavy metals" is an inexact term used to describe more than a dozen elements that are metals or metalloids (elements that have both metal and nonmetal characteristics). Examples of heavy metals include chromium, arsenic, cadmium, lead, mercury, and cobalt. Generally, heavy metals have densities above 5 g/cm^3. Because they cannot be degraded or destroyed, heavy metals are persistent in all parts of the environment.

The primary anthropogenic sources of heavy metals are point sources such as mines, foundries, smelters, and coal-burning power plants, as well as diffuse sources such as combustion by-products and vehicle emissions. Humans also affect the natural geological and biological redistribution of heavy metals by altering the chemical form of heavy metals released to the environment. Such alterations often affect a heavy metal's toxicity by allowing it to bioaccumulate in plants and animals, bioconcentrate in the food chain, or attack specific organs of the body. Heavy metals have been used in many different areas for thousands of years. Lead has been used for at least 5,000 years with early applications including building materials, pigments for glazing ceramics, and pipes for transporting water. In ancient Rome, lead acetate was used to sweeten old wine, and some Romans might have consumed as much as a gram of lead a day. Mercury was allegedly used by the Romans as a salve to alleviate teething pain in infants, and was later (from the 1300s to the late 1800s) employed as a remedy for syphilis. Cadmium was used extensively in pigments. Although adverse health effects of heavy metals have been known for a long time, exposure to heavy metals continues and is even increasing in some areas. For example, mercury is still used in gold mining in many countries. Arsenic is still common in wood preservatives, and tetraethyl lead remains a

common additive to petrol. Since the middle of the 19th century, production of heavy metals increased steeply for more than 100 years, with concomitant emissions to the environment.

Emissions of heavy metals to the environment occur via a wide range of processes and pathways, including to air (e.g. during combustion, extraction and processing), to surface waters (via runoff and releases from storage and transport) and to the soil (and hence into groundwater and crops). Atmospheric emissions tend to be of greatest concern in terms of human health, both because of the quantities involved and the widespread dispersion and potential for exposure that often ensues.

Humans are often exposed to heavy metals in various ways – mainly through the inhalation of metals in the workplace or polluted neighbourhoods, or through the ingestion of food (particularly seafood) that contains high levels of heavy metals or paint chips that contain lead.

Because of their toxic effects on cellular metabolism, contamination of soil and ground water with heavy metals is one of the major environmental and human health problems. Despite their well-known toxicity, the basis of their toxicity is not well understood.

Heavy metals are associated with myriad adverse health effects, including allergic reactions, neurotoxicity, nephrotoxicity and cancer. Cadmium has been officially listed as a pulmonary carcinogen for rats and humans by the International Agency for Research on Cancer (IARC) (IARC, 1993). Cd can enhance the peroxidation of lipids because of disturbances in glutathione and metallothionein levels after the exposure to cadmium. Free radicals such as $HO^{\cdot}$ and $O^{2\cdot}$ are released. They can attack double bonds in membrane lipids and result in an increase in lipid peroxidation. In addition Cd can also cause alteration in antioxidant enzyme activities (Ercal et al., 2001).

Mercury exhibits toxic effects, including neurotoxicity, nephrotoxicity and gastrointestinal toxicity (Ercal et al., 2001). Lead causes neurological, haematological, gastrointestinal, reproductive, circulatory, and immunological pathologies (Patrick, 2006). It has also been reported to inhibit plant and microbial growth by influencing the pH of the substrate and inactivating the cell enzymes (Nies, 1999; Soares et al., 2002). Lead causes oxidative stress and increases production of reactive oxygen species (ROS), which inactivate the enzymes, damage nucleic acids and inhibit DNA repair (Patrick, 2006).

Cobalt increases lipid peroxidation and produces oxygen-derived free radicals, which leads to a greater oxidative stress damage (Llesuy and Tomaro, 1994), in addition cobalt can replace ions such as magnesium and calcium in various essential reactions (Jenette, 1981). Its toxicity

is associated with several human diseases such as contact dermatitis, pneumonia, allergic asthma, and lung cancer (Barceloux, 1999).

Silver-ion-mediated perturbation of the bacterial respiratory chain has raised the possibility of reactive oxygen species (ROS) generation (Park et al., 2008). In humans, accidental or intentional ingestion of large doses of silver nitrate has produced corrosive damage of the gastrointestinal tract, abdominal pain, diarrhea, vomiting, shock, convulsions, and death (U.S.EPA, 1985).

2.3 Oxidative stress and reactive oxygen species (ROS)

The situation when for some reason the generation of free radicals is higher than the rate of their detoxication is called oxidative stress (Sies, 1993). Oxidative stress cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA.

In humans, oxidative stress is involved in many diseases, such as atherosclerosis, Parkinson's disease, Heart Failure, Myocardial Infarction, Alzheimer's disease. In chemical terms, oxidative stress is a large rise (becoming less negative) in the cellular reduction potential, or a large decrease in the reducing capacity of the cellular redox couples, such as glutathione (Schafer and Buettner, 2001) The effects of oxidative stress depend upon the size of these changes, with a cell being able to overcome small perturbations and regain its original state. However, more severe oxidative stress can cause cell death and even moderate oxidation can trigger apoptosis, while more intense stresses may cause necrosis (Lennon et al., 1991) However, under the severe levels of oxidative stress that cause necrosis, the damage causes ATP depletion, preventing controlled apoptotic death and causing the cell to simply fall apart (Lelli et al, 1998; Lee et al., 1999).

A particularly destructive aspect of oxidative stress is the production of reactive oxygen species, which include free radicals and peroxides. Some of the less reactive oxygen species (such as superoxide) can be converted by oxidoreduction reactions with transition metals or other redox cycling compounds (including quinones) into more aggressive radical species that can cause extensive cellular damage (Valko et al., 2005). ROS are formed as a natural by product of the normal metabolism of oxygen and have important roles in cell signaling and in the defense against micro-organisms (Lee et al., 1998). However, during times of environmental stress (e.g., UV, heavy metals, or heat exposure) ROS levels can increase dramatically, which can result in significant damage of cell structures. ROS may

spontaneously react with nucleophilic centers in the cell and thereby covalently bind to DNA, RNA and protein (Figure 1). Such a reaction may lead to cytotoxicity, allergy, mutagenicity and/or carcinogenicity, depending of the properties of the epoxide in question (Matés and Sánchez-Jiménez , 1999).

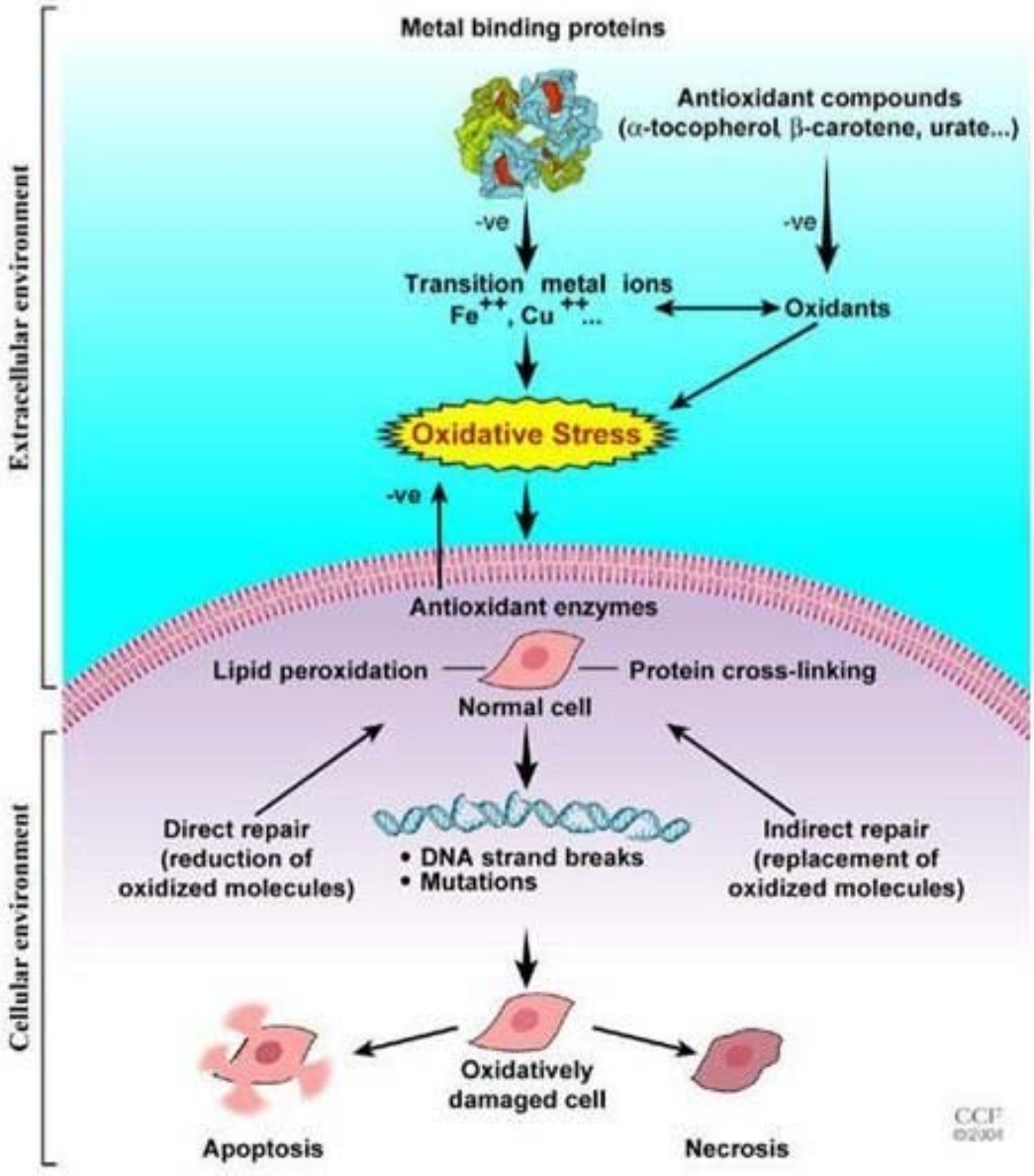


Figure 1. Mechanisms of oxidative stress-induced cell damage (Agarwal et al., 2005)

2.4 *Model organism Corynebacterium glutamicum*

Corynebacterium glutamicum is gram-positive and non-pathogenic bacterium. It is used for the industrial production of the amino acids lysine and glutamine (Leuchtenberger, 1996). *C. glutamicum*, a soil bacterium of the actinomycetes family which is abundant in contaminated

soils, has probably developed highly specific defense mechanisms to cope with heavy metals exposure. This is in contrast to other pro- and eukaryotic model organisms, such as *Escherichia coli* and *Saccharomyces cerevisiae*, which are naturally less exposed to heavy metals concentrations, and whose stress reaction is supposedly less specific. *C. glutamicum* was also chosen based on the fact that besides of having a short generation time, the bacterium is safe to handle and its genome has been completely sequenced (Kalinowski et al., 2003), and furthermore has been the subject of biological research for several decades (Silberbach and Burkovski, 2006).Corynebacteriaceae can be divided into three groups:

1. Parasite and pathogen in human and animal such as *C.diphtheriae*
2. Plant pathogenic forms such as *C. michiganese* and *C. fascians*
3. Non- pathogenic forms such as *C. glutamicum*, *C.herculis* or *C.acetophilum*

The bacteria have a rod or clubbed shape (Figure 2). They are immovable, aerobic and have no spores.

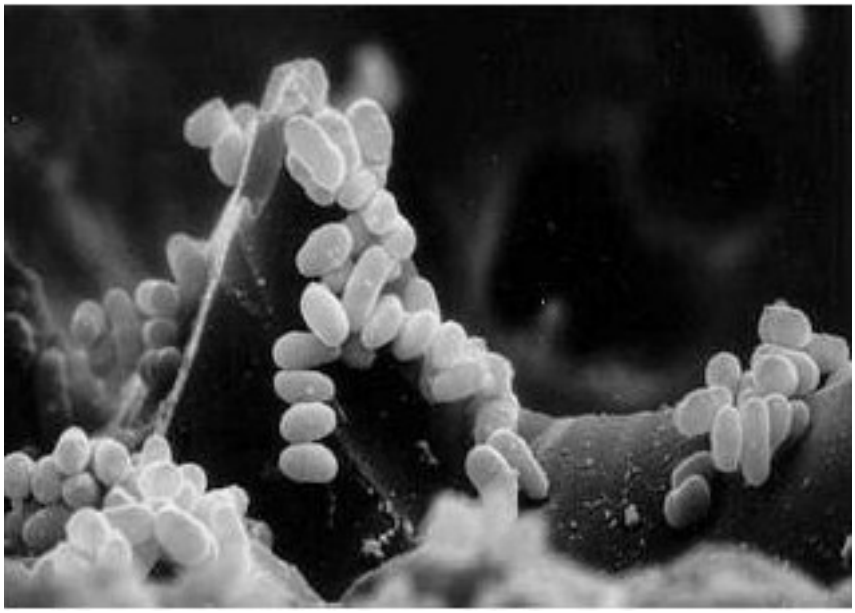


Figure 2. *Corynebacterium glutamicum* cells (www.fz-juelich.de/ibt/coryne.html.)

The proteome analysis of *C. glutamicum* was performed by using 2-D PAGE; the proteins were identified by means of mass spectrometry (MS). The application of this approach enables to establish 2-D maps for *C. glutamicum*, which are important tools for fundamental

microbial and biochemical investigations of the metabolism of *C. glutamicum*, as well as for the increase of the efficiency of amino acid production (Herrmann et al., 1998).

In order to see the protein distribution, pH gradients between pH 3 and 10 were used (Figure 3).

Complete *C. glutamicum* genome of approx. 3.3 MT codes about 3000 different proteins (Herrmann et al., 2001). The hypothetical 2D-map, which derived from genome data, shows the protein distribution with a gap in the neutral range. Additionally too many proteins arise in the range of pH 4-5, which are called cluster. In practice it is difficult to see all these proteins on the gel because not all proteins are expressed at the same time; it depends on the cultural conditions of the microorganisms. In addition some proteins are posttranslational modified and are then recognizable as multiple spots. Other proteins are expressed in small quantities so that they could not be detected with the help of the silver staining, or they have a low solubility (e.g. membrane proteins).

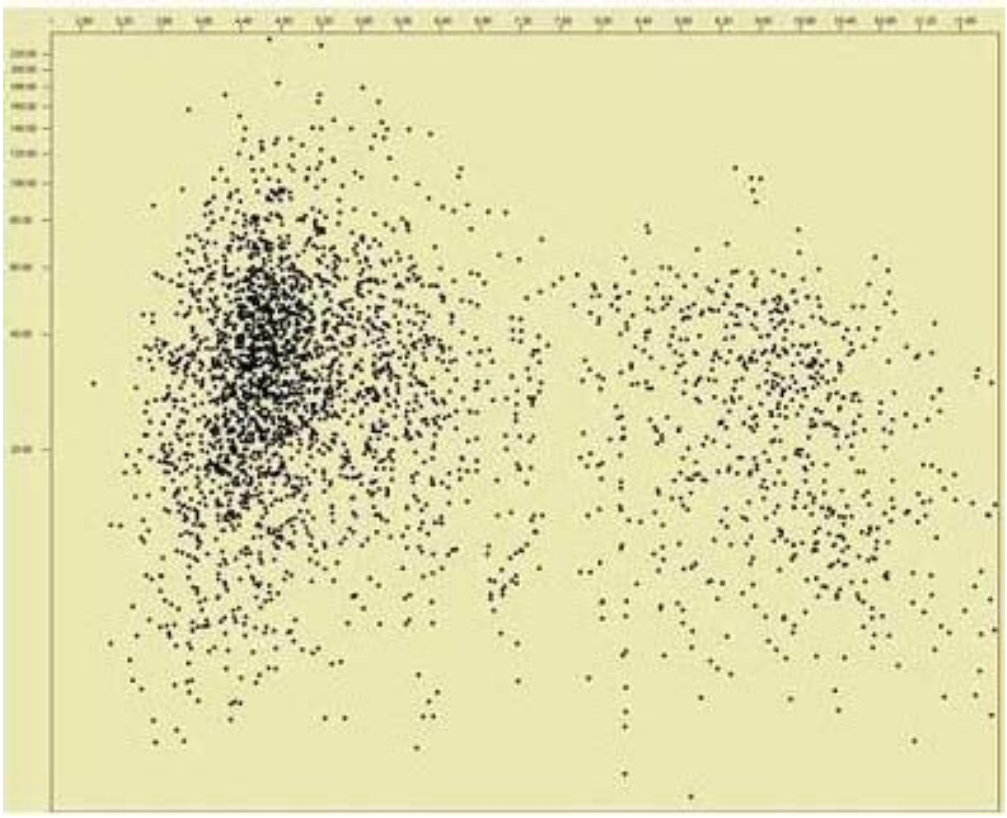


Figure 3. Theoretical protein distribution of the proteom of *C. glutamicum* (Hiller et al, 2003)

2.5 *Previous studies of proteome analysis of model organism treated with heavy metals*

Chemical pollution of the environment increases heavily worldwide, and one of the most significant indicator of this increasing pollution is the accumulation of heavy metals in the soil. A major problem with heavy metals is that they cannot be biodegraded and therefore reside in the environment for long periods of time if they are not removed. In mobile forms they can enter the food chain, damaging the environment seriously and posing a risk to human health. Studies in order to understand the toxic mechanism of cadmium on a molecular level have been performed using *e.g., Saccharomyces cerevisiae* (Vido et al., 2001) and *Schizosaccharomyces pombe* (Bae and Chen, 2004). The authors observed that in yeast many stress proteins were induced, especially antioxidant enzymes such as catalase, thioredoxin, Cu/Zn superoxidase and heat shock proteins. In another important work the gene expression of *Caulobacter crescentus* cells exposed to cadmium sulfate (6 μM) was studied; many genes were up regulated, which are responsible for expression of antioxidant enzymes, DNA repair proteins and efflux pump proteins. Surprisingly, the treated cells did not induce heat-shock proteins (Hu et al, 2005). Croute et al., 2000, who worked with the lung cell-line A549, observed that heat-shock protein (hsp) 72 was over expressed and considered as a sensitive and early biomarker of environmental pollution by cadmium.

When mercury was applied as stress factor to investigate its influences on rat kidney (0.25, 0.5, or 1 mg Hg/ kg, i.v.) for 4, 8, 16 or 24 h, two proteins, namely heat-shock protein hsp72, and glucose regulated protein grp 94 were induced. The authors found that the induction of these proteins is important for cells in order to counteract the toxic effect of mercury (Goering et al, 2000). Devlin and Clary, 1998 exposed Fathead Minnow cells to MeHg; they found the production of hsp70 was increased under MeHg exposure. Despite of extensive research, the toxic mechanism remains poorly understood and further research in this area is needed.

Lead was intensively studied on a molecular level using terrestrial woodlouse, *Oniscus asellus* (Kohler and Eckwert, 1997). The authors observed that heat shock protein-70 (hsp70) and heat shock protein-60 (hsp60, chaperonin 60) were strongly expressed after the treatment with lead. In another important work the protein expression in rat fibroblasts and kidney epithelial cells exposed to lead glutamate (1000 μM) for 3 hours was studied, three proteins were induced; two have been termed glucose-regulated proteins and the third is indicated herein as metal-induced protein.

Chen and Wang investigated the response of *S. cerevisiae* to different concentrations of lead (Chen and Wang, 2007). The authors demonstrated that the growth of *S. cerevisiae* in the presence of lead showed a lag phase much longer than that in the absence of lead. The inhibition was dependent upon lead concentrations. The lead concentration of 5 μM inhibited the microbial growth by approximately 30% with regard to control, whereas lead at concentration of 2 μM din not have a significant effect on the microbial growth, on the other hand, lead at a concentration of 4 μM had a significant inhibitory effect on protein biosyntheses.

Many studies were performed to investigate the toxic mechanism of cobalt on a molecular level, e.g., yeast cells (Stadler and Schweyen, 2002) and *Escherichia coli* (Ranquet et al., 2007). The authors indicate that cobalt might compete with iron for specific binding sites in certain protein, thereby impairing their functions, the cells tried to counteract the cobalt stress by up-regulating their iron transport system and increasing their intracellular iron content. Gonzales et al have demonstrated that the level of antioxidant enzymes was decreased in rat liver after $CoCl_2$ injection for 12 hours.

When silver was applied as stress factor to investigate its effects on *E. coli* and *Staphylococcus aureus* the inhibition of bacterial growth was attributed to reactive oxygen species (ROS) especially superoxide-radical (Park et al., 2008). Lock et al., (2006) exposed *E. coli* cells to silver nanoparticles; they found that the expression of heat shock proteins and envelope proteins was increased.

2.6 Two dimensional polyacrylamide gel electrophoresis (2-D PAGE)

2-D PAGE is a method introduced by O'Farrell in 1975 (O'Farrell, 1975). The method described a new way to separate proteins from biological samples in two dependent dimensions, and is still the method of choice for the majority of differential protein expression studies. The basic principle for the technology is to separate the proteins in each dimension using two independent high resolution properties: isoelectric point (*pI*) and molecular mass (Mr). Before separation all proteins must be completely solubilized to break the interaction between proteins and to remove non-protein components. After this, separation by isoelectric point is carried out by a procedure called isoelectric focusing (IEF). A pH gradient is applied, and the proteins are allowed to migrate in the first dimension until their net charge is zero. The pH at which a protein has zero net charge is called the isoelectric point *pI*-value. Since proteins have differint charges, they will occupy different locations in the isoelectric

dimension after the isoelectric focusing. However, several proteins have similar *pI*-value, so the separation on isoelectric focusing alone is not sufficient to identify individual proteins in a sample under investigation. Thus a second dimension protein migration is caused by applying a second electric field in the presence of SDS. When SDS is present, the electric field will cause the proteins to migrate to positions in the second dimension proportional to their molecular mass. Because few proteins have both identical *pI* and Mr value, it is possible to create a two dimensional map, where most individual proteins are located with unique (*pI*, Mr) coordinates. It should thus be possible to identify most proteins in biological sample by the described procedure.

2D electrophoresis according to Görg et al (1988, 2000) is one of the core technologies in proteomics and utilizes immobilines for establishing a pH gradient in the 1st dimension (Bjellqvist et al., 1982). In contrast to the application of carrier ampholytes, the pH gradient is established while casting the gel and subsequently fixed by cross-linking to the acrylamide matrix. The immobilized pH gradient (IPG) gel can even be dried and easily distributed (Görg and weiss, 2000). Nowadays, pre-cast IPG strips enable the access to 2D electrophoresis for a broader scientific community. However, IPGs are not calculated, cast and applied from sketch. Buffer capacities in IPGs, solvents for proteins and customized protocols for sample entry or steady state focusing are just a few of many critical factors in isoelectric focusing (IEF) and needed optimization. In this field, Görg et al., established many protocols for casting IPG gels, sample preparation and IEF (e.g. (Görg et al., 1988; Görg et al., 2000)).

One major advantage of utilizing immobilines is the possibility to zoom into overcrowded areas of protein patterns by the application of narrow pH gradients. Limitations of protein loading onto such narrow gradients have recently been overcome by prefractionation of the sample by IEF with Sephadex as matrix (Görg et al., 2002). Finally, the resolving power of narrow IPGs successfully meets the demand in proteomics to cover thousands of proteins for the analysis of complete proteomes.

2.7 MALDI- mass spectrometry in proteomics

Since the 1970's laser devices have been employed in mass spectrometric analysis to achieve a direct desorption of intact molecule ions from condensed phases by a suitable primary excitation. On these initial attempts a thin layer of a sample was placed on a metal surface to be irradiated by a laser pulse. However, mass spectra resulting from these approaches were of less intensity, as the laser energy was sufficient enough to cause molecular fragmentation of

the sample molecules. Therefore the laser desorption / ionization mass spectrometry (LDI-MS) was of less practical use for the analysis of biomolecules since only ions of a molecular weight below 1000 Da could have been detected.

This situation changed entirely as M. Karas and F. Hillenkamp from the University of Münster inspected the correlation process between UV laser irradiation and organic molecules. They figured out that embedding the sample into suitable matrix material, consisting of small organic molecules, the laser light was strongly absorbed and thus not only higher intensities of the analyte were obtained but also sample fragmentation was reduced to a minimum. This method denoted MALDI-MS (Matrix Assisted Laser Desorption/Ionization) allows now detecting large molecules such as proteins.

The principle of MALDI-ionization based on that the mixing an analyte on a metal sample probe with a suitable matrix compound of a 1.000 up to 10.000 times molar excess absorbing at the used laser wavelength, causes a co-crystallization process of both matrix and analyte material after evaporation of the solvent. The incorporation of the sample molecules into the lattice structure of the matrix is supposed to be precondition of the functioning of the laser desorption/ionization process (XE “Photo of a matrix”).

The crystallized surface of the prepared sample is then exposed to an intensive pulse of short waved laser irradiation in the high vacuum area inside the ion source of the mass spectrometer (XE “MALDI process”). The coupling of the energy, which is necessary for the ions, is performed on UV radiation by resonant excitation of the matrix molecules, e.g., into the π-electron system of aromatic compounds. Theoretical calculations let suppose that the excitation energy being stored in the matrix molecules relaxes in extremely short time periods into the solid-state lattices causing here a strong distortion of the expansion. This process is followed by transition into a phase in which a part of the solid-state surface is vaporized explosively long before a thermal balance occurs. In this step matrix molecules and sample molecules as well are released into the gas phase (Figure 44). Obviously the internal degree of freedom of the molecules sharing this process is low enough that even thermal labile macromolecules such as proteins endure this process. However this is only valid as a limited range of irradiation power on the sample between 10^6 and 10^7 W/cm^2 is concerned. Too a high radiation destroys the sample to a great extent. A lot of experiments indicate that the matrix is also considered an agent ionizing the sample molecules. Accordingly photo ionized, radical matrix molecules cause by transferring protons a high yield of electrically charged sample molecules. An electrode, which is mounted some mm apart opposite to the sample

position, is used to generate an electrostatic field in the range of some 100 V/cm to some 1000 V/cm. Depending on the polarity positively or negatively charged ions are accelerated from the sample surface towards the analyzer.

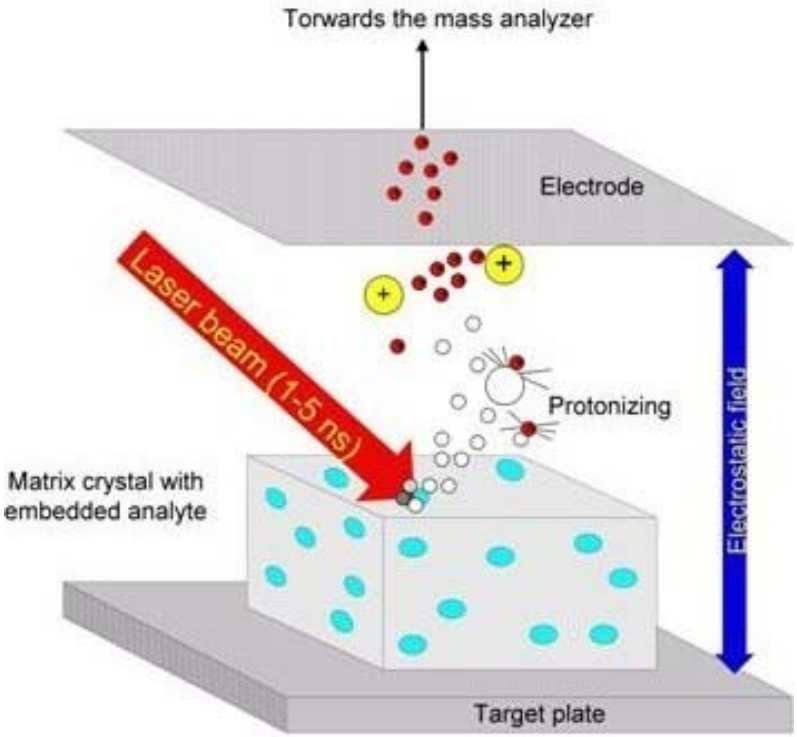


Figure 4. Principle of the MALDI process.

In many cases, analyzers combined with MALDI experiments are Time-Of-Flight instruments where the mass determination in the high vacuum area is performed by a very precise measurement of the period of time after acceleration process of the ions in the source and impact on the detector (XE "TOF analysers"). An electro-static field accelerates ions formed during a short laser pulse inside the source to a kinetic energy of some keV. After leaving the source the ions pass a field-free drift region in which they are separated due to their m/z ratio. This takes place because at fixed kinetic energy ions with different m/z values are accelerated in the ion source to different velocities. Knowing the acceleration voltage and the length of the drift region the m/z ratio can be determined by measuring the flight time.

In combination with 2D electrophoresis, MALDI MS is in particular applied as downstream analytical methods for protein identification. In the present study, peptide mass fingerprinting with MALDI-TOF MS was performed. Peptide mass fingerprinting by MALDI-TOF MS provides a high throughput method for protein identification. Peptide mass fingerprints (PMF) are produced by chemical or enzymatic digestion of individual proteins and subsequent MS

analysis of the obtained peptide fragments. The PMF is characteristic for each protein and thus, can be used for protein identification by comparison with predicted peptide masses (Henzel et al., 1993; Pappin et al., 1993). As a result, the analysis of PMFs requires known protein sequences and is most successful for organisms with completely sequenced genomes. PMF is commonly performed with MALDI-TOF MS, which utilizes time of flight (TOF) mass analyzers. The TOF of ions in the mass analyzer is proportional to their molecular weight. High mass accuracies are achieved by delayed ion-extraction technique in combination with so-called reflectrons , which reflect ions in the mass analyzer by an ion mirror (Jensen et al., 1996) (Fig. 5).This high performance makes it possible to identify proteins with fewer peptide masses than before and thus, was used in the present thesis.

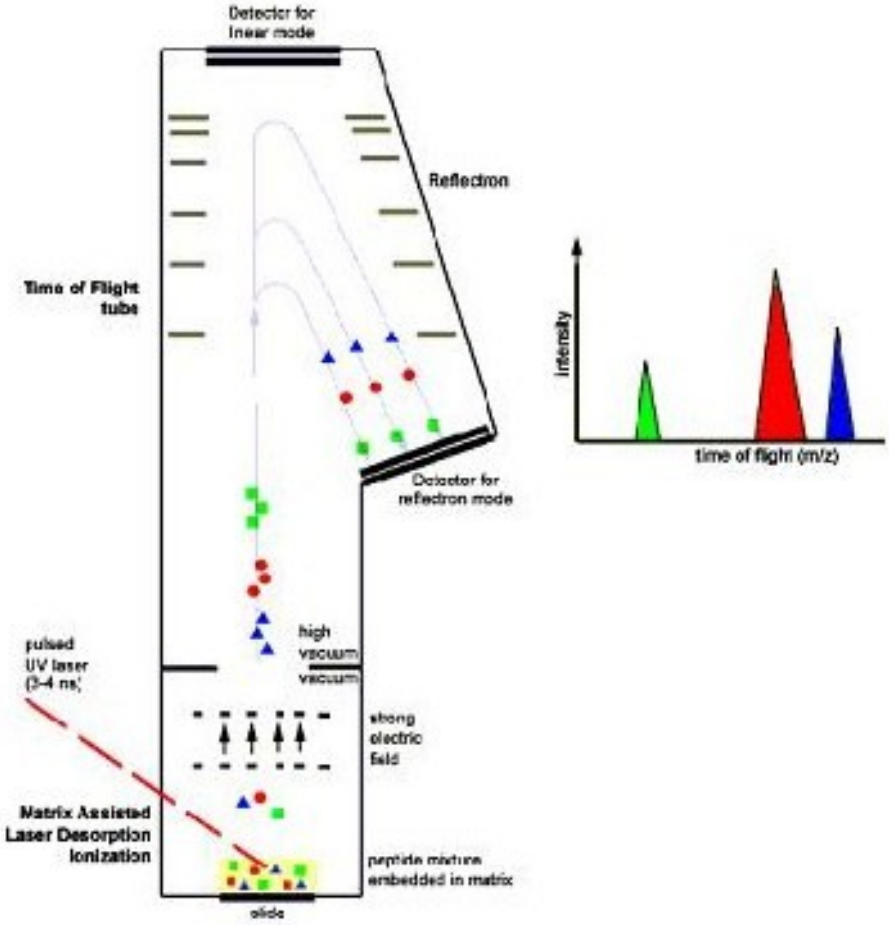


Figure 5. MALDI- TOF with reflectrons (www.proteomicsnijmegen.nl/Maldi-about.html)

3 Materials and Methods

3.1 Bacterial strain, media and growth conditions

C. glutamicum cells (ATCC 13032) were grown aerobically on a rotary shaker at 30ºC in brain heart infusion (BHI) medium (Merck KGaA, Germany), composed of (g/L) nutrient substrate (extracts of brain heart, and peptones) 27.5, D(1)-glucose 2.0, sodium chloride 5.0, and di-sodium hydrogen phosphate 2.5.Media was prepared by dissolving 37g of the powder in 1 L of purified water and sterilized by autoclaving at 121°C for 15min.

Before stress treatment, *C. glutamicum* was grown over night in 100 ml at 30°C in the (BHI) medium. Then, an appropriate volume of (BHI) medium was inoculated with the overnight culture. Growth was monitored spectrophotometrically (*i.e.*, measurement of the OD) at 600 nm.

3.2 Stress treatments

Metal stock solutions were prepared by dissolving the compounds $HgCl_2$ (VWR, Ismaning, Germany), $CdCl_2$ (Alfa Aesar; Karlsruhe, Germany), $Pb(NO_3)_2$ (VWR, Rue Carnot, France), $CoCl_2$ (Merk, Hohenbrunn, Germany), $AgNo_3$ (AppliChem, Darmstadt, Germany) in water to 10 mM for $HgCl_2$, 100 mM for $CdCl_2$, 100 mM for $Pb(NO_3)_2$,100 mM for $CoCl_2$ and 10 mM for $AgNo_3$. Metal stocks were sterilized by filtration through a 0.2-µm membrane. *C. glutamicum* overnight cultures were diluted in BHI medium, with various concentrations of heavy metals added. Growth was monitored spectrophotometrically (*i.e.,* measurement of the optical density) at 600nm. The heavy metal concentration which exerted approximately 50% growth inhibition was selected as stress concentration for the subsequent proteomic studies. Particular emphasis was placed on harvesting both unstressed controls and stressed *C. glutamicum* samples in the same (*i.e.*, mid-logarithmic) growth phase, as otherwise the differentially expressed proteins might not only reflect heavy metal-induced stress, but also different growth phases, and thus hamper the identification of specific heavy metal-induced *C. glutamicum* proteins.

In this study 10 µM $HgCl_2$, 5 mM $CdCl_2$, 6 mM $Pb(NO_3)_2$, 0,75 mM $CoCl_2$ and 120 µM $AgNo_3$ were applied as sublethal concentrations.

3.3 Protein extraction

The proteomes of two samples were compared: The first sample served as the control, corresponding to the *C. glutamicum* population grown in the absence of heavy metals, and the second sample was *C. glutamicum* population adapting to heavy metals. Populations were harvested at the mid-log phase (OD600 = 6.5). Harvesting took place by centrifugation (9000×*g*, 3 min) and by briefly washing the pellets twice with preheated (30ºC) PBS. The bacteria pellet (dry weight 1 mg) was afterwards suspended in 250 mL of SDS (Serva,Heidelberg, Germany) buffer (100 mM Tris/HCl pH 9.5,1% SDS w/v). While ice-cooling the sample, cell disruption was performed with an ultrasonic probe (two times, 30 pulses; interval: 1 Hz; pulse duration: 0.3 s; 20 kHz homogeneous sound; power output: 60 W) (Bandelin Sonoplus HD 60; Berlin, Germany). The cell debris was pelleted by centrifugation at 14 000×*g*, 4ºC for 30 min. Thereafter the protein extract was aliquoted and frozen at -78ºC. All fluids and devices that came in contact with the bacterial culture prior to the protein extraction had been sterilized to prevent contamination, and preheated to 30ºC to avoid other stresses such as cold shock.

3.4 Determination of protein concentration

The protein concentration was determined with the 2D Quant Kit (Amersham Biosciences, Uppsala, Sweden). The 2-D Quant Kit is appropriate for the accurate determination of protein concentration in samples to be analyzed by high resolution electrophoresis techniques such as 2-D electrophoresis, SDS PAGE or IEF. Many of the reagents used in the preparation of such samples, including detergents, reductants, chaotropes and carrier ampholytes, are incompatible with other protein assays.

The procedure works by quantitatively precipitating proteins while leaving interfering substances in solution. The assay is based on the specific binding of copper ions to protein. Precipitated proteins are resuspended in a copper-containing solution and unbound copper is measured with a colorimetric agent. The colour density is inversely related to the protein concentration. The assay has a linear response to protein in the range of 0–50 μg. The procedure is compatible with such common sample preparation reagents as 2% SDS, 1% DTT, 8 M.

3.5 2-DE with Immobilized pH-Gradients (IPGs)

3.5.1 Preparation of immobilized pH gradient (IPG) gels

In the present study, the following IPG slab gels were used: pH 3-12, 3.4-5.4, 4.9-6.9 All IPG gels were cast according to Görg et al (2004). The IPG gels were cast on the hydrophilic side of Gelbond PAGfilms by using a 260 x 200 mm2 gel cassette. The glass plate with the U-frame was treated with repelsilane to facilitate the detachment of the gel, when disassembling the gel cassette.

An acidic and an alkaline pH solution were prepared for each pH gradient according to the published recipes, e.g. IPG 3.4-5.4 and IPG 4.9-6.9 in Tab.1 and Tab 2. For homogenous polymerization, the acidic and alkaline solutions were adjusted to pH 7 with sodium hydroxide and hydrochloric acid, respectively. The acidic, dense solution was pipetted into the mixing chamber and mixed with TEMED and ammonium persulfate using a magnetic follower. After ventilation of the connecting tube, the basic, light solution was pipetted into the reservoir of the gradient mixer. Again, TEMED and ammonium persulfate were added and mixed, this time with a spatula, which remained in the reservoir as equivalent for the magnetic follower in the mixing chamber. The gradient was poured at a reproducible speed of the magnetic follower into the precooled mold (refrigerator, 4°C) of the gel cassette. Next, the gel cassette was kept at room temperature for 15 min to allow adequate leveling of the density gradient prior to polymerization for one hour at 50°C. After polymerization, the gel cassette was kept at room temperature for at least 15 min. Then, the IPG gel was removed from the mold and washed six times for 10min with MilliQ H2O. Finally, it was impregnated with 2% glycerol for 30 min, and dried at room temperature over night in a dust-free cabinet. The IPG gels were covered with a plastic film and cut into individual IPG strips and if not immediately used stored at -20°C.

Table 1. Recipe for casting IPG 3.4-5.4.

	pH 3.4		pH 5.4	
	mMol/L	Vol. (µl)	mMol/L	Vol. (µl)
Immobiline pK 1.0	1.16	87.44	0.00	0.00
Immobiline pK 3.6	6.97	522.73	0.997	74.7
Immobiline pK 4.6	2.66	199.8	9.94	745.92
Immobiline pK 7.0	3.75	281.77	10.001	750.04
Acrylamide/Bis	2 ml		2 ml	
H2O	9.87 ml		11.4 ml	
Glycerol (100%)	3.75 g		0.00 g	
TEMED (100%)	9 µl		9 µl	
APS (40%)	15 µl		15 µl	

Table 2: **Recipe for casting IPG 4.9-6.9.**

	pH 4.9		pH 6.9	
	mMol/L	Vol. (µl)	mMol/L	Vol. (µl)
Immobiline pK 3.6	4.778	87.44	1.984	148.78
Immobiline pK 4.6	6.398	479.88	2.208	165.62
Immobiline pK 6.2	9.539	715. 43	6.653	498.95
Immobiline pK 7.0	0.00	0.00	3.508	263.07
Immobiline pK 8.5	0.00	0.00	1.001	75.082
Acrylamide/Bis	2 ml		2 ml	
H2O	9.4 ml		11.8 ml	
Glycerol (100%)	3.75 g		0.00 g	
(100%)TEMED	9 µl		9 µl	
APS (40%)	15 µl		15 µl	

For effective polymerization, acidic and basic solutions were adjusted to pH 7with 4N NaOH and 25% HCl, respectively, before adding TEMED and APS.

3.5.2 IPG strip rehydration and sample application

Prior to IEF, the IPG dry strips must be rehydrated (usually overnight) to their original thickness of 0.5 mm with a rehydration buffer containing 8M urea (or, alternatively, 2M thiourea and 6M urea), 1- 2% non-ioninc or zwitterionic detergents (*e.g.*, 2% CHAPS), a reductant (typically 0.4% DTT) and 0.5% (v/v) carrier ampholytes (*e.g.*, IPG buffer or Pharmalyte 3-10). Rehydration buffer should be prepared fresh before use, or stored frozen in aliquots at –70°C. It is important to deionize the urea with an ion exchange resin prior to adding the other components, because urea in aqueous solution exists in equilibrium with ammonium cyanate which can react with protein amino groups and introduce charge artifacts, giving rise to additional spots on the IEF gel. Carrier ampholytes are added for improved protein solubility, but also as a cyanate scavenger.

IPG dry strips are either rehydrated with sample already dissolved in rehydration buffer ("sample in-gel rehydration"), or with rehydration buffer without sample, followed by sample application by "cup-loading". Rehydrated IPG strips can be stored at –70°C for instantaneous use. For *cup-loading*, IPG drystrips are reswollen in rehydration buffer, either in a reswelling cassette or, more convenient, in a reswelling-tray, however without sample. After IPG strip rehydration, samples (150 µl) dissolved in lysis buffer are applied into disposable plastic or silicone rubber cups placed onto the surface of the IPG strip. Best results are obtained when the samples are applied at the pH extremes, *i.e.*, either near the anode or cathode. Sample application near the anode proved to be superior to cathodic application in most cases. When using basic pH gradients such as IPGs 6-12 or 9-12, anodic application is mandatory for all kinds of samples investigated (Görg et al., 2000). Approximately 150 µg of protein extract was loaded onto analytical gels, which were subsequently stained with silver nitrate. On preparative gels submitted to MALDI-TOF MS approximately 500 µg protein extract was loaded. Such gels were stained with Coomassie Blue R-250.

3.5.3 First dimension IPG-IEF

IPG-IEF for 2D electrophoresis can be simplified by the use of an integrated instrument. The IPGphor includes a Peltier element for precise temperature control (between 19.5°C and 20.5°C) and a programmable power supply. The central part of this instrument are so-called strip holders made from an aluminium oxide ceramic, in which IPG strip rehydration with

sample solution and IEF are performed without further handling after the strip is placed into the strip holder.

The IPGphor can handle up to 12 strip holders of different lengths (7, 11, 13, 18 or 24 cm). Alternatively, the IPGphor can also be used with a cup loading procedure using cuploading strip holders or a multiple cup-loading strip holder (’Manifold’), which allow(s) the application of quantities up to 100 µl. The strip holder platform regulates temperature and serves as the electrical connector for the strip holders. Besides easier handling, a second advantage of the IPGphor is shorter focusing time, since IEF can be performed at rather high voltage (up to 8000 V).

The IPGphor is programmable and can store nine different programs. A delayed start is also possible, which allows the user to load the strip holders with sample dissolved in rehydration buffer in the afternoon, and then automatically start IEF during the night so that IEF is finished the next morning. Typical running conditions for IEF using the IPGphor are given in the **Table 3**. As indicated earlier, low voltage (30-50 V) is applied during the rehydration step for improved sample entry of high Mr proteins into the polyacrylamide gel which otherwise can be a problem with sample in-gel rehydration. Then voltage is stepwise increased up to 8000 V (when IPG strips with separation distances < 11 cm are used, voltage should be limited to 5000 V only). For optimum results for samples with high salt concentrations, or when narrow pH intervals are used, it is beneficial to insert moist filter paper pads (size: 4 x 4 mm2) between the electrodes and the IPG strip prior to raising voltage to 8000 V. After termination of IEF, the IPG strips are stored as described above.

Table 3. Conditions for isoelectric focusing of immobilized pH gradients 3-12, 3.4-5.4 and 4.9-6.9

IPG	Sample entry			IEF	Total volthours
pH 3-12 (18 cm)	1 h	1 h	1:30 h	7 h	56000 Vh
pH 3,4-5,4 (18 cm)	1 h	1 h	1:30 h	8 h	64000 Vh
pH 4,9-6,9 (18 cm)	1 h	1 h	1:30 h	8 h	64000 Vh
Voltage	150 V	300 V	600 V	8000 V	-
current per IPG strip	50 µA	50 µA	50 µA	50 µA	-
max. power	5 W	5 W	5 W	5 W	-

3.5.4 IPG strip equilibration for proper protein transfer and pattern quality

Prior to the second-dimension separation (SDS-PAGE), it is essential that the IPG strips are equilibrated to allow the separated proteins to fully interact with SDS. Due to the observation that the focused proteins bind more strongly to the fixed charged groups of the IPG gel matrix than to carrier ampholyte gels, relatively long equilibration times (10–15 minutes), as well as urea and glycerol to reduce electroendosmotic effects are required to improve protein transfer from the first to the second dimension (Görg et al,1988). Thiourea is sometimes recommended for more efficient transfer of hydrophobic proteins (Pasquali et al, 1997), but may cause vertical streaks in the 2-DE pattern. The by far most popular protocol is to incubate the IPG strips for 10–15 minutes in the buffer originally described by Görg et al. (1987) [50 mM Tris-HCl (pH 8.8), containing 2% (w/v) SDS, 1% (w/v) dithiothreitol (DTT), 6 M urea and 30% (w/v) glycerol]. This is followed by a further 10–15 minute equilibration in the same solution containing 4% (w/v) iodoacetamide instead of DTT. The latter step is used to alkylate any free DTT, as otherwise it migrates through the second-dimension SDS-PAGE gel, resulting in an artifact known as point-streaking that can be observed after silver staining. More importantly, iodoacetamide alkylates sulfhydryl groups and prevents their reoxidation.

This two-step reduction/alkylation procedure is highly recommended, since it considerably simplifies downstream sample preparation (protein in-gel digestion) for spot identification by mass spectrometry. After equilibration, the IPG strips are applied onto the surface of the second-dimension horizontal or vertical SDS-PAGE gels.

3.5.5 Second Dimension: SDS-PAGE on horizontal and vertical systems

Due to simplified handling and higher reproducibility, in most cases, SDS gels of a homogeneous polyacrylamide concentration are preferred, but polyacrylamide concentration (“pore”) gradients, which extend the range over which proteins of different molecular mass can be effectively separated, are also used **Tab.4**. SDS-PAGE can be performed on horizontal or vertical systems (Görg et al, 1995). Horizontal setups (Görg & Weiss, 1999) are ideally suited for ready-made gels (*e.g.*, ExcelGel SDS; *GE Healthcare Lifesciences*), whereas vertical systems are preferred for multiple runs in parallel, in particular for large-scale proteome analysis which usually requires simultaneous electrophoresis of batches of second-dimension SDS-PAGE gels for higher through-put and maximal reproducibility (Anderson & Anderson 1978). The most commonly used buffers for the second dimension of 2-DE are the discontinuous buffer system of Laemmli (1970) and modifications thereof, although for

special purposes other buffer systems are employed, such as borate buffers for the separation of highly glycosylated proteins (Patton et al, 1991). Typically, gel sizes of 20 x 25 cm2 and a gel thickness of 1.0 mm are employed. In contrast to horizontal SDS-PAGE systems, it is not necessary to use stacking gels with vertical setups, as the protein zones within the IPG strips are already concentrated and the nonrestrictive, low polyacrylamide concentration IEF gel acts as a stacking gel (Dunn, 1993).

The analysis of low Mr (< 15 kDa) and high Mr (> 150 kDa) proteins is somewhat intricate since there is no "standard" 2-DE system which effectively allows separation of proteins over the entire Mr range between 5 kDa and 500 kDa. A common approach is to combine several gels optimized for the approximate Mr ranges 5-30 kDa, 15-200 kDa, and > 150 kDa instead of using a single "standard" 2-DE system, or to use polyacrylamide „pore" gradient gels. Conventional Tris-glycine gels do not allow efficient separation of proteins below 15 kDa.

The major problem associated with high Mr proteins is that a significant proportion of these proteins is rather hydrophobic, and, consequently, will not readily dissolve in "standard" urea lysis and/or rehydration solutions used for sample solubilization and IEF.

Even though these proteins were solubilized, they will not always enter the IEF gel matrix, or are not transferred from the first to the second dimension. Several strategies have been proposed to overcome at least some of these obstacles. For example, it has been demonstrated that sample application of high Mr proteins to IPG gels *via* cup-loading is more efficient than "passive"sample application by sample in-gel rehydration If samples are applied by in-gel rehydration, "active"reswelling by applying low voltage (30 - 50V) during the rehydration step is superior to "passive" loading and improves the entry of high Mr proteins into the polyacrylamide matrix (Görg et al., 2000). The transfer of high Mr proteins from the IPG strip onto the SDS gel is enhanced by sufficiently long equilibration steps (2 x 15 min). The same holds true for application of low voltages during the transfer step, *i.e.*, 50 V for vertical SDS gels, and 100 V for horizontal SDSPAGE systems, respectively (Görg et al., 2000).

Table 4. Recipe for casting vertikal SDS-Gels.

	IsoDalt	EttanDalt
	T = 13 %	T = 13 %
Acrylamide/ Bisacrylamide [mL]	422	379,8
Glycerol (100%) [g]	50	45
Gel Buffer [mL]	250	225

Deionized water [mL]	283	254,7
APS (10%) [mL]	7	6
TEMED [µL]	55	100
Final volume [mL]	1000	900

3.5.5.1 Running SDS gels

The second dimension can be performed on horizontal or vertical electrophoresis systems (Görg et al., 1988, 1995). Horizontal setups are ideally suited for ready-made gels on film supports (*e.g.*, ExcelGel SDS), whereas vertical systems (*e.g.,* the Ettan- DALT multiple slab gel unit) are preferred for multiple runs in parallel. However, readymade SDS gels are also available for vertical systems.

For horizontal setups, laboratory-made or ready-made SDS polyacrylamide gels (0.5 mm thick on GelBond PAGfilm) are placed on the cooling plate of the horizontal electrophoresis unit. Electrode wicks or buffer strips made from polyacrylamide are then applied. The equilibrated IPG strip(s) is (are) simply placed gel side down onto the surface alongside the cathodic electrode wick or polyacrylamide buffer strip without any embedding procedure. In the vertical setup, the equilibrated IPG gel strips are placed on top of the vertical SDS gels and embedded in hot agarose.

3.5.6 Protein fixation

After termination of the second dimension run (SDS-PAGE), fixing is necessary to immobilize the separated proteins in the gel and to remove any non-protein components which might interfere with subsequent staining. Depending on gel thickness, the gel is submersed in the fixative for one hour at least, but usually overnight, with gentle shaking.

Widely used fixatives are either 20% (w/v) trichloroacetic acid (TCA), or methanolic (or ethanolic) solutions of acetic acid (*e.g.* methanol / distilled water / acetic acid 45/45/10). A disadvantage of the latter procedure is that low molecular weight polypeptides may not be adequately fixed. Several authors also recommend aqueous solutions of glutardialdehyde for covalently cross-linking proteins to the gel matrix (*e.g.* for diamin silver staining). However, in this case subsequent protein identification, *e.g.*, by mass spectrometry, is impractical.

3.6 Protein gel staining techniques

After 2-D PAGE, the separated proteins have to be visualized, either by “universal” or by "specific" staining methods. Since the concentrations of individual proteins in a single cell differ between six or seven orders of magnitude, ranging from several millions of copies / cell for some highly abundant proteins (*e.g.*, glycolytic enzymes) to a few copies / cell for very low abundant proteins, these enormous variations in protein concentrations are a major challenge for almost all currently available protein detection methods. The most important properties of protein visualization methods are high sensitivity (low detection limit), high linear dynamic range (for quantitative accuracy), reproducibility, and compatibility with post-electrophoretic protein identification procedures, such as mass spectrometry. Unfortunately, currently no staining method for 2-D gels meets all requirements for proteome analysis.

Universal detection methods of proteins on 2-D gels include staining with anionic dyes

(*e.g.*, Coomassie Blue), negative staining with metal cations (*e.g.*, zinc imidazole), silver staining, fluorescence staining or labelling, and radioactive isotopes, using autoradiography, fluorography, or Phosphor-imaging. For most of these staining procedures, the resolved polypeptides have to be fixed in solutions such as in ethanol/acetic acid/ H2O for at least several hours (but usually overnight) before staining to remove any compounds (*e.g.*, carrier ampholytes, detergents) that might interfere with detection.

3.6.1 Silver staining

Silver staining methods are far more sensitive than CBB or imidazole-zinc stains (detection limit is as low as 0.1 ng protein/spot). They provide a linear response with over a 10-40 fold range in protein concentration, which is slightly worse than with CBB staining. However, silver staining methods are far from stoichiometric, and are much less reproducible than CBB stains due to the subjective end-point of the staining procedure which makes them less suitable for quantitative analysis. Silver staining methods are quite laborious and complex. Silver staining methods using aldehyde-based fixatives/sensitizers are the most sensitive ones, but prevent subsequent protein analysis (*e.g.*, by MS) due to protein cross-linkage. If aldehydes are omitted in the fixative and in the subsequent gel impregnating buffers (except in the developer), microchemical characterization by peptide mass fingerprinting (PMF) is possible, however at the expense of sensitivity.

Analytical gels were silver stained according to a modified protocol of Blum et al., (1987).

Table 5. Silver staining protocol according to Blum et al. (modified).

Step	Reagent	Time
Fixing	Fixing solution	> 3 h
wash	Deionized water, ethanol (30%), ethanol (15%)	3× 20 min
Sensitizer	Sensitive solution	1 min
Wash	Deionized water	3× 20 sec
Incubation in silver nitrate solution	Silver nitrate solution	20 min
Wash	Deionized water	3× 20 sec
Development	Developer solution	2-5 min
Stop the staing	Stop solution	>10 min
Wash	Deionized water	3× 10 min

3.6.2 Coomassie Brilliant Blue staining

Coomassie Brilliant Blue (CBB) staining methods have found widespread use for the detection of proteins on 2-D gels, last not least because of their low price, ease of use and compatibility with most subsequent protein analysis and characterization methods such as MS. However, in terms of the requirements for proteome analysis, the principal limitation of CBB stains lies in their insufficient sensitivity, which does not permit the detection of low abundance proteins (the detection limit of CBB stains is in the range of 200-500 ng protein per spot). Hence, typically no more than a few hundred protein spots can be visualized on a 2 D gel, even if milligram amounts of protein had been loaded onto the gel.

Coomassie Brilliant Blue (CBB) is an anionic triphenylmethane dye that binds noncovalently to the lysyl residues of proteins. After fixation at least for one hour in 40 % ethanol and 10 % acetic acid, the gel was washed 30 min in 25 % ethanol and 8 % acetic acid. Then, it was saturated with dye solution (0.1 % CBB R-250 (w/v) in 45 % ethanol (v/v) and 10 % acetic acid (v/v)) for three hours, and finally destained in several steps with 25 % ethanol and 8 % acetic acid (Candiano et al., 2004). The detection limit of this stain is better than one

microgram of protein per spot. In general, CBB staining was chosen if spots were subjected to further protein analysis methods, such as peptide mass fingerprinting with MALDI-TOF MS.

3.7 Image analysis after silver or CBB staining using Progenesis Samespots

One of the key objectives of proteomics is to identify the differential expression between control and experimental samples run on a series of 2-D gels. That is, the protein spots that have been inhibited (disappeared), induced (appeared) or have changed abundance (increased or decreased in size and intensity). Once these gel features have been found, the proteins of interest can be identified using MS. This goal is usually accomplished with the help of computerized image analysis systems. The first step in computerized image analysis of 2-DE protein patterns is capture of the gel images in a digital format. A range of devices, including modified document scanners, laser densitometers, charge-coupled device and (CCD) cameras are available for the acquisition of 2-D gel images. The saved images are then subjected to computer assisted image analysis.

The images from stained gels were digitized with a flatbed scanner (Epson expression 1680 Pro). The computer based analysis of the 2-D gels for protein detection, spot matching between gels, and change of protein expression levels was performed with SameSpots software (Molecular Dynamics, Newcastle, UK) according to the manufacturer's instructions.

Before the image analysis, the gel images were rotated to the same orientation to minimize the time for protein pattern matching of the gels. Then, the gel images were cut on the edges, because the gels are wider than the IPG strips. By this, detection of non-protein spots outside of the protein pattern was avoided. After the images were loaded into the Progenesis Samespots, at first the images were be automatic aligned. Next, the gel with the highest spot number within the reference group was chosen for the creation of a reference gel. The reference gel was used to match same spots of all gels belonging to the reference group. This will be done automatically. After that the artefact spots can be removed based on normalised volume or spot area. We can also select areas of the gel to filter out, for example a damaged area, noisy area, spots on the edge of the gel or spots on the scanner bed. The next step is setting up groups, such as control vs. treated. This means we can measure differential expression patterns between any groups at the review stage. Any spot IDs remain the same across different groups. After that complete analysis of a whole experiment can be done in single step, which takes only minutes per gel. This incredibly rapid process includes

SameSpots detection, background subtraction, normalisation and matching. Results are ranked, based on ANOVA p-value and fold change ready for validation. We can review statistically ranked lists of spots using a range of displays. Spots are ranked by p-value from the one way ANOVA analysis, with maximum fold change based on spot normalised volume displayed. This can be done to compare any chosen groups. Data were ranked according to *p*-value and maximum fold change observed across all the groups compared. For the control and treated sample, only protein spots showing a statistically significant up- or down-regulation (*p*, 0.05) were considered as differentially expressed. The normalized spot volumes of the image in the match set were compared (if the data were identical, all ratios equal 1; for different data, the ratios were < 1 or > 1). Finally Statistical Analysis can be done automatically. It allows us to easily apply powerful statistical analysis and make reliable conclusions.

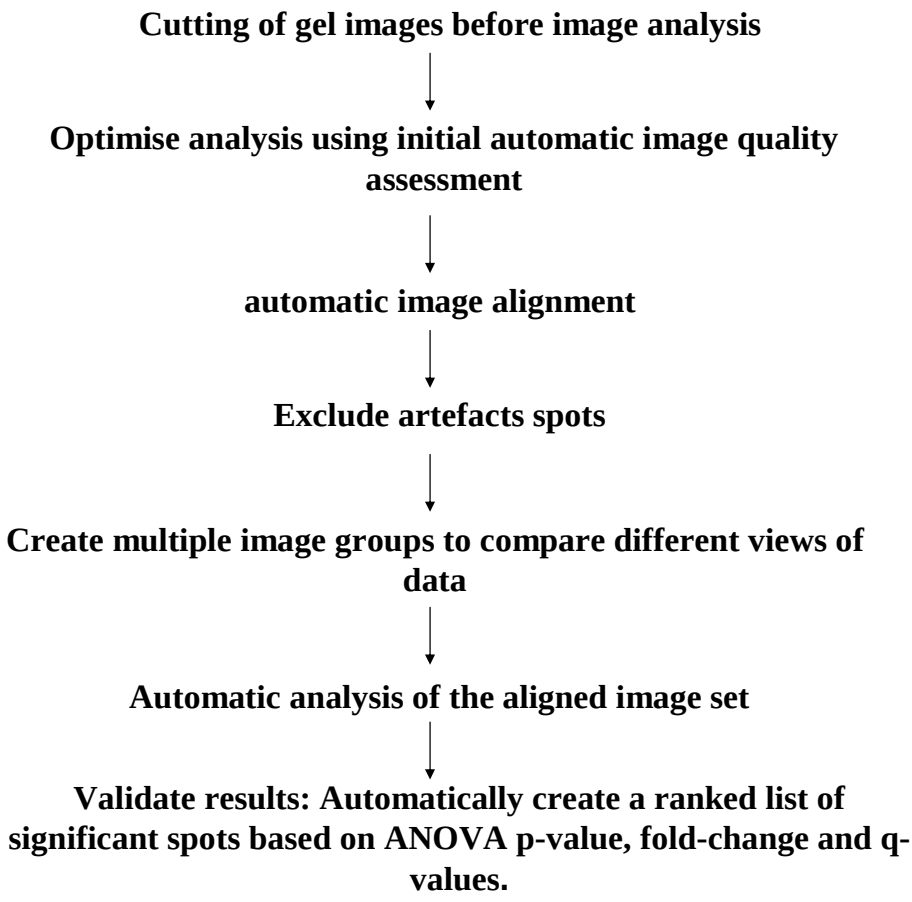


Figure 6. Workflow of image analysis using Progenesis Samespots

3.8 Protein identification

Mass spectrometry has become the technique of choice for identification of proteins from excised 2-D gel spots as these methods are very sensitive, require small amounts of sample (femtomols to attomols) and have the capacity for high sample throughput.

Recent advances in mass spectrometry also allow the investigation of post translational modifications including phosphorylation and glycosylation. **Peptide mass fingerprinting (PMF*)*** is typically the primary tool for protein identification. This technique, which is user-friendly and quite fast, is based on the finding that a set of peptide masses obtained by MS analysis of a protein digest (usually trypsin) provides a characteristic mass fingerprint of that protein. The protein is then identified by comparison of the experimental mass fingerprint with theoretical peptide masses generated *in silico* using protein and nucleotide sequence databases. This approach proves very effective when trying to identify proteins from species whose genomes are relatively small, completely sequenced, and well annotated, but is not so reliable for organisms whose genomes have not been completed. A second problem is to identify proteins that are extensively postranslationally modified, since the peptides generated from these proteins may not match with the unmodified protein in the database. A third problem is that PMF does not work very well if several different proteins are present in the same spot.

3.8.1 Peptide mass fingerprinting MALDI-TOF MS

Spots were excised from Coomassie stained gels and destained for 30 minutes using 100 µl acetonitrile (50%) and 5mM (NH4)HCO3 (50%); Next, spots were dehydrated with acetonitrile and dried at 40°C for 30 minutes using a Speedvac.

For the tryptic digestion, 20 µg trypsin (Promega, Madison, WI, USA) was dissolved in 100 µl 1mM HCl and directly before use, 150 µl 5mM (NH4)HCO3 were added to the trypsin solution (final concentration:12.5 ng/µl). 10 µl of this solution was pipetted on each dried protein spot and incubated for 30 minutes at 20°C. The supernatant was discarded to minimize auto-digestion of trypsin. Then 20 µl 5mM $(NH_4)\ HCO_3$ was added and the sample was incubated for 8h at 37°C. On the sample slides 0.25 µl α-cyano-4-hydroxycinnamic acid in acetonitrile (1mg/ml) were pipetted and air dried (seed-layer). Then 0.5 µl digested sample was added to the slide and mixed with 0.5 µl matrix solution (15 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile + 0.5% trifluoroacetic acid) and air dried for 10 minutes.

Mass spectrometry was performed by using the Ettan z² MALDI-ToF (Amersham Biosciences, Uppsala, Sweden) with UV nitrogen laser (337 nm) and harmonic reflectron; Mode: Positive-ion-reflectron-mode at 20 kV with delayed extraction mode und low mass rejection; Calibration: Peptide-Samples (Angiotensine II, ACTH 1-39) and internal standard (trypsine auto-digestion fragments). For each spectrum, 200 single shots were accumulated.

Proteins were identified by searching the monoisotopic masses against the database of curated by NCBI (accession: NC_002662) with the implemented software ETTAN MALDI-TOF Pro Evaluation Module Ver. 2.0 (Amersham Biosciences). One missed cleavage per peptide was allowed, and a mass tolerance of 10 ppm was used in all searches. Partial modifications of proteins by carbamidomethylation of cysteines and oxidization of methionines were taken into account. Proteins were considered as identified, when at least five peptides were matched, the expectation value was smaller than 0.01 and the result could be reproduced with a corresponding spot picked of a parallel gel. Agreement of theoretical and approximate experimental isoelectric point and molecular weight were generally included in the identification process of proteins too.

4 Results and Discussion

In this study we analysed the proteome of *C. glutamicum* before and after the exposure to heavy metal by using 2DE and MALDI- TOF- MS. Proteome analysis can deliver more accurate and comprehensive information than genomic/ transcriptomic, because protein expressions are regulated not only at transcriptional but also at translational levels, resulting in more details about mature proteins and their interactions than genome-based prediction (Humphery-Smith et al., 1997). After the treatment of *C. glutamicum* cells with heavy metal many proteins were up or down regulated, these proteins are called stress proteins. Some of them are general stress proteins, but others are specific stress proteins, which can be considered as biomarkers. In this study we used IPG strips 3.4-5.4 and 4.9-6.9 because the most proteins of *C. glutamicum* concentrate in pH gradient 4-7 (Figure 7).

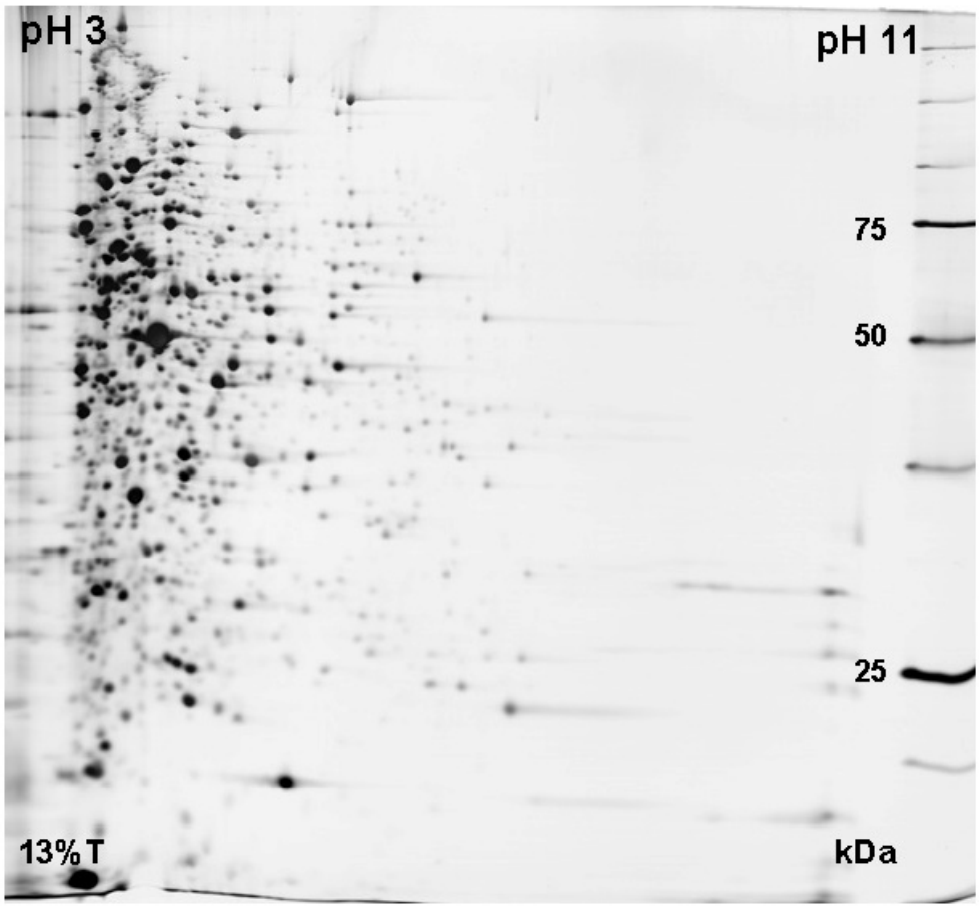


Figure 7. Protein map of *C. glutamicum* using IPG strip 3-11

In this study we used sub-lethal concentrations of heavy metals as stress concentration and for each heavy metal, three analytical replicates of two independent biological experiments were run on pH-ranges of 3,4-5,4 and 4,9–6,9.

C. glutamicum was chosen based on the fact that besides of having a short generation time, the bacterium is safe to handle and its genome has been completely sequenced (Kalinowski et al., 2003), and furthermore has been the subject of biological research for several decades (Silberbach and Burkovski, 2006).

The *C. glutamicum* proteome was separated in three distinct fractions, (i) cytoplasmic proteins, (ii) proteins of the membrane fraction, and (iii) cell wall-associated proteins. The Proteome of *C. glutamicum* is similar to the proteome of other bacteria (Himmelreich et al., 1996; Link et al., 1997). Compared to *E. coli* protein map, the number of proteins with a p*I* higher than 8 is decreased and the gap between proteins with acidic and basic p*I* is more pronounced in *C. glutamicum*.

Although many proteins putatively involved in heavy metals stress-response are not amenable to a 2-DE-based proteomic approach due to their low solubility (*e.g.*, membrane proteins) or low abundance, our focus on the "soluble" protein fraction would only be a shortcoming if the major goal of our study had been to elucidate the detoxification mechanisms, in many of which membrane proteins are involved. Yet, the current study was primarily designed to identify a set of heavy metal stress-regulated proteins that may be utilized as proteomic signatures, *e.g.*, for the development of rapid diagnostic tools for heavy metal exposure.

4.1 A proteome analysis of C. glutamicum after exposure to mercury

4.1.1 Effect of mercury on the growth of *C. glutamicum* cells

To study the effect of mercury ions on the growth of *C. glutamicum*, the cells were cultivated in the presence of various concentrations of $HgCl_2$. The measured optical densities of control and mercury-treated cells over time are shown in Figure. 8.

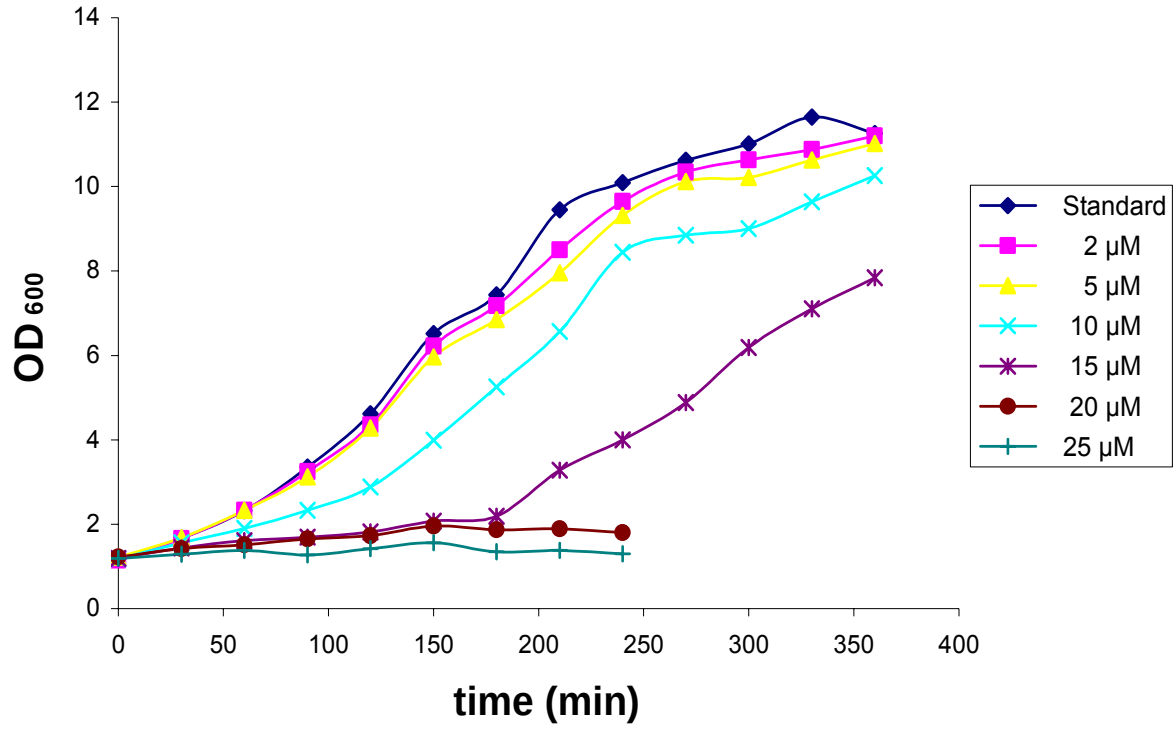


Figure 8. Effect of mercury on the growth of wild-type *C. glutamicum* cells. C. glutamicum grown in BHI medium for about 5h at 30°C in BHI medium supplemented with different concentrations (0 - 25 µM) $HgCl_2$. Cells were harvested at OD = 6.5 for proteomic analysis.

C. glutamicum cells could survive and grow at mercury concentrations from 2–15 µM, whereas 20 µM led to growth arrest. At lower mercury concentrations, the cells were able to adapt and resume growth after a long lag phase or a period of stasis. Figure 8 indicates that the stress-free cells reached the mid-log phase (OD_{600} = 6.5) faster than the stressed cells which needed a longer time period for adaptation.

4.1.2 Differential protein expression

The exposure of *C. glutamicum* cells to low concentration of mercury resulted changes in protein synthesis. We analyzed the proteomic response to mercury to identify differentially expressed proteins, which could be important for the resistance to this toxic compound. *C. glutamicum* cells were treated with 10 µM mercury. Extracts from untreated as well as treated cells were then subjected to comparative two-dimensional gel electrophoresis (Figure. 9. 10). Changes in spot intensity between untreated and treated cells were quantified by image analysis software (see Materials and Methods). Differences by a fold change > 1.5 between treated and untreated cultures and a p value (expectation) < 0.005 were considered as

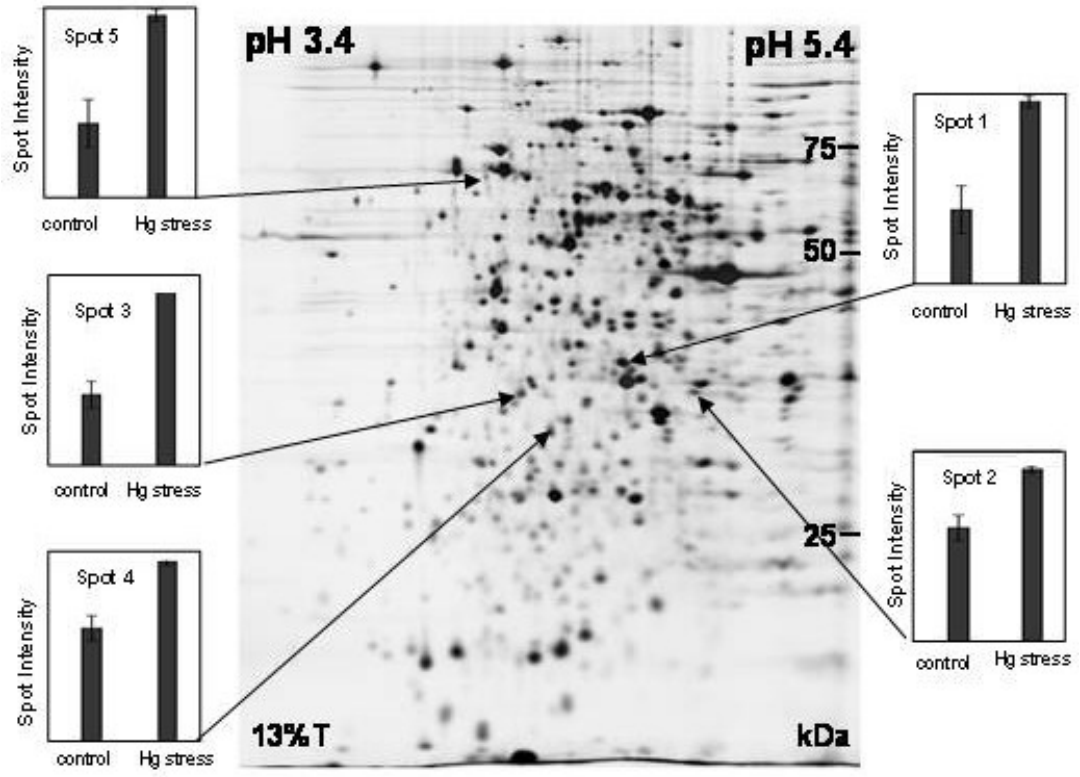
significant. The intensity of 13 spots changed after the exposure to mercury, all 13 proteins were up regulated by mercury.

The identity of six of the thirteen proteins induced by mercury is given in Table 6. The identity of the remaining differentially expressed proteins could not be determined unequivocally because the concentration of these proteins on the Coomassie Blue stained gels was too low for MALDI-MS PMF. Mercury-responsive proteins were sorted into three different functional classes: (i) Enzymes with antioxidant properties. This class includes NADPH-quinone reductase and related Zn-dependent oxidoreductases and thioredoxin reductase. (ii) Proteins involved in the transport system. This class contains only one protein ABC-type dipeptide transport system, periplasmic component. (iii) Other proteins: These include enzymes involved in inositol metabolism, a conserved hypothetical protein and Zn-dependent alcohol dehydrogenases.

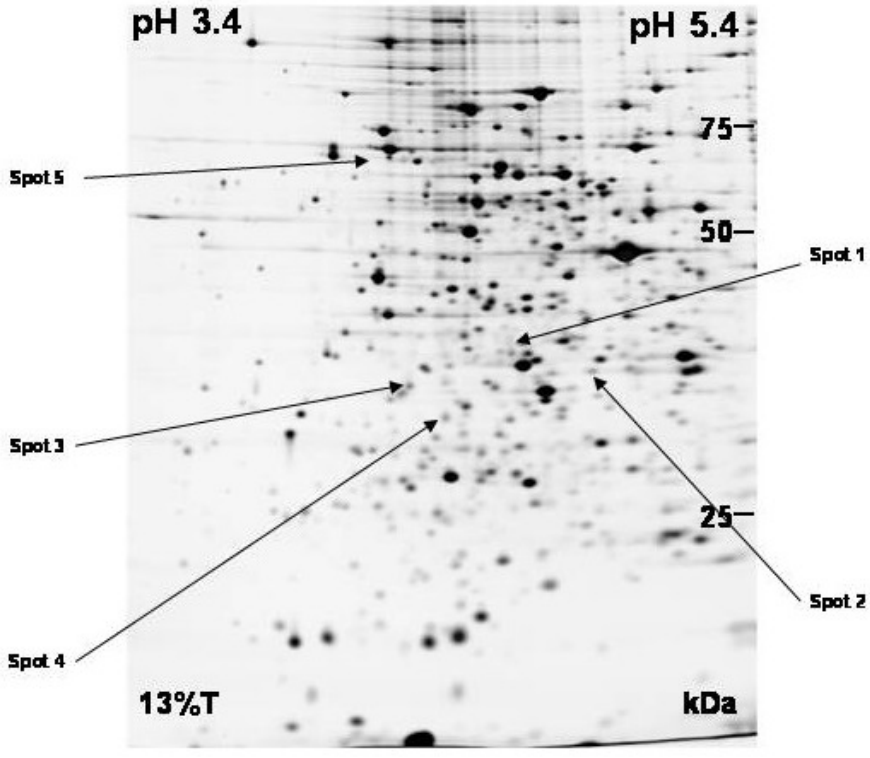
Table 6. **List of identified *C. glutamicum* proteins whose expressions were stimulated by $HgCl_2$**

Spot	Protein information	Expectation	Induction	Coverage (%)	p*I*	Mass (kDa)	Match
1	Cg3405–[C]: NADPH quinone reductase or Zn-dependent oxidoreductase) NCgl2971 (COG0604) [CR]: NADPH: quinone reductase and related Zn-dependent oxidoreductases	0.00	2.3	56.9	4.8	33.88	13/26
2	Cg0201–(iolB) [C]: enzyme involved in inositol metabolism NCgl0158 (COG3718) [G]: uncharacterized enzyme involved in inositol metabolism	0.00	1.7	58.4	4.9	32.49	11/26
3	Cg3422–(trxB) [C]: thioredoxin reductase (EC 1.6.4.5) NCgl2984 (COG0492) [O]: thioredoxin reductase	0.00	2.1	69.1	4.6	34.24	18/26

4	Cg0198–[C]: conserved hypothetical protein NCgl0156	0.00	1.9	32.6	4.7	32.9	11/24
5	Cg2937–[S]: ABC-type dipeptide/oligopeptide/nickel transport system, secreted component NCgl2562 (COG0747) [E]: ABC-type dipeptide transport system, periplasmic component	0.001	2.3	16.6	4.5	57.69	6/20
6	Cg3107–(adhA) [C]: Zn-dependent alcohol dehydrogenase (EC 1.1.1.1) NCgl2709 (COG1064) [R]: Zn-dependent alcohol dehydrogenases	0.00	3.1	23.8	5.2	36.79	7/19

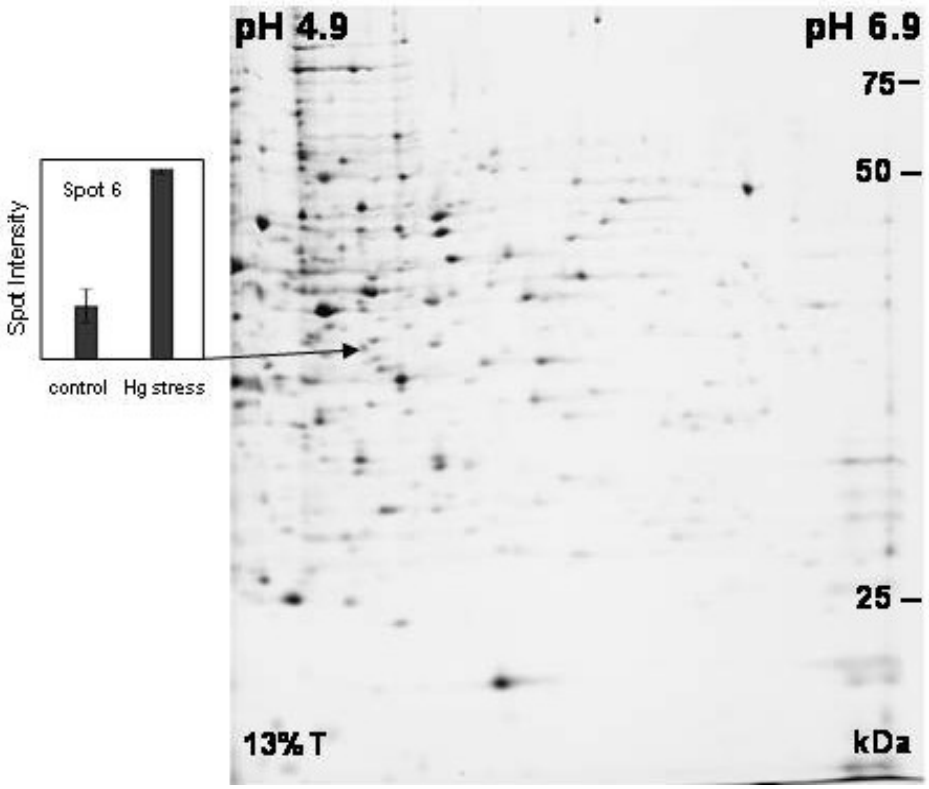


(A)

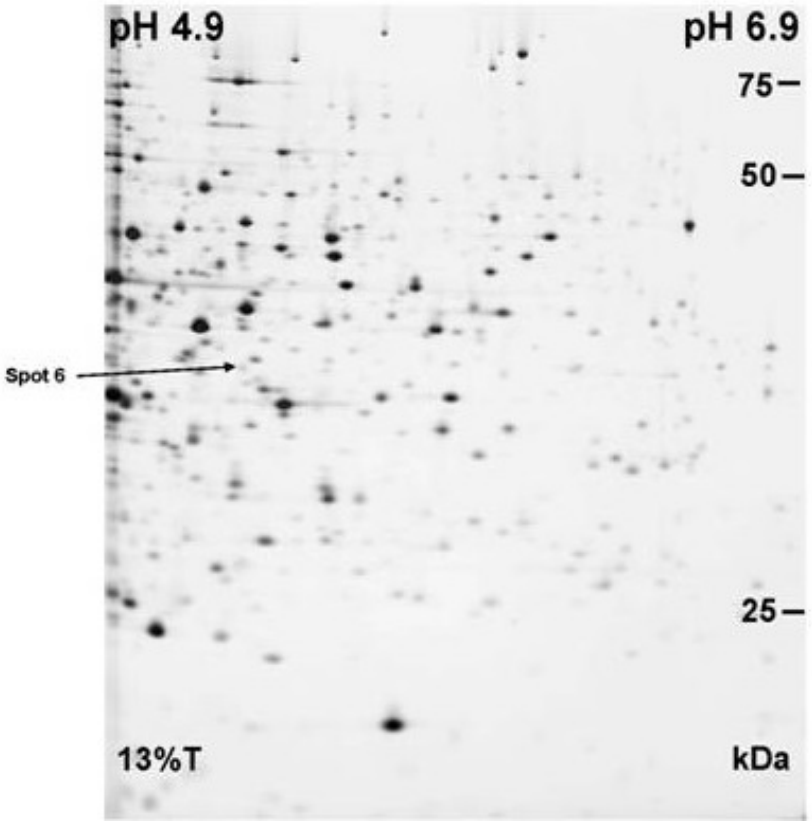


(B)

Figure 9. 2D gel images of *C. glutamicum* cells ranging from 3.4 to 5.4 treated with 10 µM $HgCl_2$ (A) or untreated (=control) (B). The stress proteins are numbered and their identities listed in Table 6.



(A)



(B)

Figure 10. 2D gel images of *C. glutamicum* cells ranging from 4.9 to 6.9 treated with 10 µM $HgCl_2$ (A) or untreated (=control) (B). The stress proteins are numbered and their identities listed in Table 6.

The production of reactive oxygen species (ROS) is known as one of the major mechanisms of the toxicities exerted by heavy metals. In this study we found that two antioxidant proteins were significantly up-regulated. These data indicate that mercury causes oxidative stress and *C. glutamicum* cells respond against mercury stress by enhancing the antioxidant proteins. Reactive oxygen species (ROS) which are produced by heavy metals as a result of oxidative stress cause severe damage to biological macromolecules (Lledías et al., 1998).They bind to lipids, proteins and DNA; they can cause DNA damage and may lead to cytotoxicity, mutagenicity and carcinogenicity (Tampo et al., 1998). Thioredoxin reductase was overexpressed 2.1 fold upon mercury stress (Figure 11).

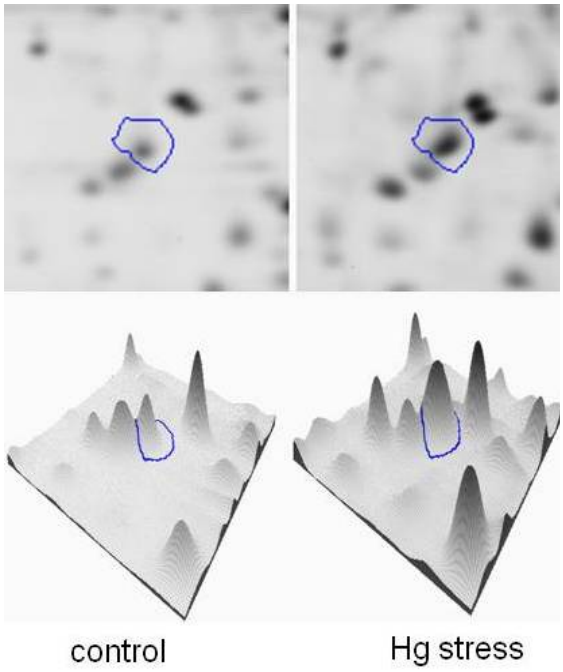


Figure 11. Overexpression of thioredoxin reductase (spot 3) after treatment with mercury. "Zoom in" from IPG 3.4-5.4 (silver staining).

Thioredoxin reductase (Trx) is considered as an important antioxidant in the protection of cells from oxidative stress (Figure 12). Thioredoxin reductase utilizes NADPH to catalyse the conversion of oxidized (ox.) Thioredoxin into reduced (red.) Thioredoxin, and to reduce the oxidized forms of ascorbate into reduced ascorbate. Reduced Thioredoxin provides reducing equivalents to (i) Thioredoxin peroxidase, which breaks down H2O2 to water, (ii) ribonucleotide reductase, which reduces ribonucleotides to deoxyribonucleotides for DNA synthesis, and (iii) transcription factors, which leads to their increased binding to DNA and

altered gene transcription. Thioredoxin reductase may play an additional role through increasing cell growth and inhibits apoptosis. (Mustacich and Powis, 2000).

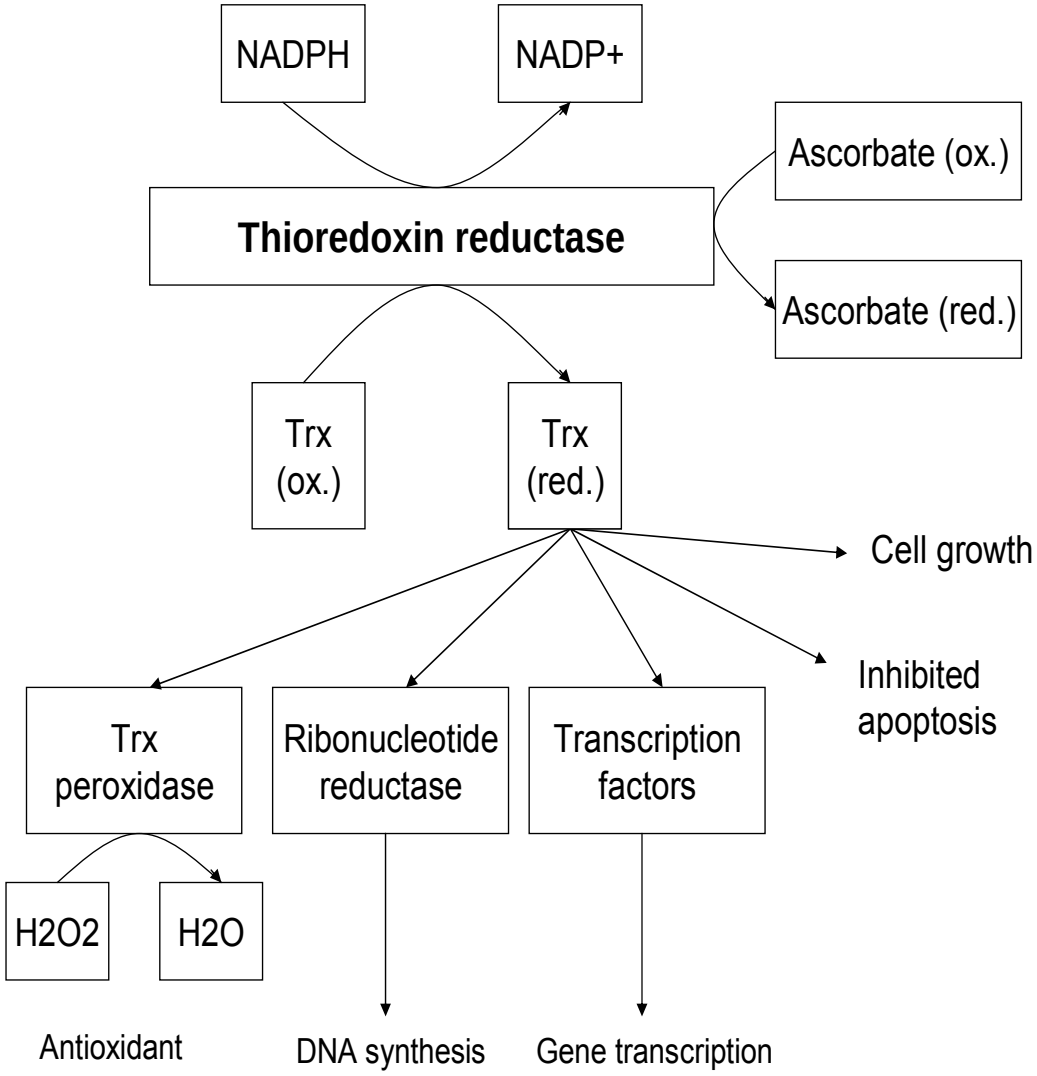


Figure 12. Reactions and functions of thioredoxin reductase in the cell (Mustacich and Powis, 2000).

NADPH-quinone reductase and related Zn-dependent oxidoreductases is also induced 2.1 fold after the treatment with mercury (Figure 13). This protein is an antioxidant enzyme, which is NADH-dependent and requires either FMN or FAD.

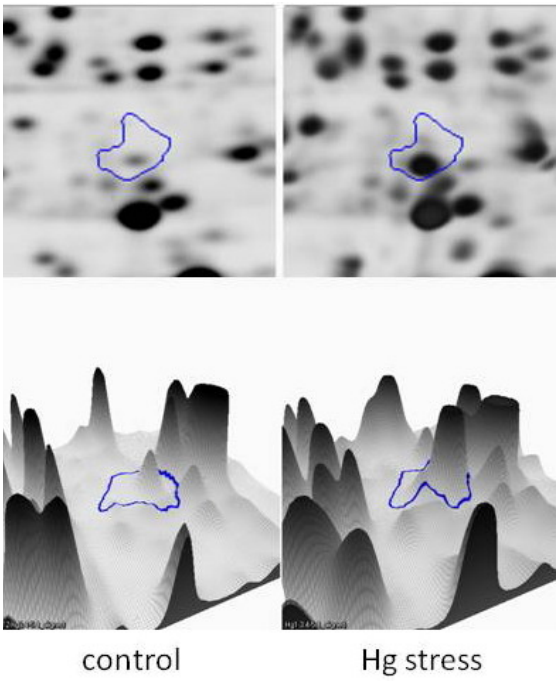


Figure 13. Overexpression of NADPH-quinone reductase and related Zn-dependent oxidoreductases (spot 1) after treatment with mercury. "Zoom in" from IPG 3.4-5.4 (silver staining).

Stress inducible defense or adaptive response mechanisms act to protect cells from these oxidative threats (Storz et al., 1990; Crawford et al., 1994). The antioxidant enzymes play an important role in adaptive response mechanisms and have a dramatic effect on the resistance of cells to oxidative damage of lipids, proteins and DNA.

The ABC-type dipeptide transport system subunit was induced upon Hg^{2+} stress (Figure 14).

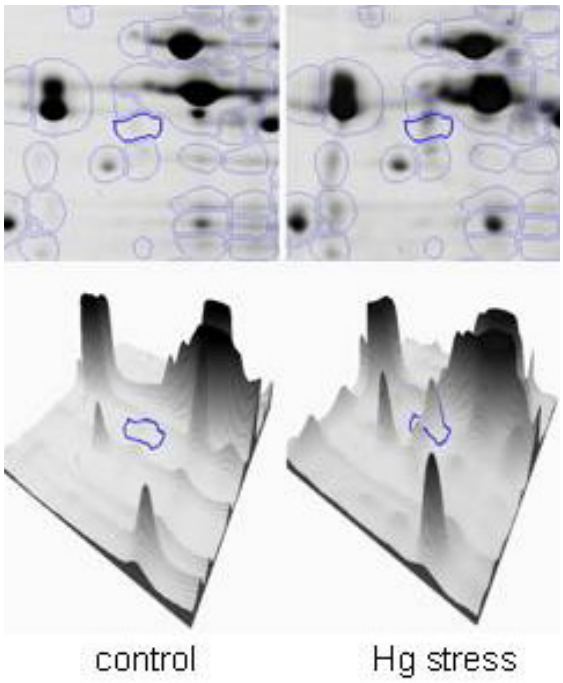


Figure 14. Overexpression of ABC-type dipeptide transport system, periplasmic component (spot 5) after treatment with mercury. "Zoom in" from IPG 3.4-5.4 (silver staining).

ABC (`ATP-Binding Cassette') transport systems comprise an extremely diverse class of membrane transport proteins that couple the energy of ATP hydrolysis to the translocation of solutes across biological membranes **(**Higgins, 1992**)**. Typically, they transport ligands across cellular lipid membranes, which are critical for most aspects of cell physiology, including the uptake of nutrients and elimination of waste products, energy generation, and cell signalling.

ABC transporters require a minimum of four domains. Two transmembrane domains (TMDs) bind ligand, and transport is driven by ATP binding and hydrolysis by the two nucleotide binding domains (NBDs). The TMDs from different subfamilies of ABC transporters are not necessarily homologous. The NBDs are homologous throughout the family. Each NBD has seven highly conserved, but not invariant, motifs. A simple ATP-switch mechanism ABC transporter is shown in Figure 15. Ligand binding to a high-affinity pocket formed by the TMDs induces a conformational change in the NBDs resulting in a higher affinity for ATP. Two molecules of ATP bind to the NBDs. The energy released by the formation of the closed NBD dimer causes conformational change in the TMDs (which is sufficient for P-glycoprotein to extrude the anticancer drug vinblastine, although subsequent conformational changes may drive translocation of different drugs). ATP hydrolysis (an inexorable consequence of the closed conformation) triggers dissolution of the closed NBD dimmer

resulting in further conformational changes in the TMDs. Finally, phosphate and then ADP release restores the transporter to the open NBD dimer conformation ready for the subsequent cycle **(**Linton, 2007).

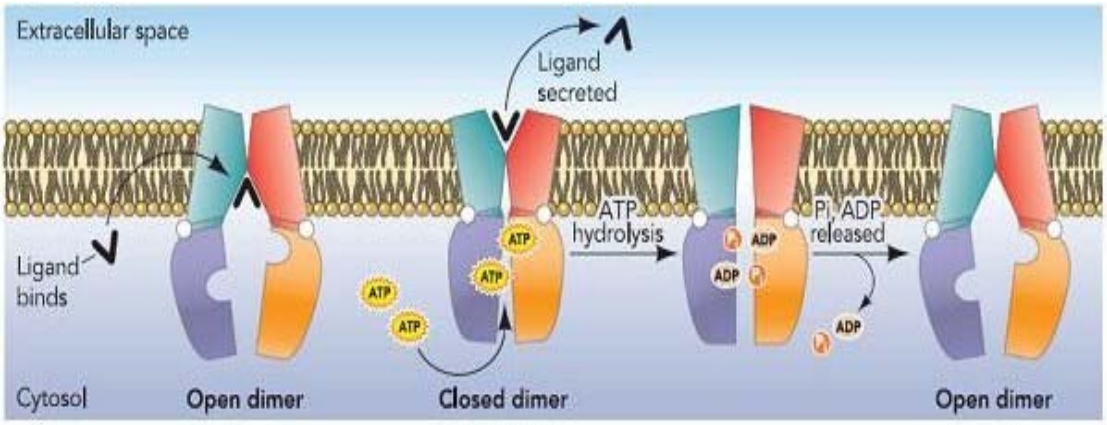


Figure 15. A simple ATP-switch mechanism powers ABC transporters (Linton, 2007).

The expression of this protein was also observed when the yeast *S. cerevisiae* was treated with ethanol or weak acids like sorbic acid (Ferianc et al., 1998); the protein may play an important role in the elimination of toxic metabolites deriving from Hg^{+2}.

The *C. glutamicum* cells also expressed Zn-dependent alcohol dehydrogenase after the exposure to Hg^{2+} (Figure16).

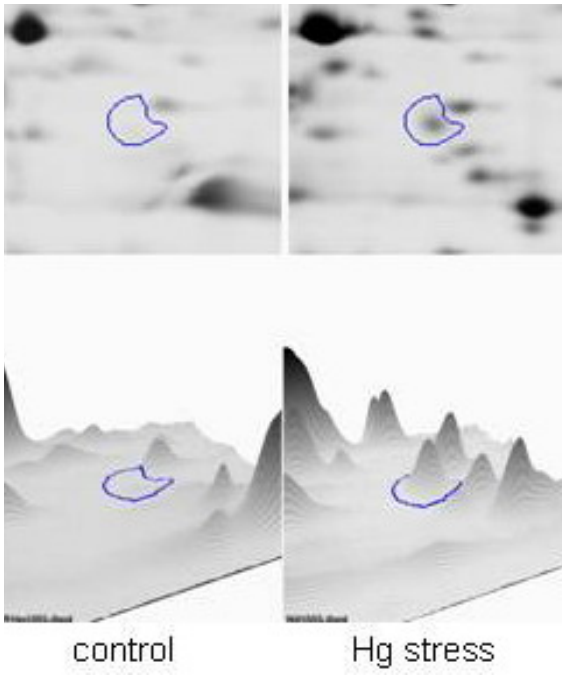


Figure 16. Overexpression of Zn-dependent alcohol dehydrogenase (spot 6) after treatment with mercury. "Zoom in" from IPG 4.9-6.9 (silver staining).

To the best of our knowledge, the protein has not been previously reported as a stress protein in bacteria after the exposure to Hg^{2+}, but An et al. found that this enzyme is a prominent stress protein in obligatory fermentative, gram-negative bacterium *Zymomonas mobilis* after the exposure to ethanol (An et al., 1991). The physiological role of this enzyme with respect to stress is unknown. Perhaps this enzyme could serve to re-establish an NADH-NAD balance after exposure to stress conditions or act as a chaperonin to stabilize other essential enzymes (An et al., 1991).

C. glutamicum is able to induce many defence mechanisms upon mercury stress: Up-regulation of antioxidant enzymes and mercury ion elimination mechanism are the most important defence mechanism of the cells against mercury stress. Surprisingly the cells did not up-regulate heat shock proteins after the exposure to Hg^{2+}, although these proteins were expressed in rat kidney and Fathead Minnow cells upon Hg^{2+} stress (Goering et al., 2000; Devlin and Clary, 1998).

4.2 *A proteome analysis of C. glutamicum after exposure to cadmium*

4.2.1 Effect of cadmium on the growth of *C. glutamicum* cells

The *C. glutamicum* cells were cultivated in the presence of various concentrations of $CdCl_2$. The measured optical densities of control and cadmium-treated cells over time are shown in Figure 17.

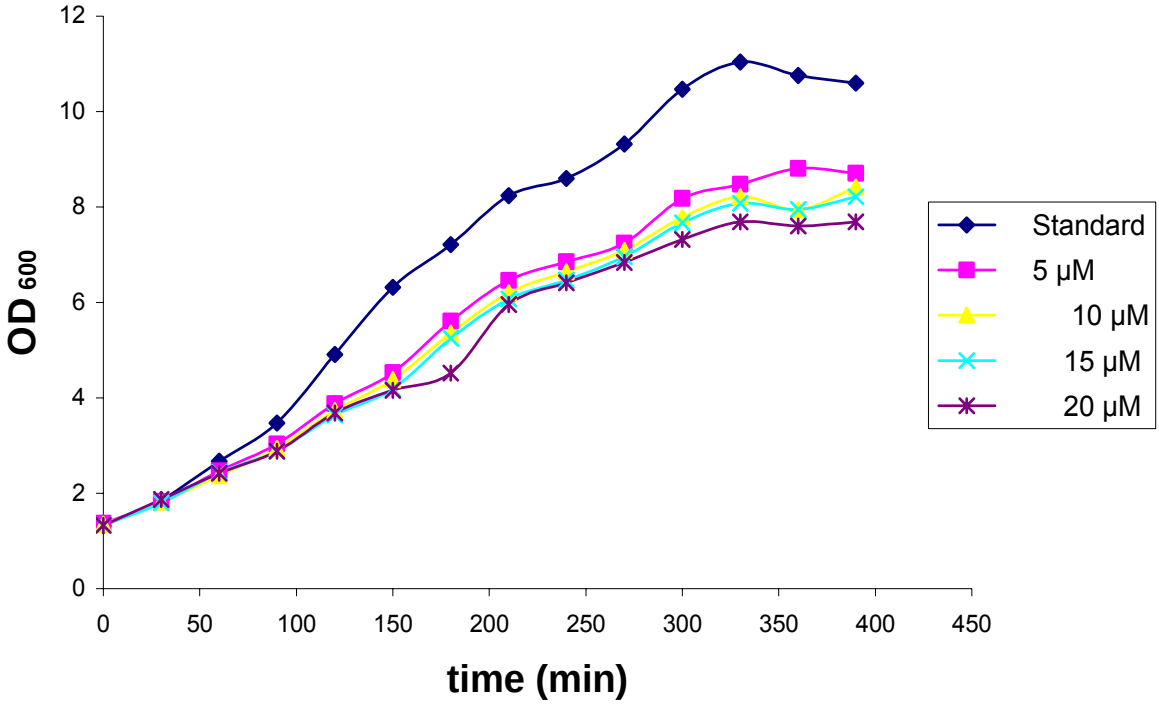


Figure 17. Effect of cadmium on the growth of wild-type *C. glutamicum* cells. *C. glutamicum* grown in BHI medium for about 5h at 30°C in BHI medium supplemented with different concentrations (0 - 20 µM) $CdCl_2$. Cells were harvested at OD = 6.5 for proteomic analysis.

Higher cadmium concentrations (>10 µM) inhibited the growth of *C. glutamicum.* Interestingly, *C. glutamicum* cells have more resistance to cadmium than many other bacteria such as *E. coli*, for which 10 µM of cadmium was lethal (Ferianc et al., 1998)

At lower cadmium concentrations, the cells were able to adapt and resume growth after a long lag phase or a period of stasis. Figure 17 indicates that the stress-free cells reached the mid-log phase (OD_{600} = 6.5) faster than the stressed cells which needed a longer time period for adapatation. Gad El-Rab et al., (2006) made similar observations when *Rhodobacter*

capsulatus B10 was exposed to 150 µM $CdCl_2$. The growth rate of stressed *R. capsulatus* cells was also delayed compared to unstressed cells, but the *R. capsulatus* seems to have higher resistance to cadmium compared to *C. glutamicum*.

4.2.2 Differential protein expression

At a low concentration cadmium can cause dramatic changes in protein synthesis of *C. glutamicum. C. glutamicum* cells were treated with 5 µM mercury. Extracts from untreated as well as treated cells were then subjected to comparative two-dimensional gel electrophoresis (Figure. 18, 19). Changes in spot intensity between untreated and treated cells were quantified by image analysis software (see Materials and Methods). The intensity of 43 spots changed after the exposure to cadmium, 35 proteins were induced and 8 proteins were repressed by cadmium. The identity of 26 proteins out of the 35 proteins induced, and one protein out of eight repressed by cadmium is given in Table 7. Cadmium-responsive proteins were sorted into eight different functional classes (i) Enzymes with antioxidant properties: This class includes flavin-dependent oxidoreductases, manganese superoxide dismutase, NADPH-quinone reductase and related Zn- dependent oxidoreductases, thioredoxin reductase, putative thioredoxin and putative glutathione reductase. (ii) Proteins involved in repair and biogenesis of Fe-S cluster proteins: This class contains two proteins: ABC-type transport system involved in Fe-S cluster assembly, permease component, and ABC-type transport system involved in Fe-S cluster assembly, ATPase component. (iii) Molecular chaperones: This class contains two proteins DnaJ-class molecular chaperone with C-terminal Zn finger domain and molecular chaperone GrpE (heat shock protein). (iv) Proteases and proteasome subunits: This class contains protease subunit of ATP-dependent Clp proteases. (v) Proteins involved in terpenoid biosynthesis: This class shows a strong stimulation index. It contains only one protein involved in the nonmevalonate pathway of terpenoid biosynthesis. (vi) Proteins involved in lipoate synthase. (vii) Proteins involved in energy metabolism: This class contains the down-regulated protein succinate dehydrogenase and phosphogluconate dehydrogenase (viii) Other proteins: These include predicted Fe-S cluster redox enzyme, nitroreductase, aldo/keto reductasese, uncharacterized protein, uroporphyrinogen III decarboxylase, and putative dithiol-disulfide isomerase involved in polyketide biosynthesis.

Table 7. **List of identified *C.glutamicum* proteins whose expressions were stimulated by $CdCl_2$.**

Spot	Protein information	Expectation	Induction	Repression	Coverage (%)	pI	Mass (kDa)	Match
1	Cg1762 - (sufC) [C]: Iron-regulated ABC transporter ATPase subunit | NCgl1501 (COG0396) [O]: ABC-type transport system involved in Fe-S cluster assembly, ATPase component	0.001	1.8	-	43.3	5	27.62	8/25
2	Cg1127 - [C]: Uncharacterized proteins, LmbE homolog | NCgl0948 (COG2120) [S]: Uncharacterized proteins, LmbE homologs	0.000	2.1	-	59.5	5	33.04	13/34
3	Cg3405 - [C]: NADPH quinone reductase or Zn-dependent oxidoreductase | NCgl2971 (COG0604) [CR]: NADPH:quinone reductase and related Zn-dependent	0.000	2.7	-	45.5	4.8	33.88	9/16

	oxidoreductases							
4	Cg2645 - (clpP1) [C]: ATP-DEPENDENT CLP PROTEASE PROTEOLYTIC SUBUNIT CLPP1 (EC 3.4.21.92) | NCgl2328 (COG0740) [OU]: Protease subunit of ATP-dependent Clp proteases	0.000	1.9	-	53.3	4.9	21.13	8/24
5	Cg2644 - (clpP2) [C]: ATP-DEPENDENT CLP PROTEASE PROTEOLYTIC SUBUNIT CLPP2 (EC 3.4.21.92) | NCgl2327 (COG0740) [OU]: Protease subunit of ATP-dependent Clp proteases	0.000	1.7	-	44.2	4.8	32.02	4/9
6	Cg3344 - [C]: Nitroreductase | NCgl2913 (COG0778) [C]: Nitroreductase	0.001	6.9	-	56.2	4.5	22.05	9/17
7	Cg2661 - [C]: putative dithiol-disulfide isomerase involved in polyketide biosynthesis |	0.000	3.1	-	44.8	4.6	22.73	7/18

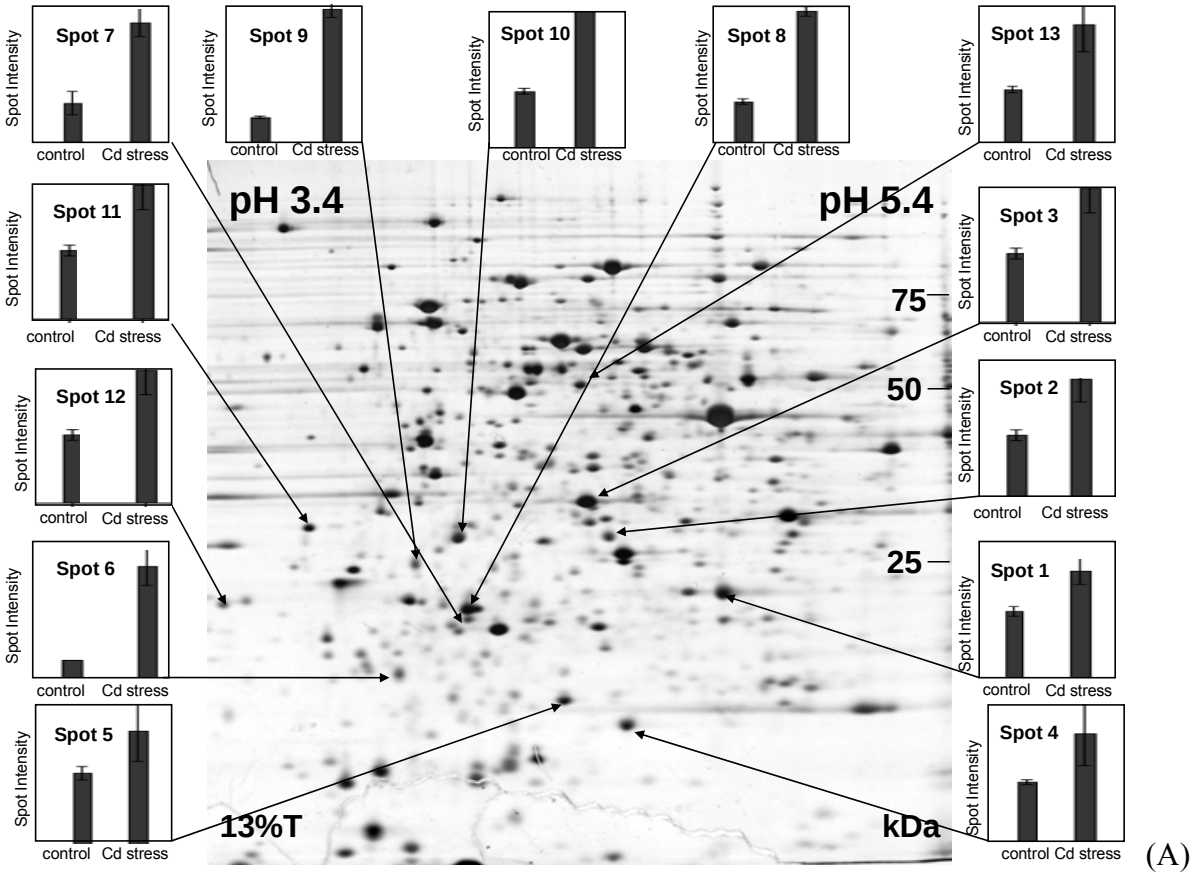
	NCgl2339							
8	Cg2661 - [C]: putative dithiol-disulfide isomerase involved in polyketide biosynthesis | NCgl2339	0.005	3.3	-	45.8	4.6	22.73	9/21
9	Cg2838 - [C]: Predicted dithiol-disulfide isomerase | NCgl2478 (COG2761) [Q]: Predicted dithiol-disulfide isomerase involved in polyketide biosynthesis	0.000	5.5	-	42.5	4.8	29.57	9/34
10	Cg3422 - (trxB) [C]: THIOREDOXIN REDUCTASE (EC 1.6.4.5) | NCgl2984 (COG0492) [O]: Thioredoxin reductase	0.000	2.7	-	38.5	4.6	34.24	10/27
11	Cg1375 - [C]: PUTATIVE THIOREDOXIN | NCgl1172 (COG3118) [O]: Thioredoxin domain-containing protein	0.000	2.2	-	29.6	4.3	32.35	7/25

12	Cg3099 - (grpE) [C]: Molecular chaperone GrpE (heat shock protein) | NCgl2701 (COG0576) [O]: Molecular chaperone GrpE (heat shock protein)	0.002	2.8	-	46.3	4.1	23.66	7/22
13	Cg2194 - (gor) [C]: PUTATIVE GLUTATHIONE REDUCTASE (EC 1.6.4.2) | NCgl1928 (COG1249) [C]: Pyruvate/2-oxoglutarate dehydrogenase complex, dihydrolipoamide dehydrogenase (E3) component, and related enzymes	0.002	2.3	-	20.4	4.8	50.13	8/38
14	Cg2214 - [C]: Predicted Fe-S-cluster redox enzyme | NCgl1944 (COG0820) [R]: Predicted Fe-S-cluster redox enzyme	0.000	2.3	-	47.8	6.1	40.01	19/40
15	Cg2538 - [C]: ALKANAL MONOOXYGENASE ALPHA CHAIN (EC 1.14.14.3) | NCgl2229 (COG2141) [C]:	0.004	11	-	45.4	5.4	35.66	8/38

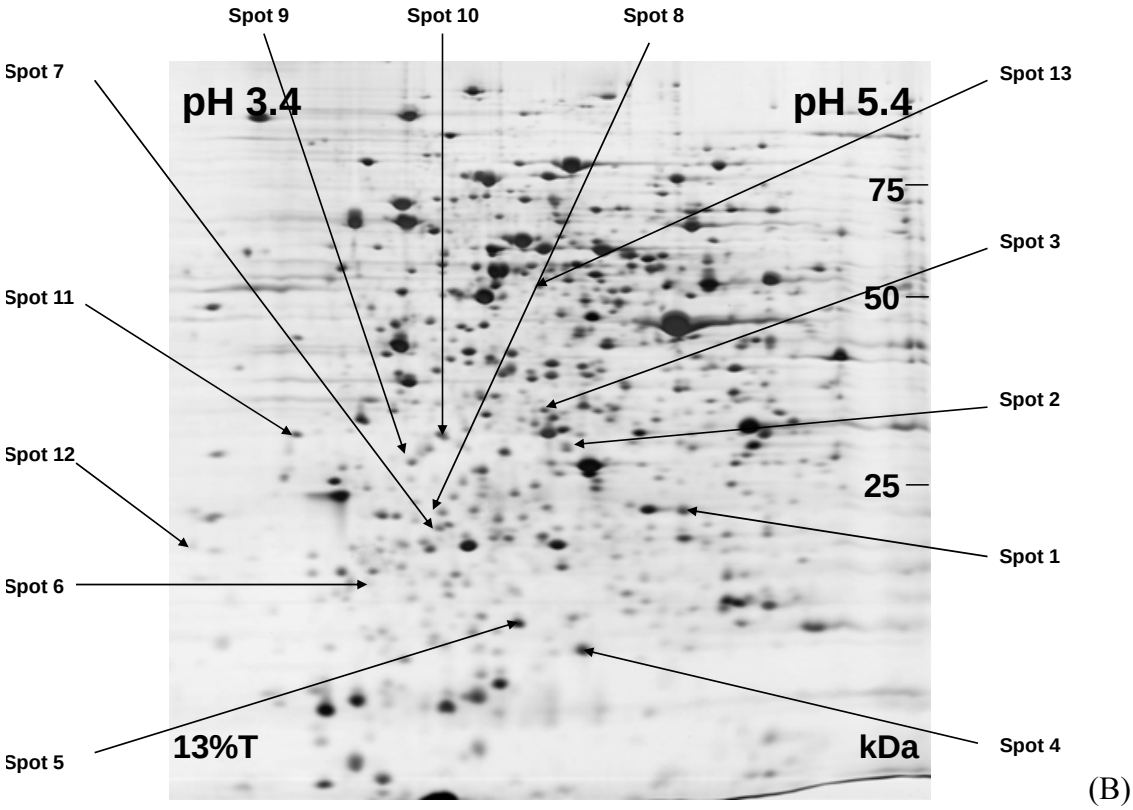
	Coenzyme F420-dependent N5,N10-methylene tetrahydromethanopterin reductase and related flavin-dependent oxidoreductases							
16	Cg2423 - (lipA) [C]: LIPOIC ACID SYNTHASE | NCgl2128 (COG0320) [H]: Lipoate synthase	0.000	8	-	46.6	5.4	39.29	14/47
17	Cg2515 - (dnaJ2) [C]: Molecular chaperone (contain C-terminal Zn finger domain), putative transcriptional repressor | NCgl2210 (COG0484) [O]: DnaJ-class molecular chaperone with C-terminal Zn finger domain	0.005	2.5	-	22.8	5.4	40.64	6/32
18	Cg1432 - (ilvD) [C]: DIHYDROXY-ACID DEHYDRATASE (EC 4.2.1.9) | NCgl1219 (COG0129) [EG]:	0.000	2.4	-	35.4	5.2	64.66	16/29

	Dihydroxyacid dehydratase/phosphogluconate dehydratase							
19	Cg1763 - (sufD) [C]: components of an uncharacterized iron-regulated ABC-type transporter | NCgl1502 (COG0719) [O]: ABC-type transport system involved in Fe-S cluster assembly, permease component	0.002	2	-	33.7	5.2	42.26	8/26
20	Cg3237 - (sod) [C]: MANGANESE SUPEROXIDE DISMUTASE (EC 1.15.1.1) | NCgl2826 (COG0605) [P]: Superoxide dismutase	0.001	1.4	-	54.5	5.1	22.08	6/18
21	Cg0516 - (hemE) [C]: UROPORPHYRINOGEN DECARBOXYLASE (EC 4.1.1.37) | NCgl0420 (COG0407) [H]:	0.000	1.5	-	9.2	5.3	38.4	3/6

	Uroporphyrinogen-III decarboxylase							
22	Cg1528 - (dkgX) [C]: putative 2,5-DIKETO-D-GLUCONIC ACID REDUCTASE (EC 1.1.1.-) | NCgl1302 (COG0656) [R]: Aldo/keto reductases, related to diketogulonate reductase	0.004	4.3	-	23.4	4.7	32.35	4/15
23	Cg2206 - (ispG) [C]: involved in the nonmevalonate pathway of terpenoid biosynthesis | NCgl1938 (COG0821) [I]: Enzyme involved in the deoxyxylulose pathway of isoprenoid biosynthesis	0.000	6.3	-	40	5.5	42.56	14/36
24	Cg0447 - (sdhB) [C]: succinate dehydrogenase B | NCgl0361 (COG0479) [C]: Succinate dehydrogenase/fumarate reductase Fe-S protein	0.002	-	1.6-	20.1	5.4	26.63	6/18

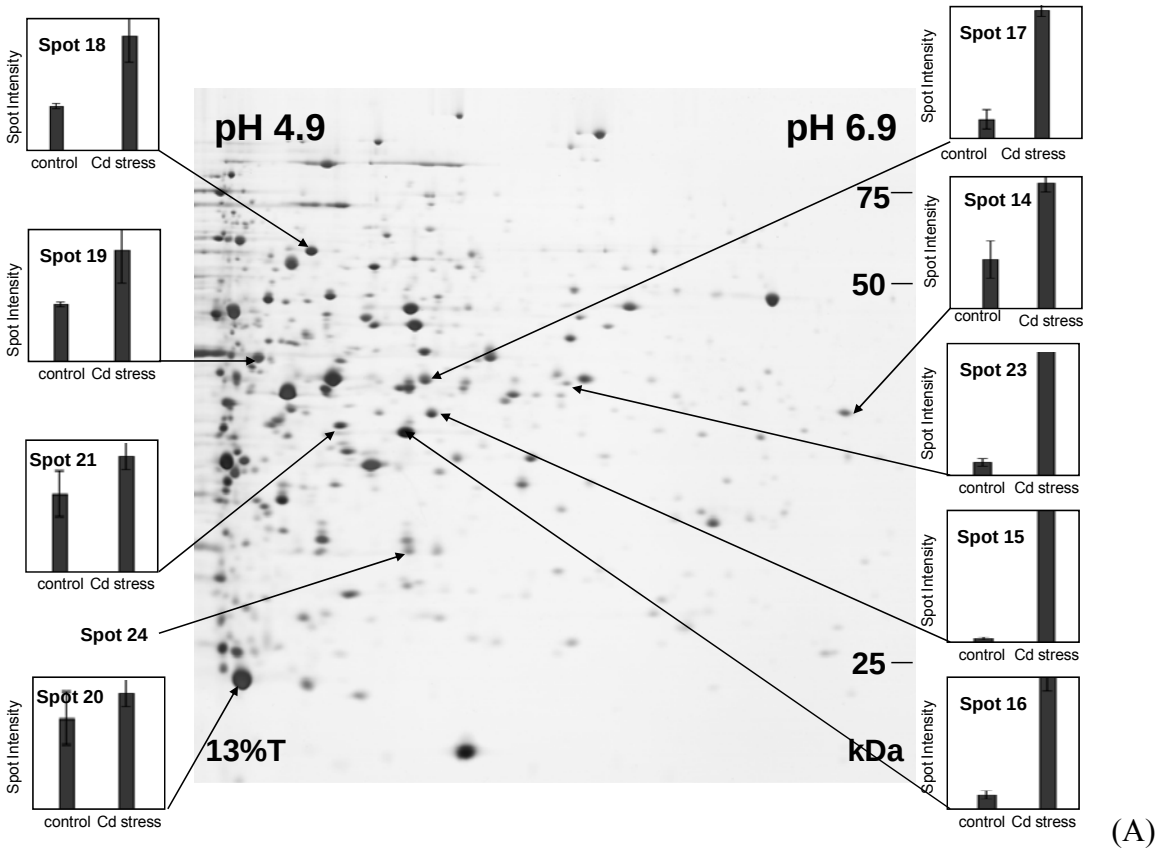


(A)

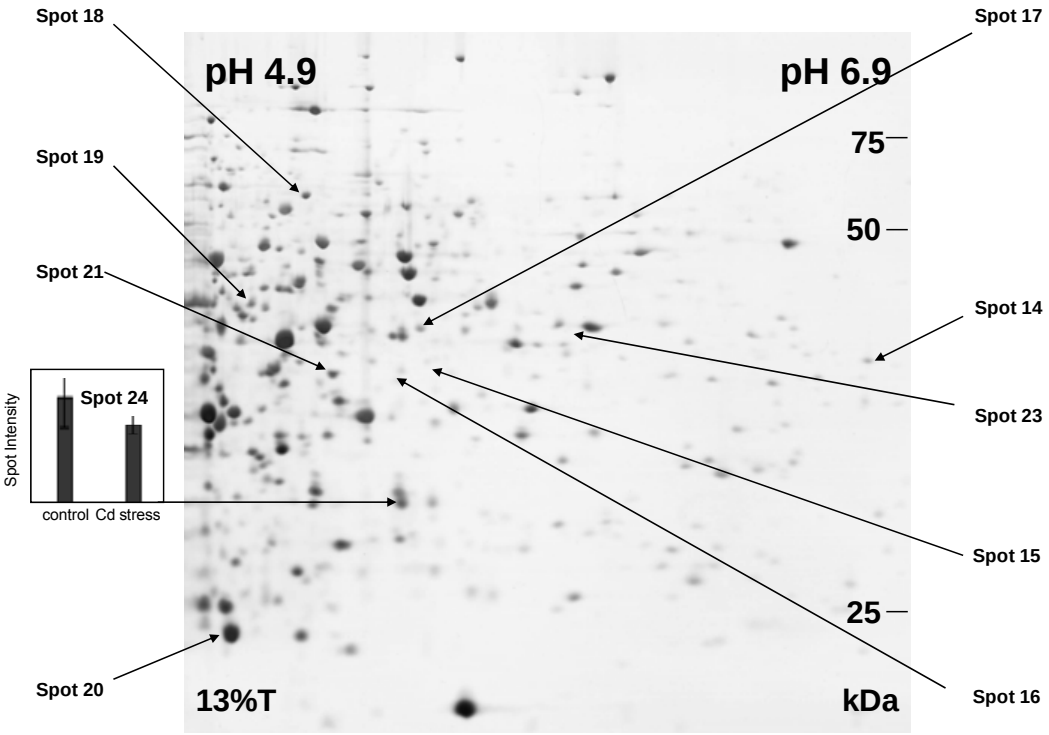


(B)

Figure 18. 2D gel images of *C. glutamicum* cells ranging from 3.4 to 5.4 treated with 5 µM $CdCl_2$ (A) or untreated (=control) (B). The stress proteins are numbered and their identities listed in Table 7.



(A)



(B)

Figure 19. 2D gel images of *C. glutamicum* cells ranging from 4.9 to 6.9 treated with 5 µM $CdCl_2$ (A) or untreated (=control) (B). The stress proteins are numbered and their identities listed in Table 7.

C. glutamicum cells up-regulated three antioxidant enzymes upon cadmium stress. The first one is manganese Superoxide dismutase (Figure 20).

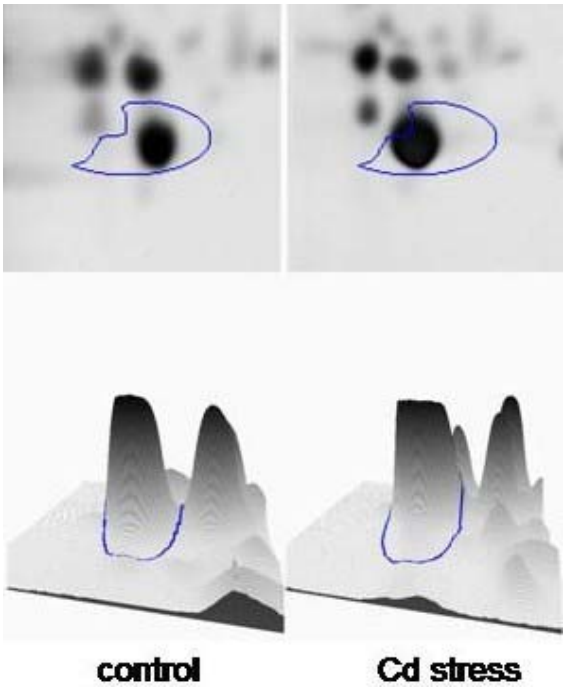


Figure 20. Overexpression of Mn-Superoxide dismutase (spot 20) after treatment with cadmium. "Zoom in" from IPG 4.9-6.9. (silver staining).

Superoxide dismutase (SOD) is metalloenzyme that catalyzes the conversion of the superoxide anion into hydrogen peroxide and dioxygen (McCord and Fridovich, 1969), SOD converts the highly reactive superoxide radical to the less reactive H_2O_2. Another function of superoxide dismutase is to protect dehydratases (dihydroxy acid dehydratase, aconitase, 6-phosphogluconate dehydratase and fumarases A and B) against inactivation by the free radical superoxide (Benov and Fridovich, 1998).Thus, the expression of Mn-SOD is essential for the survival of aerobic life and the development of cellular resistance to oxygen radical mediated toxicity.

The antioxidant proteins thioredoxin reductase and NADPH-quinone reductase and related Zn-dependent oxidoreductases are also induced after the treatment with cadmium (Figure 21).

Glutathione (GSH) binds to heavy metal ions and detoxifies them. The toxic effects of cadmium were inhibited in two ways: the first way by direct scavenging of free Cd^{+2} ions and detoxification of toxic effects produced by Cd^{+2} ions; free cellular Cd^{+2} ions are scavenged initially by GSH. Although GSH is absent in all actinomycetes, mycothiol (MSH) has been identified as a major thiol instead. MSH is even more resistant than GSH to heavy metal ion-

catalyzed oxidation, and it seems likely to be the antioxidant thiol used by actinomycetals. The second way is indirect detoxification of Cd^{+2} ions.

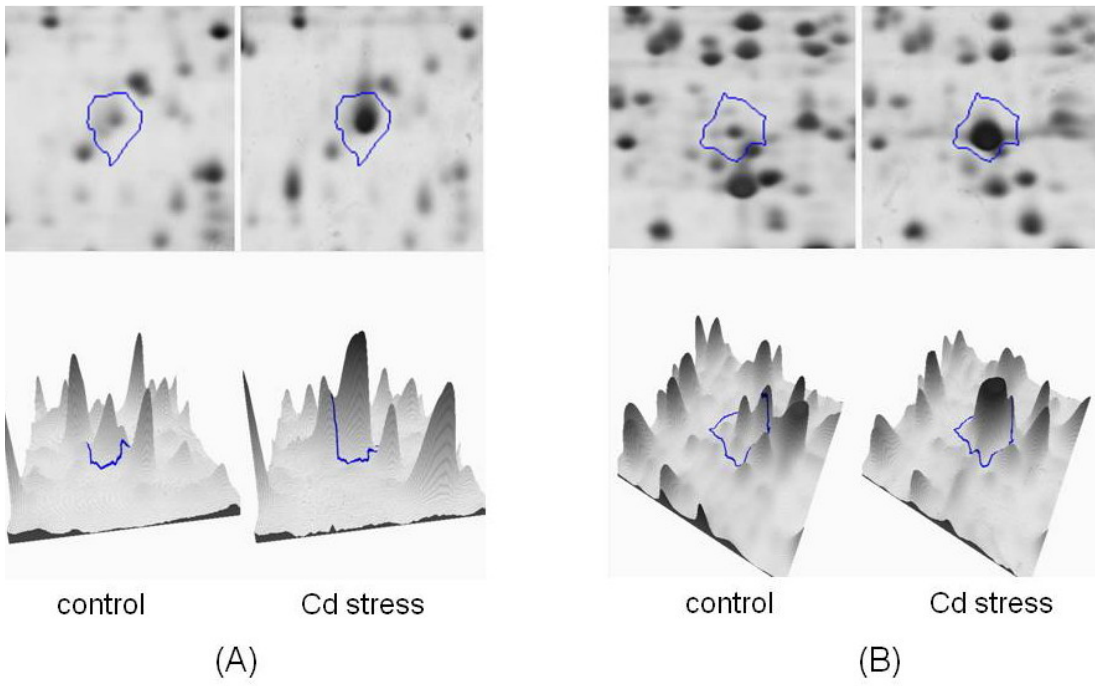


Figure 21. Overexpression of thioredoxin reductase (spot 10) (A) and Zn-dependent oxidoreductases (spot3) (B) after treatment with cadmium. "Zoom in" from IPG 4.9-6.9. (silver staining)

Cd^{+2} ions produce ROS, the cells overexpress superoxide dismutases and other antioxidant enzymes to remove the ROS and overexpress detoxification proteins to restore the damaged macromolecules (Bae and Chen, 2004).

Two ABC-type subunits involved in Fe-S cluster repair and assembly were over-expressed upon Cd^{2+} stress (Figure 22).

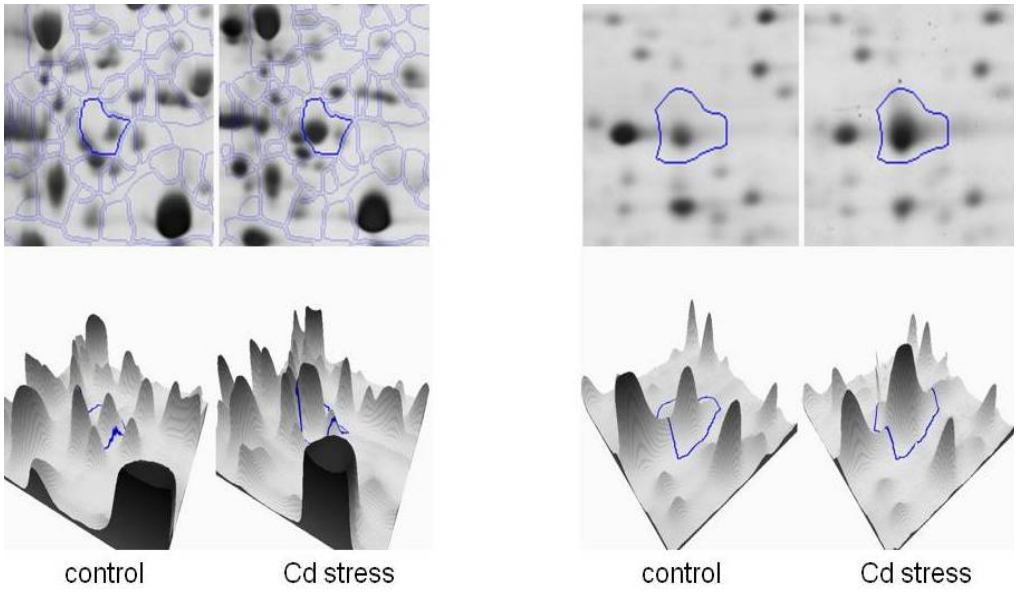


Figure 22. Overexpression of Two ABC-type subunits involved in Fe-S cluster repair (spot 1) after treatment with cadmium. "Zoom in" from IPG 4.9-6.9 (silver staining).

The stressed cells need Fe-S cluster proteins, because they play key roles in electron transport, as active site cofactors in TCA cycle enzymes; in addition they are involved in oxidative stress protection (Almeida et al., 2006).

The Cd^{2+} induction of heat shock proteins and proteases is also consistent with protective activity against oxidative stress (Figure 23).

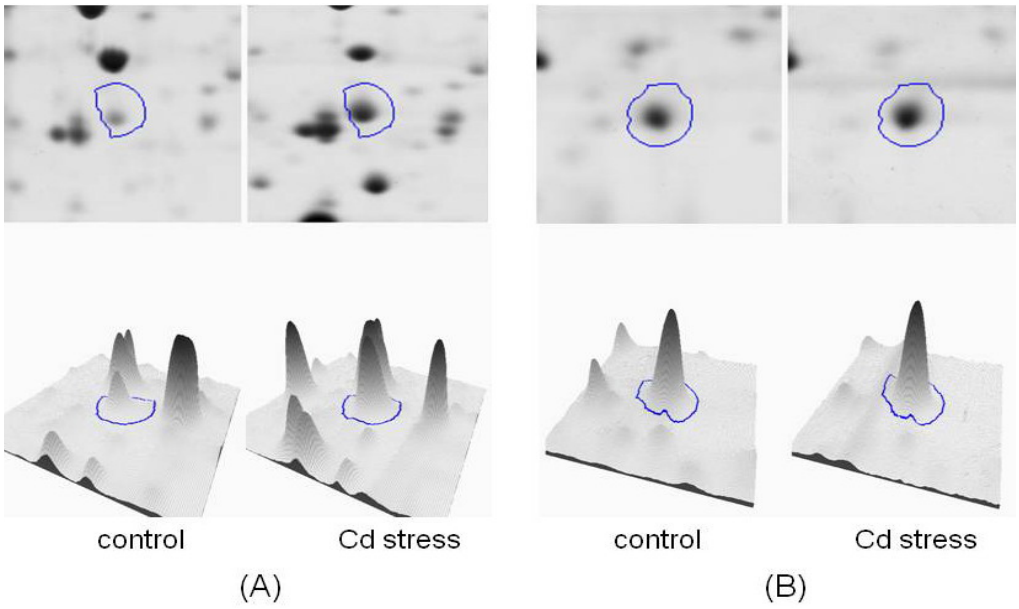


Figure 23. Overexpression of Molecular chaperone (spot 17) (A) and Protease subunit of ATP-dependent (spot 5) (B) after treatment with cadmium. "Zoom in" from IPG 4.9-6.9 and 3.4-5.4 (silver staining).

Chaperones are ubiquitous, highly conserved proteins, which utilize a cycle of ATP-driven conformational changes to fold or refold their targets, and which probably played a major role in the molecular evolution of modern enzymes (Hartl, 1996; Csermely, 1997) Environmental stress (a sudden change in the cellular environment, to which the cell is not prepared to respond, such as oxidative stress or heat shock) leads to the expression of most chaperones, which therefore are called heat-shock, or stress proteins.

A rapid oxidation would result in the formation of numerous incorrect disulfide bridges, which would lock the protein in a distorted conformation. Chaperones can serve as cytoplasmic "antioxidants", they protect their target proteins by covering their sensitive sites. Chaperones help other proteins fold correctly (or prevent them to aggregate into non-functional protein junk) (Figure.24). Without the chaperone the protein would not achieve an active conformation and end up being degraded. If the oxidative damage is prevails. In this case chaperones capture denatured proteins and hold them until their refolding or degradation (Papp et al., 2003)

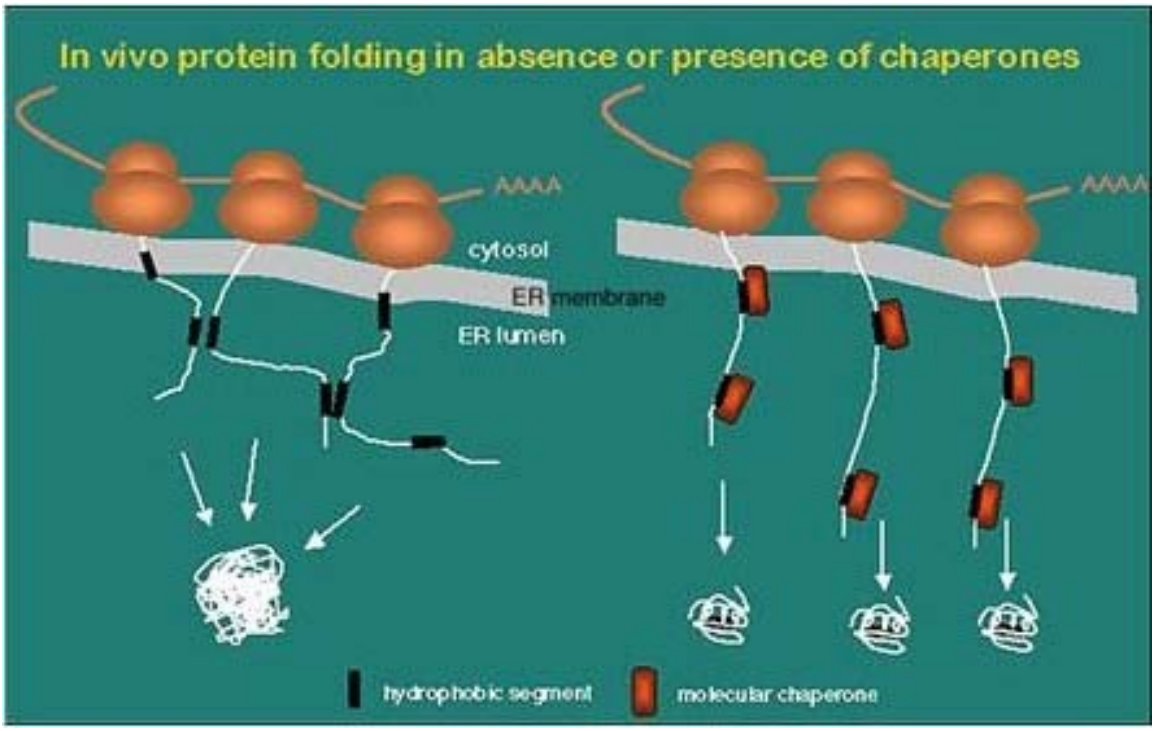


Figure 24. In the absence of molecular chaperones (left) exposed hydrophobic segments of nascent polypeptides associate and lead to aggregation. Molecular chaperones (right) interact with hydrophobic segments in regulated cycles of binding and release, preventing aggregation and allowing productive folding. The chaperone does not actually direct folding pathways but rather prevents non-productive aggregation reactions. (www.nurseminerva.co.uk/cellbiology.htm)

Induction of heat shock proteins has been reported as defense mechanisms against a wide range of stress conditions, including heat shock, oxidative stress, and heavy metals (Hall, 2002; Beyersmann and Hechtenberg, 1997). Most of chaperones and proteases, in addition to their protective functions, they may help to reorchestrate the cell metabolism to the needs of the oxidative stress response (Godon et al., 1998).

Enzyme involved in biosynthesis of terpenoid and Lipoic acid synthase were significantly up-regulated (6.3 and 8-fold), respectively (Figure 25). Terpenoids are hydrocarbons resulting from the combination of several isoprene units. It has been reported that many enzymes of the terpenoid pathway were induced in *Nicotiana tabacum* leaves, which acclimated to oxidative stress, although metabolites synthesized by these enzymes have been mainly implicated in defense reactions against pathogens and UV, many of them can act as antioxidants as well (Vranová et al., 2002). This protein may have a protective effect against Cd^{2+} in *C. glutamicum* as well.

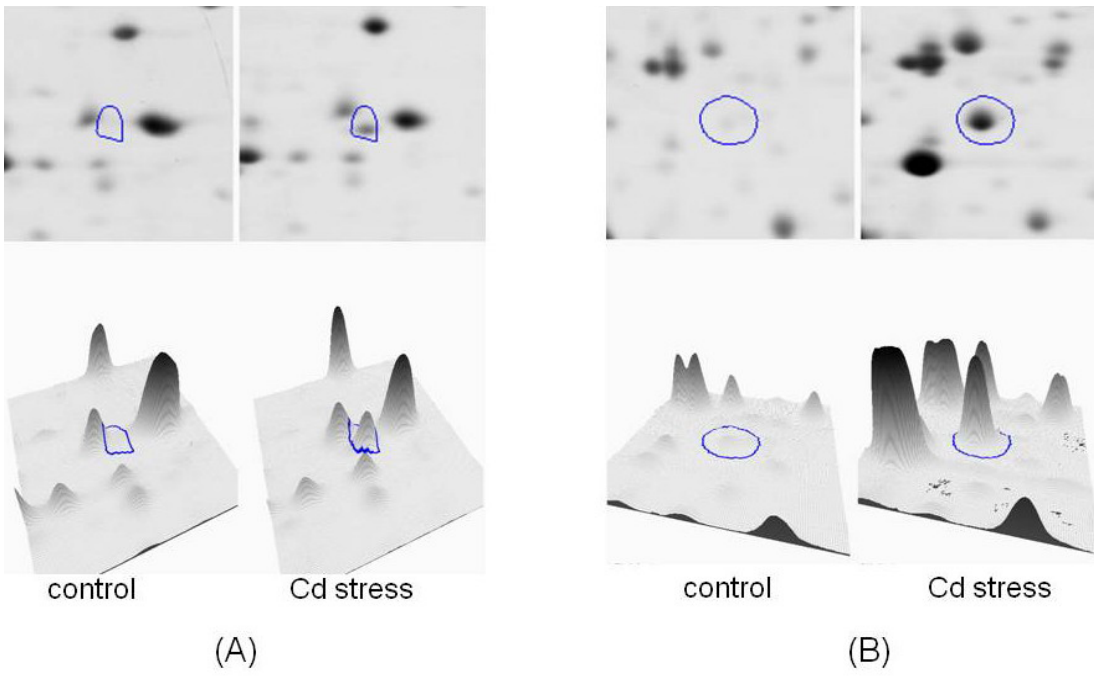


Figure 25. Overexpression of enzyme involved in biosynthesis of terpenoid (spot 23) (A) and Lipoic acid synthase(spot 16) (B) after treatment with cadmium. "Zoom in" from IPG 4.9-6.9 (silver staining).

One of the most visible roles of lipoic acid is as a cofactor in aerobic metabolism, specifically the pyruvate dehydrogenase complex. Lipoic acid participates in transfer of acyl or methylamine groups in 2-oxoacid dehydrogenase and glycine cleavage complexes,

respectively (Perham, 2000). It is able to scavenge ROS. The relatively good scavenging activity of lipoic acid is due to the strained conformation of the five-membered ring in the intermolecular disulfide (Haenen and Bast, 1991). In mammalian cells, lipoic acid can be reduced to dihydrolipoic acid, which is able to regenerate (reduce) antioxidants, such as glutathione, ascorbic acid and vitamin E, maintaining a healthy cellular redox state.(Bierwenga et al., 1997; Packer, 1995). When Pirlich et al. (2002) exposed mouse hippocampal HT22 cells to ethanol, they found that α-lipoic acid could be effective in preventing ethanol- induced neurotoxicity and these results indicate that the radical scavenging properties of α-lipoic acid are effective to ameliorate ethanol- induced neurotoxicity. To the best of our knowledge, this protein has not been previously reported as a stress protein in bacteria after the exposure to heavy metals.

The induction of two proteins involved in energy metabolism has been changed upon cadmium stress (Figure 26).

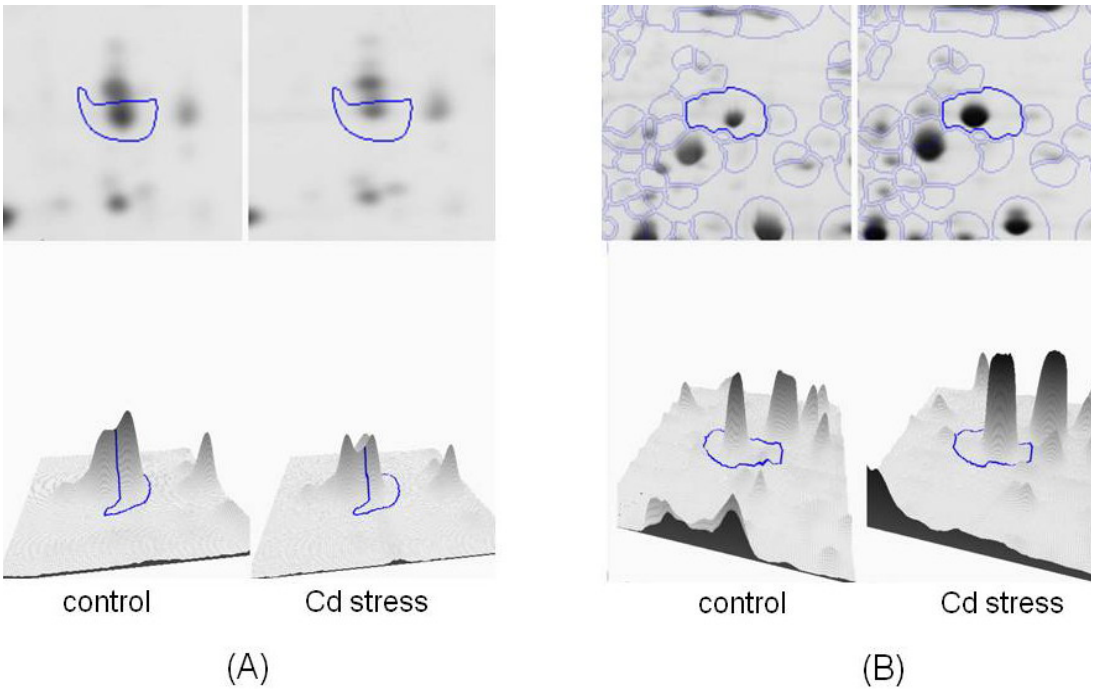


Figure 26. Down regulation of succinate dehydrogenase (spot 24) (A) and up regulation of phosphogluconate dehydratase (spot 18) (B) after treatment with cadmium. "Zoom in" from IPG 4.9-6.9 (silver staining).

The sole down-regulated protein we had been able to identify under Cd^{2+} stress is succinate dehydrogenase, which participates to the citric acid cycle. Benndorf et al (2006), in contrast made a different observation: When *Pseudomonas putida* KT2440 was under oxidative stress,

succinate dehydrogenase was up regulated. The expression of this enzyme may reflect the increased need of the cell to generate enough energy to counteract the oxidative stress, but in our study Cd^{2+} has a negative effect on this protein, in spite the cells need energy to cope the stress. The induction of phosphogluconate dehydrogenase is probably a response of the cell's necessity for energy. Phosphogluconate dehydrogenase is an enzyme of the pentose phosphate pathway (Figure 27), which is important for generating NADPH, which is a source of reducing energy. The pentose phosphate pathway (also called Phosphogluconate Pathway) is a process that serves **(i)** to generate reducing equivalents, in the form of NADPH, for reductive biosynthesis reactions within cells. **(ii)** To provide the cell with ribose-5-phosphate (R5P) for the synthesis of the nucleotides and nucleic acids.

There are two distinct phases in the pathway. The first is the oxidative phase, in which NADPH is generated, and the second is the non oxidative synthesis of 5 carbon sugars.

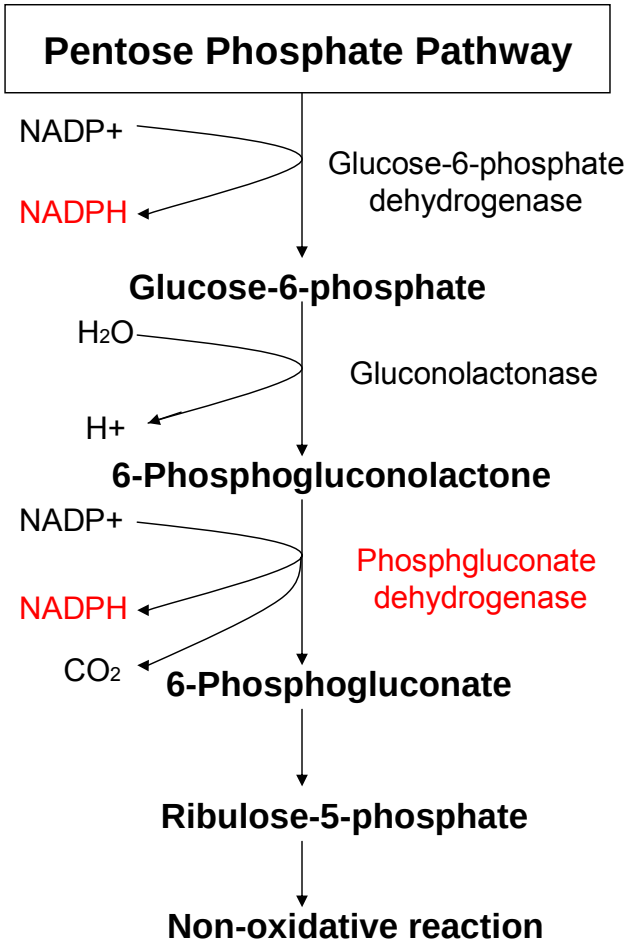


Figure 27. Pentose phosphate pathway (themedicalbiochemistrypage.org/pentose-phosphate-pathway.html)

This pathway is an alternative to glycolysis. While it does involve oxidation of glucose, its primary role is anabolic rather than catabolic. The overexpression of pentose phosphate pathway has been observed in primary rat hepatocytes after the treatment with cadmium chloride (Xu et al., 2003).

This pathway is also important for protecting cells from oxidative stress, since NADPH is an essential cofactor for gluthathione- and thioredoxin- dependent enzymes that defend cells against oxidative damage (Miosga and Zimmermann, 1996; Minard and McAlister-Henn, 2001).

The induction of Fe-S cluster redox enzyme and nitroreductase is a part of the concerted defense against the Cd^{2+} stress (Figure 28). The Fe-S cluster proteins participate to electron transportation, as active site cofactors in TCA cycle enzymes; in addition they are involved in oxidative stress protection (Almeida et al., 2006). Nitroreductase probably contributes to the defenses against oxidative stress by converting organic nitro compounds, quinines, and dyes to stable products and thus diminish the O^{2-} production due to the redox cycling of these compounds (Liochev et al., 1999).

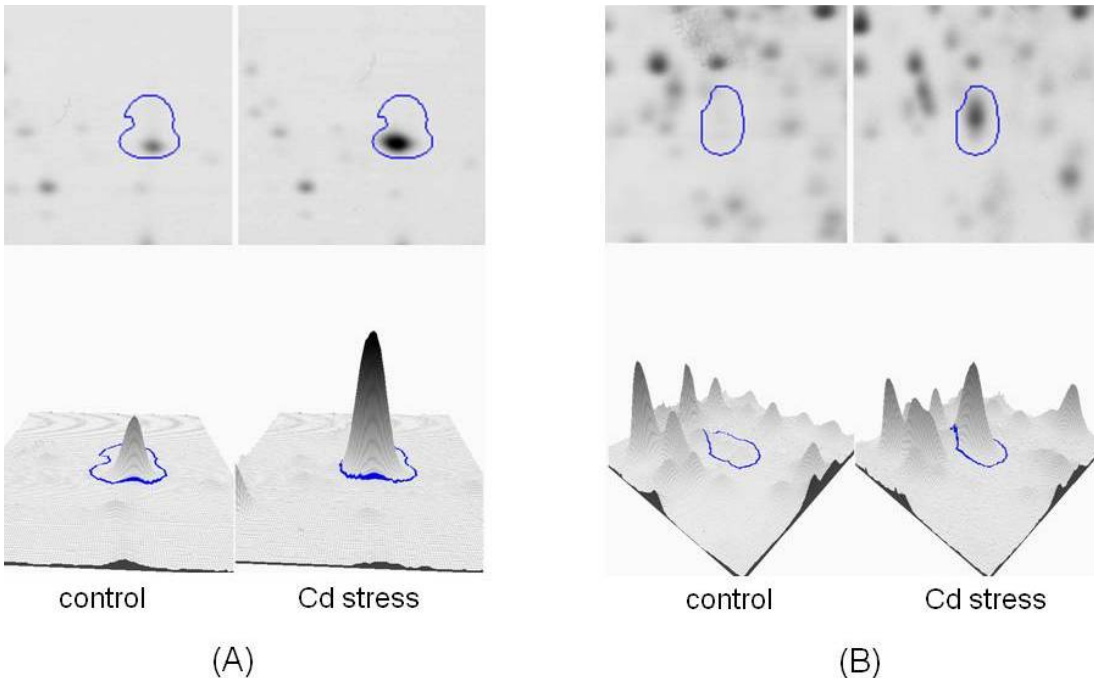


Figure 28. Overexpression of Fe-S-cluster redox enzyme (spot 14) (A) and nitroreductase (spot 6) (B) after treatment with cadmium. "Zoom in" from IPG 3.4-5.4 and 4.9-6.9 (silver staining).

Uroporphyrinogen III decarboxylase is a homodimeric enzyme which catalyzes the heme biosynthesis (Figure 29, 30). Heme and its metabolism fulfil significant roles in many

homeostatic and adaptative reactions; they control levels of several oxidative stress response proteins.

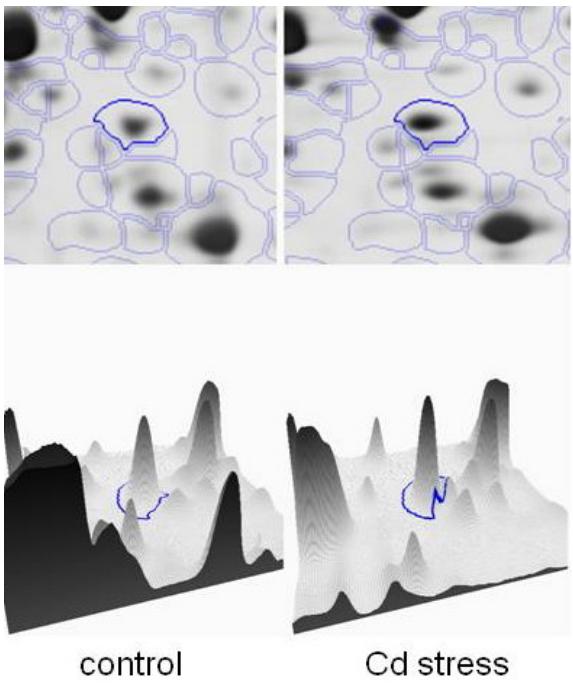


Figure 29. Overexpression of uroporphyrinogen-III decarboxylase (spot 21) after treatment with cadmium. "Zoom in" from IPG 4.9-6.9 (silver staining).

Heme oxygenase (HO) is the key enzyme in heme catabolism, which degrades heme to Fe, CO, and biliverdin, the degradation products of heme (i.e., carbon monoxide, biliverdin, and bilirubin) possess various biological activities.

Biliverdin is rapidly converted to bilirubin, which is the end product of heme catabolism in the cell prevented the decrease in antioxidant proteins (Llesuyand Tomaro,1994).The antioxidative effect of bilirubin is proposed to protect cells against reactive oxygen species (Kazuhiro, 2002).

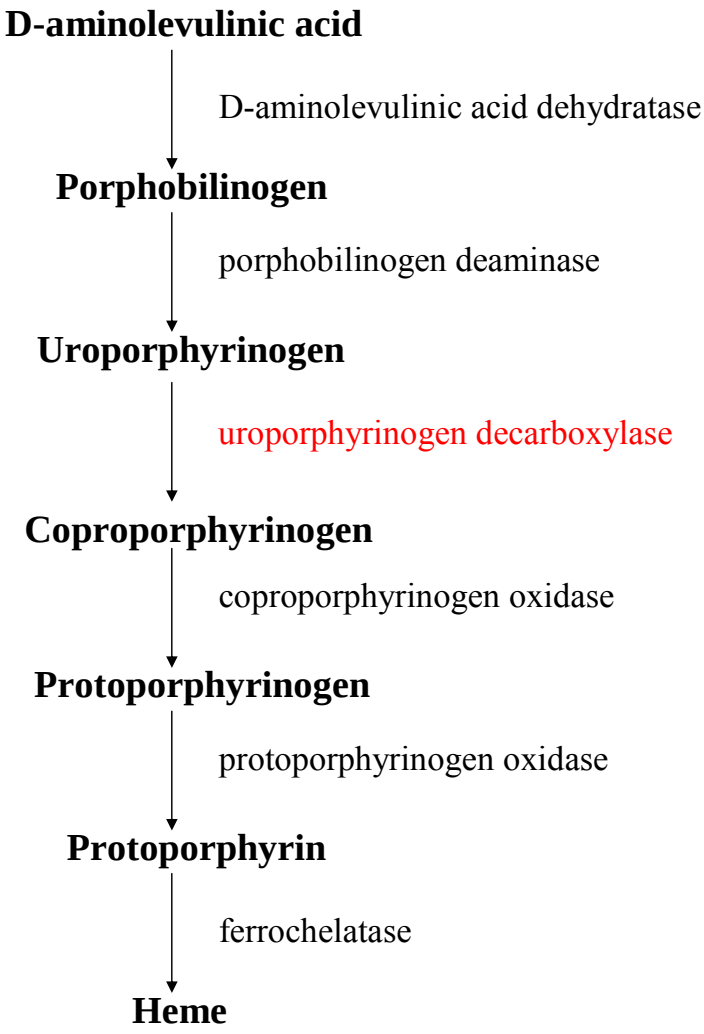


Figure 30. Heme biosynthetic pathway (Afonso et al., 1999).

Frankenberg et al (2003) has been reported that proteins carrying a prosthetic heme group are vital parts of bacterial energy conserving and stress response systems.

The cells induce four important defence mechanisms upon cadmium stress: Induction of antioxidant enzymes, induction of heat shock proteins, energy metabolization system, and induction of proteins involved in lipoic acid and terpenoid biosynthesis. The induction of stress promoters responding to cadmium could be useful as a first indicator of the presence of such pollutant. Detection of these responses could serve as an alarm signal. The protein lipoic acid synthase may have the potential to serve as biomarker in order to detect the presence of cadmium in the living environmental organisms.

4.3 *A proteome analysis of C. glutamicum after exposure to cobalt*

4.3.1 Effect of cobalt on the growth of C. glutamicum cells

The *C. glutamicum* cells were cultivated in the presence of various concentrations of $CoCl_2$. The measured optical densities of control and cobalt-treated cells over time are shown in Figure 31

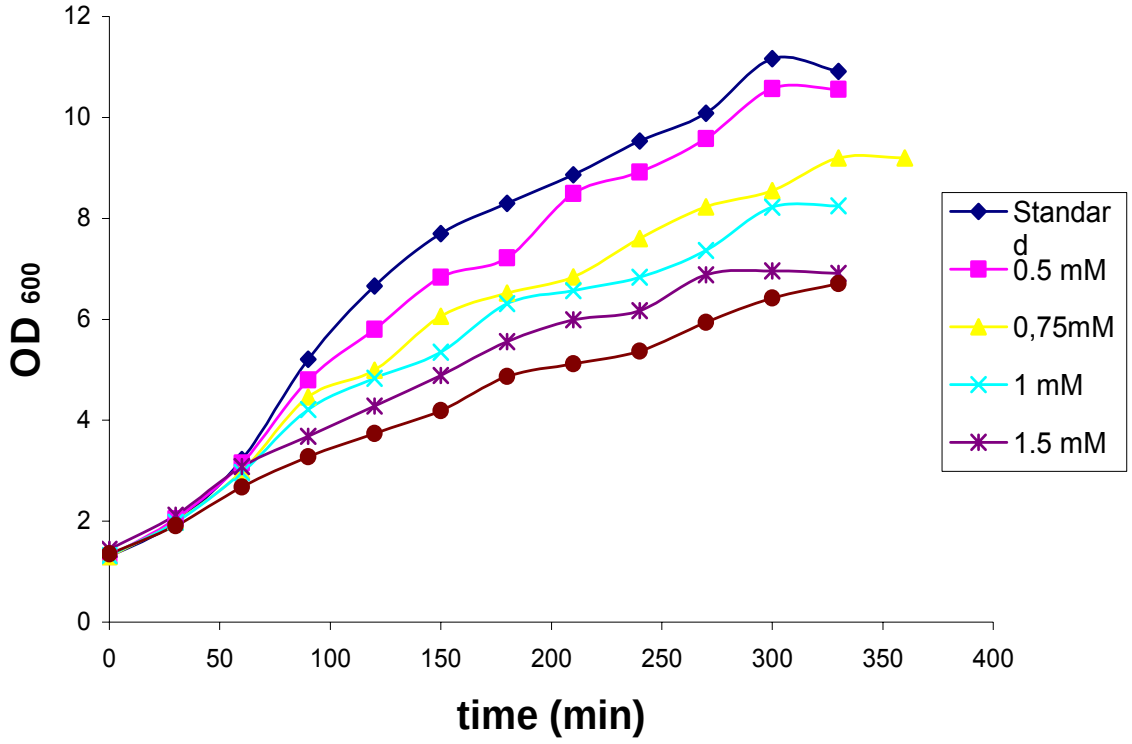


Figure 31. Effect of cobalt on the growth of wild-type *C. glutamicum* cells. C. glutamicum grown in BHI medium for about 5h at 30°C in BHI medium supplemented with different concentrations (0 - 2 mM) $CoCl_2$. Cells were harvested at OD = 6.5 for proteomic analysis.

Inhibition of *C. glutamicum* growth by cobalt Figure 31 shows a typical growth curve of *C. glutamicum* in BHI media at 30 °C in the absence or presence of various concentrations of $CoCl_2$. In the presence of $CoCl_2$ (0,75 mM) bacterial growth was dramatically impaired. Our results indicate that *C. glutamicum* cells have higher resistance to cobalt compared to many other microorganisms such as *S. cerevisiae* (Stadler et al., 2002).

4.3.2 Differential protein expression

To follow the proteomic response to cobalt, the *C. glutamicum* cells grown in BHI medium were stressed with 0,75 mM $CoCl_2$.The exposure of *C. glutamicum* cells to cobalt resulted in big changes in protein synthesis. We analyzed the proteomic response to cobalt to identify differentially expressed proteins which could be important for the resistance to this toxic compound. Extracts from untreated as well as treated cells were then subjected to comparative 2-DE (Figs. 32, 33).

The intensity of 22 spots changed after the exposure to cobalt, 15 proteins were induced and 7 proteins were repressed by cobalt. The identity of 14 proteins out of the 15 proteins induced, and three proteins out of seven repressed by cobalt is given in Table 8.

The important cobalt responsive up regulated proteins were sorted into five different functional classes: (i) Enzymes with antioxidant properties: This class includes three proteins NADPH-quinone reductase and related Zn- dependent oxidoreductases, cysteine synthase and cysteine desulfydrase (ii) Proteins involved in energy metabolism, this class contains only one protein succinate dehydrogenase (iii) Proteins involved in repair of DNA this class contains also one protein ribonucleotide reductase alpha-chain (iv) Proteins involved in repair and biogenesis of Fe–S cluster proteins: this class contains two proteins ABC-type transport system involved in Fe–S cluster assembly, permease compoponent and ABC-type transport system involved in Fe–S cluster assembly, ATPase component (v) Proteins involved in cell wall biosynthesis this class includes two proteins 1,4-alpha-glucan branching enzyme and nucleoside-diphosphate-sugar epimerase.

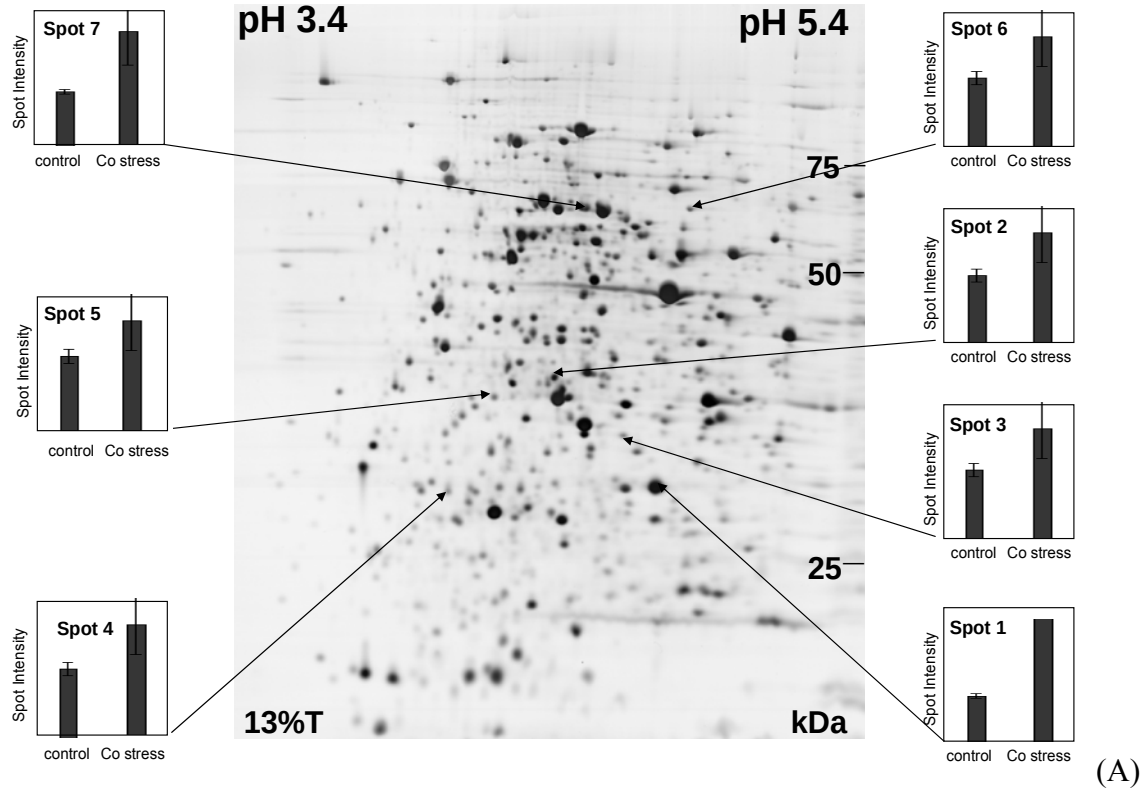
Three down-regulated proteins were identified upon cobalt stress Phosphoglycerate Dehydrogenase, FKBP-type peptidyl-prolyl cis-trans isomerase (trigger factor) and sulfit reductase hemoprotein beta-component.

Table 8. **List of identified *C.glutamicum* proteins whose expressions were stimulated by $CoCl_2$.**

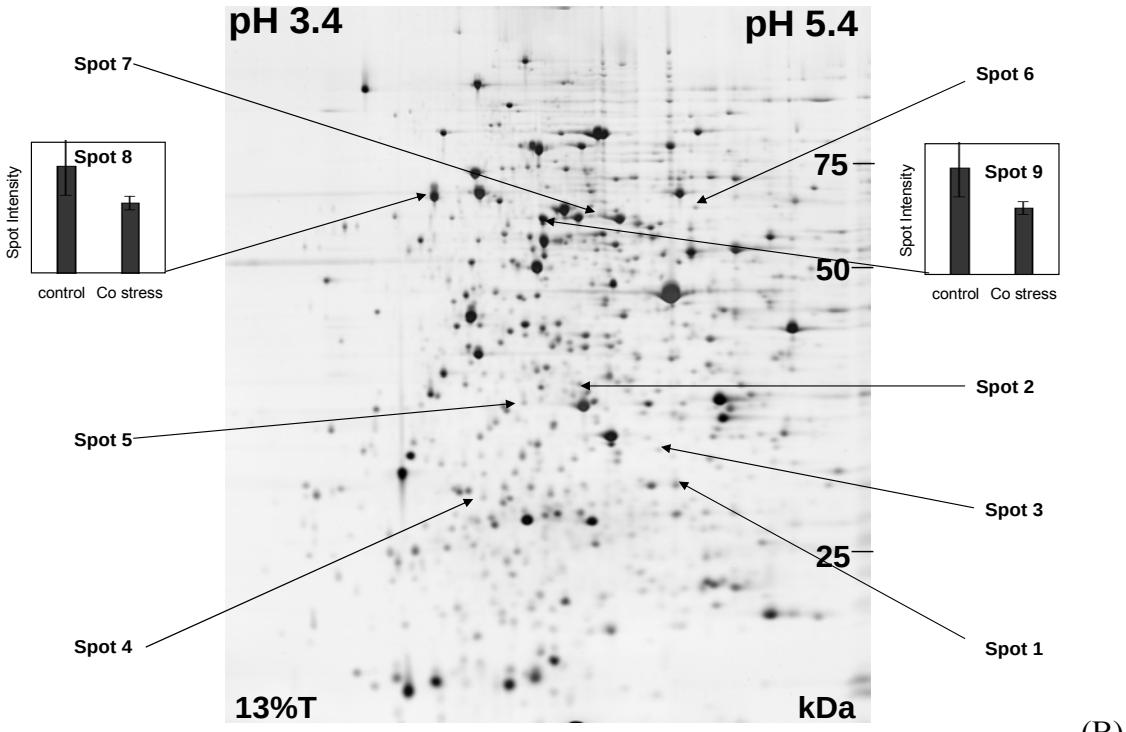
Spot	Protein information	Expectation	Induction	Repression	p*I*	Mass (kDa)
1	ABC-type transport system involved in Fe–S cluster assembly, permease	0.00	2.9	_	5.1	27.6

	compoponent					
2	NADPH quinone reductase or Zn-dependent oxidoreductase	0.00	1.9	_	4.8	45.5
3	Dehydrogenases with different specificit	0.00	1.7	_	4.6	30.8
4	Predicted nucleoside-diphosphate-sugar epimerase	0.00	1.7	_	4.1	23.4
5	Cysteine synthase	0.001	1.6	_	4.3	34.4
6	1,4-Alpha-glucan branching enzyme.	0.00	1.8	_	5.2	65.6
7	ABC-type transport system involved in Fe–S cluster assembly, ATP ase component	0.00	2.2	_	4.9	65
8	FKBP-type peptidyl-prolyl cis-trans isomerase (trigger factor).	0.00	_	- 1.7	4	69
9	Phosphoglycerate Dehydrogenase	0.00	_	- 1.8	4.2	65
10	Cysteine desulfhydrase / selenocysteine	0.00	2.4	_	5.6	45.7
11	Coenzyme F420-dependent N5,N10-methylenetetrahydro methanopterin dehydrogenase	0.00	4.5	_	5.7	43.2
12	FAD/FMN-containing dehydrogenases	0.00	1.9	_	6.4	48.3
13	Ribonucleotide reductase alpha-chain	0.00	4.3	_	5.5	70.6

14	Dehydrogenases with different specificit	0.00	2	_	5.8	30.8
15	PLP-dependent aminotransferases	0.00	2.3	_	6.1	46.7
16	Succinate dehydrogenase/fumarate reductase	0.00	3.4	_	5.6	68.3
17	Sulfite reductase hemoprotein beta-component	0.00	_	- 3.3	5.8	62.4

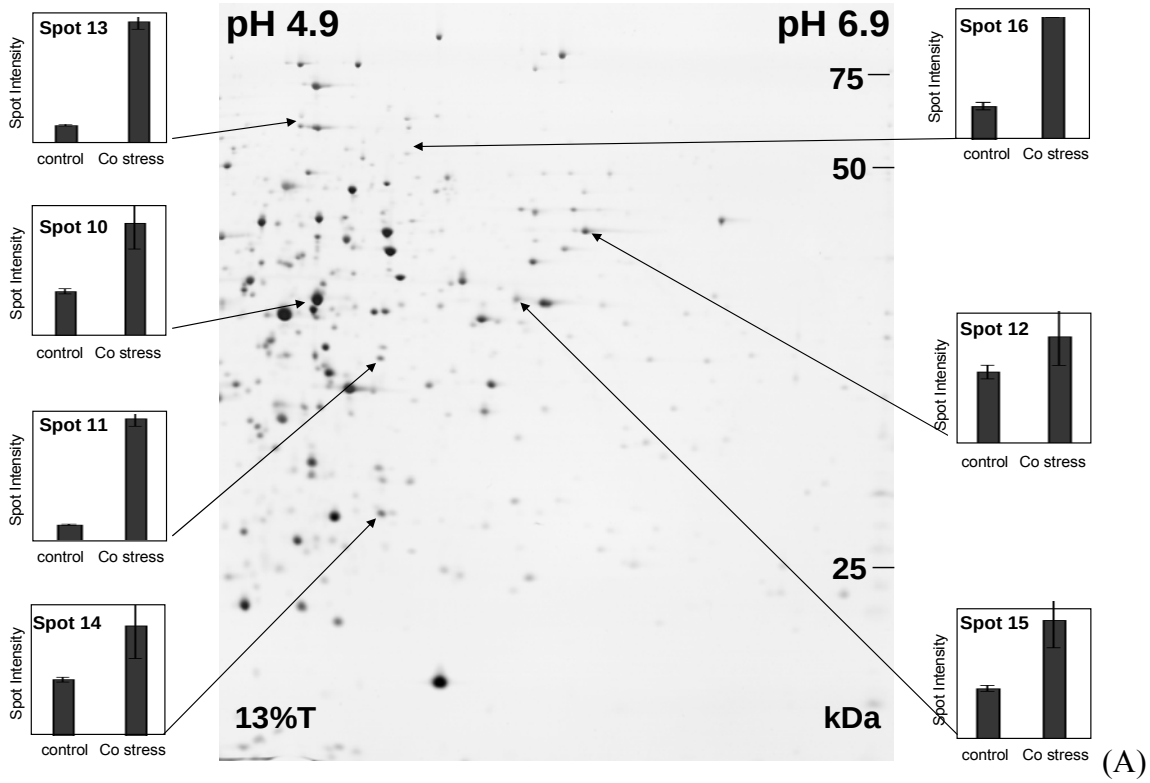


(A)

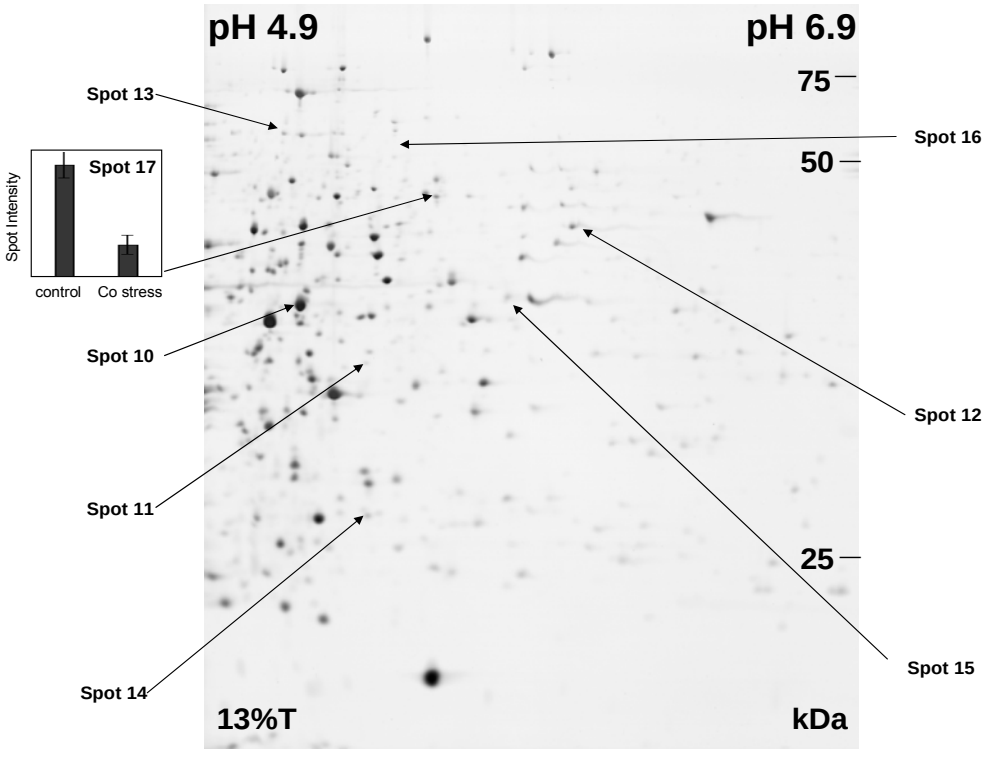


(B)

Figure 32. 2D gel images of *C. glutamicum* cells ranging from 3.4 to 5.4 treated with 0.75 mM $CoCl_2$ (A) or untreated (=control) (B). The stress proteins are numbered and their identities listed in Table 8.



(A)



(B)

Figure 33. 2D gel images of *C. glutamicum* cells ranging from 4.9 to 6.9 treated with 0.75 mM $CoCl_2$ (A) or untreated (=control) (B). The stress proteins are numbered and their identities listed in Table 8.

The induction of antioxidant enzyme NADPH quinone reductase or Zn-dependent oxidoreductase (Figure 34) indicates the cobalt can promote the production of free radicals and cause oxidative stress. This enzyme can minimize the negative effects of free radicals and help the cell to adapt to the oxidative stress.

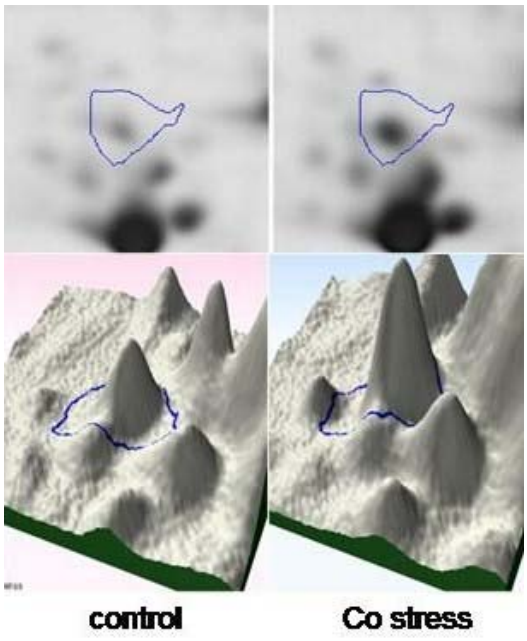


Figure 34. Overexpression of NADPH-quinone reductase and related Zn-dependent oxidoreductases (spot 2) after treatment with cobalt. "Zoom in" from IPG 3.4-5.4 (silver staining).

Cysteine synthase and Cysteine desulfhydrase were also induced upon Co stress (Figure 35)

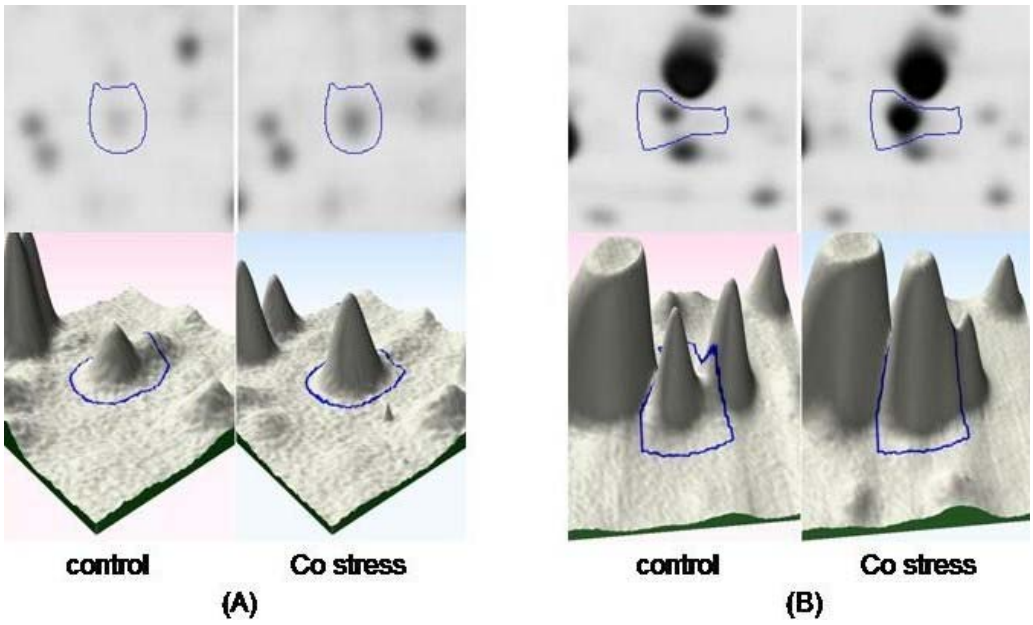


Figure 35. Overexpression of cysteine synthase (spot 5) (A) and Cysteine desulfhydrase (spot 10) (B) after treatment with cobalt. "Zoom in" from IPG 3.4-5.4 and 4.9-6.9 (silver staining).

Cysteine synthase was induced when *Moorella thermoacetica* was under oxidative stress (Das et al., 2005). Cystein synthase could play an important role in oxidative stress protection in this bacterium. In many microorganisms, cysteine-containing molecules, (e.g., glutathione and thioredoxin) play major roles in maintaining an intracellular reducing environment and in protection against oxidative and other stress conditions (Carmel-Harel and Storz, 2000; Lithgow et al., 2004). Cysteine desulfhydrase plays a secondary role in oxidative defense by its well-documented function of cysteine biosynthesis (Lo, et al., 2009).

The *C. glutamicum* cells also expressed succinate dehydrogenase after the exposure to cobalt, which is involved in energy metabolism (Figure 36).

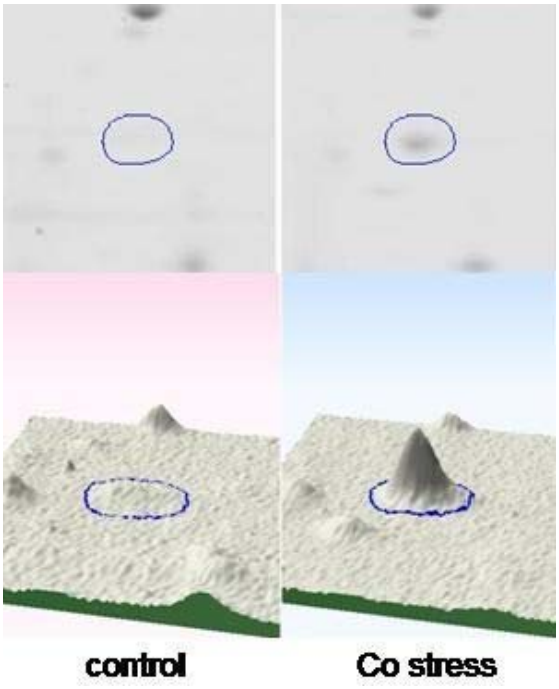


Figure 36. Overexpression of succinate dehydrogenase (spot 16) after treatment with cobalt. "Zoom in" from IPG 4.9-6.9 (silver staining).

Overexpression of proteins involved in energy metabolism has been reported as defense mechanisms against a wide range of stress conditions, including oxidative stress, and heavy metal. Succinate dehydrogenase is an important enzyme for the cell to generate energy, it participate to citric acid cycle and lead to energy gain for the cell. Interestingly this enzyme was repressed upon Cd stress in spite the cell needs energy to cope the stress; we suggest that the stressed cell with cadmium found another pathway for example pentose phosphate pathway to produce energy.

Ribonucleotide reductase was strongly induced (4.3 fold) (Figure 37), this enzyme catalyzes the formation of deoxyribonucleotides from ribonucleotides (Elledge et al., 1992).

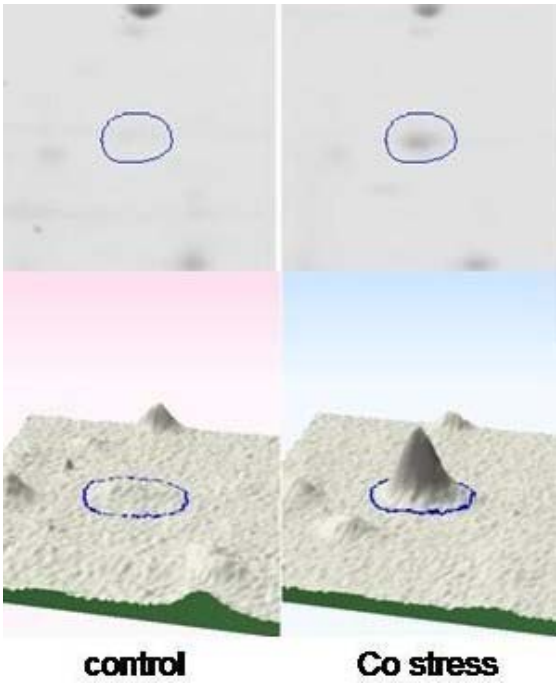


Figure 37. Overexpression of ribonucleotide reductase (spot 13) after treatment with cobalt. "Zoom in" from IPG 4.9-6.9 (silver staining).

Deoxyribonucleotides in turn are used in the synthesis of DNA. Furthermore RNR plays a critical role in regulating the total rate of DNA synthesis so that DNA to cell mass is maintained at a constant ratio during cell division and DNA repair (Herrick and Sclavi 2007).

In a good agreement with our results Gon and Beckwith (2006) have reported that this enzyme was overexpressed and activated in pro and eukaryotes upon oxidative stress.

Induction of this enzyme may be important for supplying deoxyribonucleotides to the DNA damage repair system. In addition Ribonucleotide reductase protects the mitochondrial membrane against oxidative stress (Xue et al., 2006).

Several observations indicate that cobalt might compete with iron for specific binding sites in certain proteins, thereby impairing their functions (Goldberg et al, 1988; Bunn et al, 1998).

Our results show that two types of ABC-type transport system involved in Fe–S cluster assembly were overexpressed (Figure 38), the overexpression of iron transporters leads to a slight increase of intracellular iron concentration, which is supposed to limit cobalt toxic effects by favouring iron binding in iron enzymes (Stadler and Schweyen, 2002).

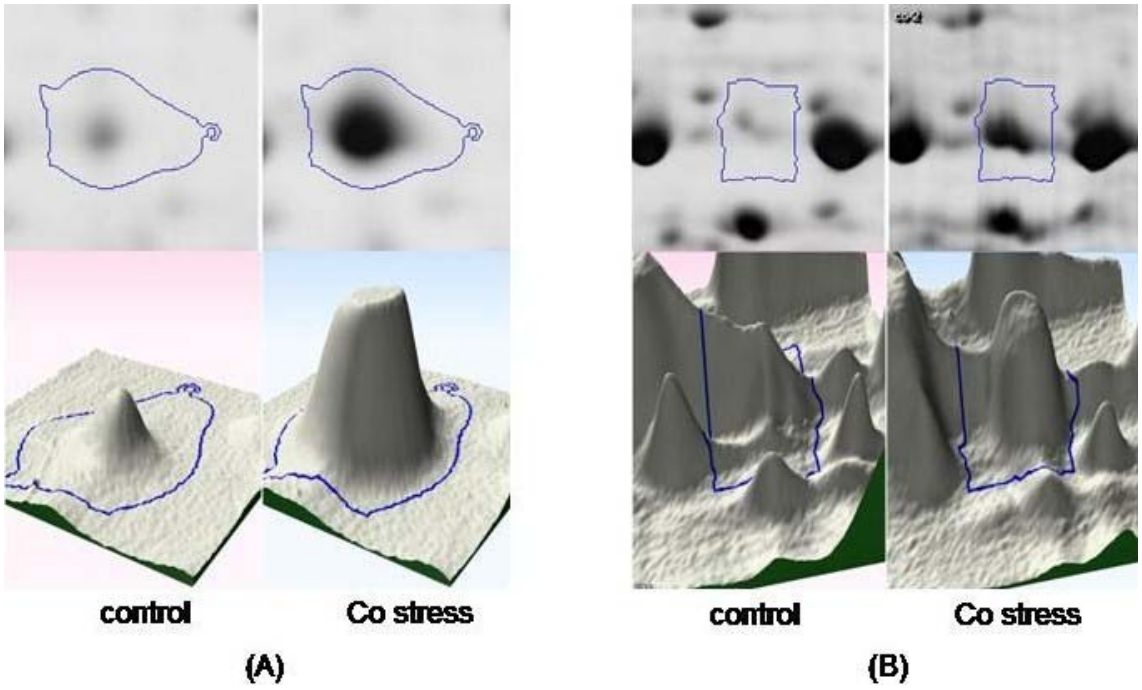


Figure 38. Overexpression of ABC-type transport system involved in Fe–S cluster assembly, permease compoponent (spot 1) (A) and ABC-type transport system involved in Fe–S cluster assembly, ATP ase component (spot 7) (B) after treatment with cobalt. "Zoom in" from IPG 3.4-5.4 (silver staining).

On the other hand stressed cells need Fe–S cluster proteins, because they play key roles in electron transport, as active site cofactors in TCA cycle enzymes; in addition they are involved in oxidative stress protection (Almeida et al, 2006). *C. glutamicum* cells appear to cope cobalt stress by up-regulating their iron transport system and increasing their intracellular iron content.

During adaptation to cobalt stress, two enzymes involved in cell wall biosynthesis

(nucleoside-diphosphate-sugar epimerase and 1,4-alpha-glucan branching enzyme) were overexpressed (Figure 39).

Nucleoside-diphosphate-sugar epimerase involves in cell wall biosynthesis. It participates to the synthesis of glucan, which is an important component of cell wall (Barbier et al., 2005).

1,4-Alpha-glucan branching enzyme is an enzyme taking part in the synthesis of glycogen by adding branches to the glycogen molecules. Glycogen is a branching polymer of large numbers of glucose units linked together. The structure is based on the chains of glucose with linkages between carbon atoms 1 and 4 of each pair of units (alpha 1,4 linkages). Glycogen plays an important role in cell wall biosynthesis (Tzvetkov et al., 2003).

Cobalt probably damages the cell membrane, and as a response, the cell up-regulated these enzymes in order to strengthen or repair the cell membrane.

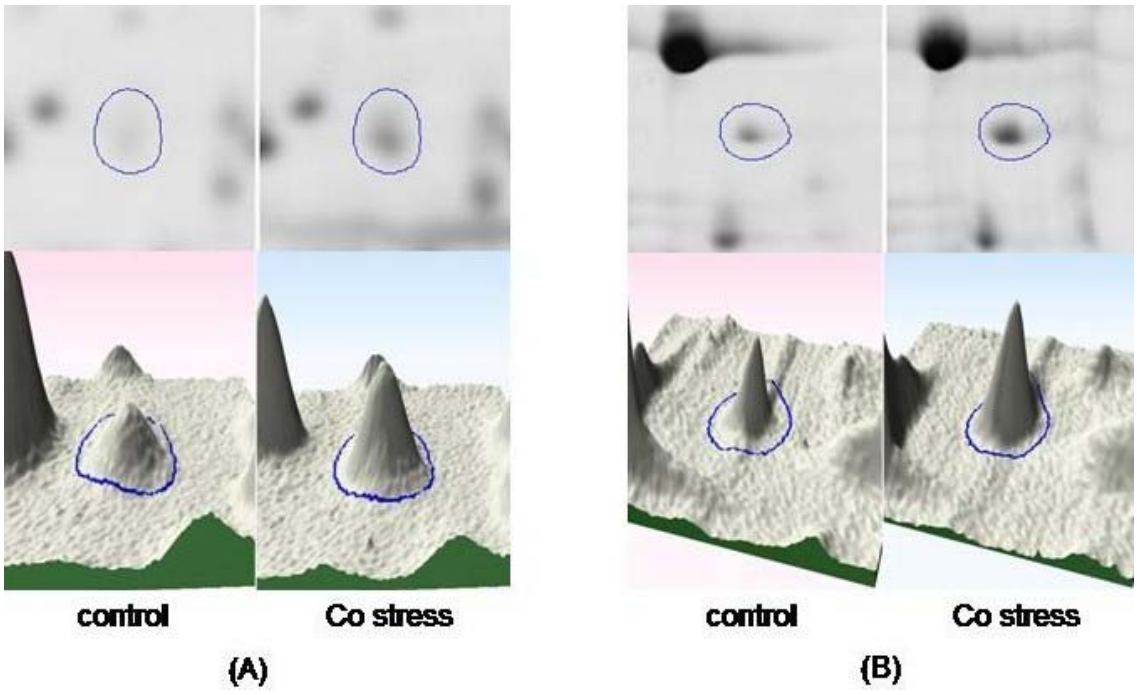


Figure 39. Overexpression of nucleoside-diphosphate-sugar epimerase (spot 4) (A) and 1,4-alpha-glucan branching enzyme (spot 6) (B) after treatment with cobalt. "Zoom in" from IPG 3.4-5.4 (silver staining).

In good agreement with our results was the work from Santos et al. (2004), who observed that the enzyme UDP-3-0-acyl N-acetylglucosamine deacetylase (LpxC) was up-regulated in *Pseudomonas putida* KT2440 upon exposure to phenol – the enzyme is also involved in the cell envelope biosynthesis. Fanous et al. (2007) made the same observation, when *C. glutamicum* was upon oxidative stress two enzymes involved in cell wall biosynthesis were strongly overexpressed.

Our results show that high concentrations of cobalt can inhibit the growth of the cells, the repression of FKBP-type peptidyl-prolyl cis-trans isomerase (trigger factor) (Figure 40) supports our results because this protein is involved in cell division and important for proliferation of cells (Lamote, Willems, 1998).

The enzyme Sulfite Reductase (Hemoprotein) was also down regulated. It is essential for the cell to transform the inorganic element into a biocompatible form (Hell, 1997), Kusch et al (2007) supported our results by finding that Sulfite Reductase was repressed in pathogenic

yeast *Candida albicans* after treatment with hydrogen peroxide. The oxidative stress may decrease the activity of this enzyme.

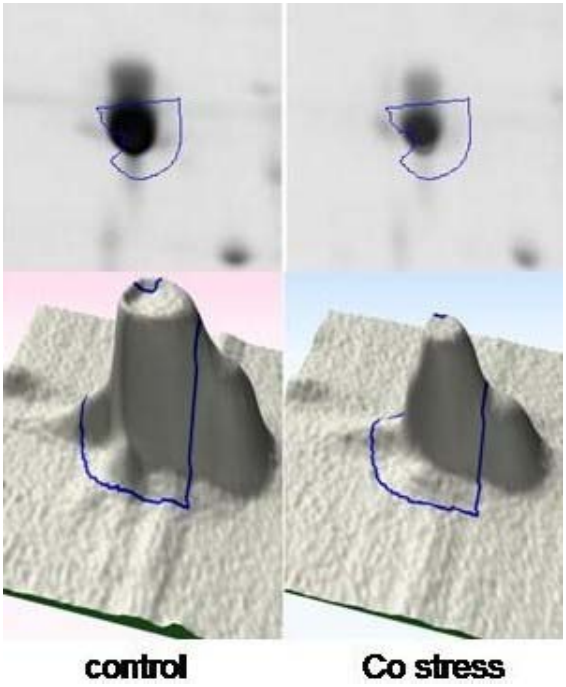


Figure 40. Downregulation of FKBP-type peptidyl-prolyl cis-trans isomerase (trigger factor) (spot 8) after treatment with cobalt. "Zoom in" from IPG 3.4-5.4 (silver staining).

Our results indicate that the cell adapted to cobalt stress by inducing five defence mechanisms: Scavenging of free radicals, promotion of the generation of energy, reparation of DNA, reparation and biogenesis of Fe–S cluster proteins and supporting and reparation of cell wall.

All stress proteins play an important role to counteract the cobalt stress but proteins involved in cell wall biosynthesis 1,4-alpha-glucan branching enzyme and nucleoside-diphosphate-sugar epimerase could be considered as biomarkers because to the best of our knowledge, these proteins have not been previously reported as a stress proteins in bacteria after the exposure to heavy metals or another kind of stress.

4.4 *A proteome analysis of C. glutamicum after exposure to silver*

4.4.1 Effect of silver on the growth of *C. glutamicum* cells

The *C. glutamicum* cells were cultivated in the presence of various concentrations of $AgNO_3$. The measured optical densities of control and silver-treated cells over time are shown in Figure 41.

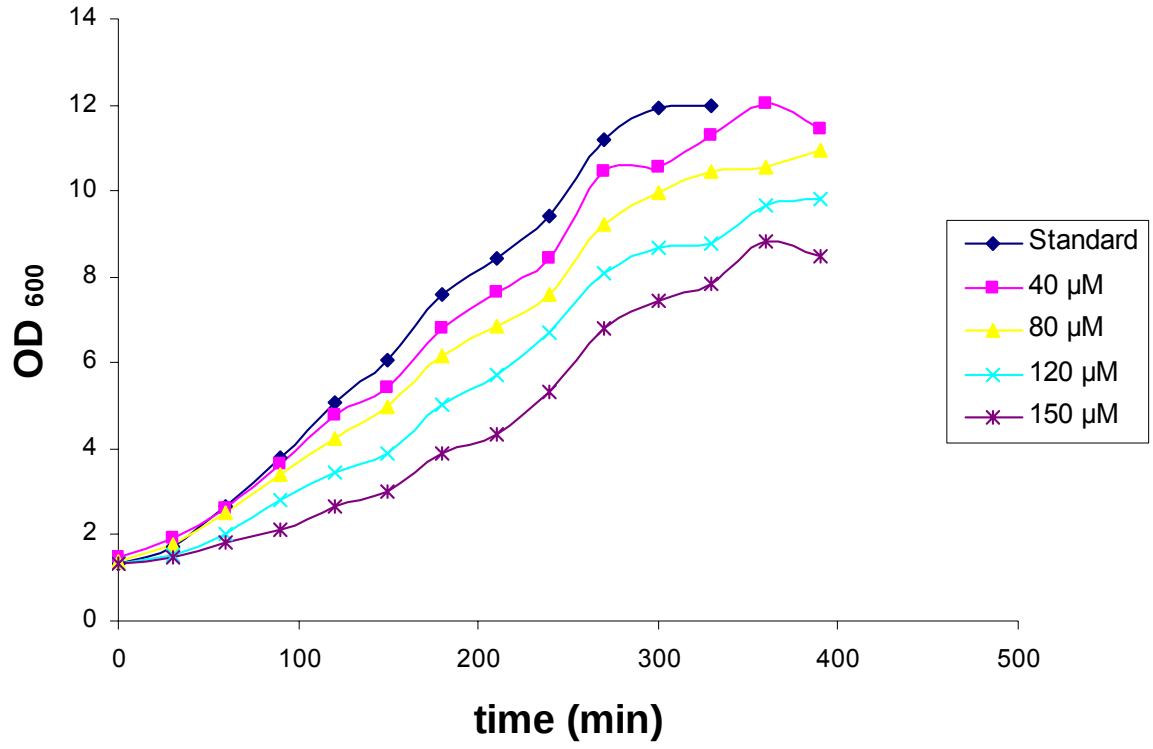


Figure 41. Effect of silver on the growth of wild-type *C. glutamicum* cells. *C. glutamicum* grown in BHI medium for about 5h at 30°C in BHI medium supplemented with different concentrations (0 - 150 µM) $AgNO_3$. Cells were harvested at OD = 6.5 for proteomic analysis.

We selected 120 µM Ag^+ to treat the cells for inducing observable cell responses at the protein level after carefully examining the cell survival curve (Figure 41). Lower silver concentrations have a negative effect on the growth of *C. glutamicum*. Our results indicate that *C. glutamicum* cells have a high ability to resist silver compared to many other microorganisms such *E. coli* (Lok et al, 2006).

4.4.2 Differential protein expression

Silver treatment produced a global change at the proteomic level (Figure 42, 43). After the cell exposure to silver many proteins showed a change even with different ratios, either up- or down-regulation. The change in the expression level of these proteins indicates that they might be involved in the overall cellular survival mechanism. The intensity of 21 spots changed after the exposure to silver, 17 proteins were induced and 4 proteins were repressed by silver. The identity of 16 proteins out of the 17 proteins induced, and three proteins out of four repressed by silver is given in Table 9.

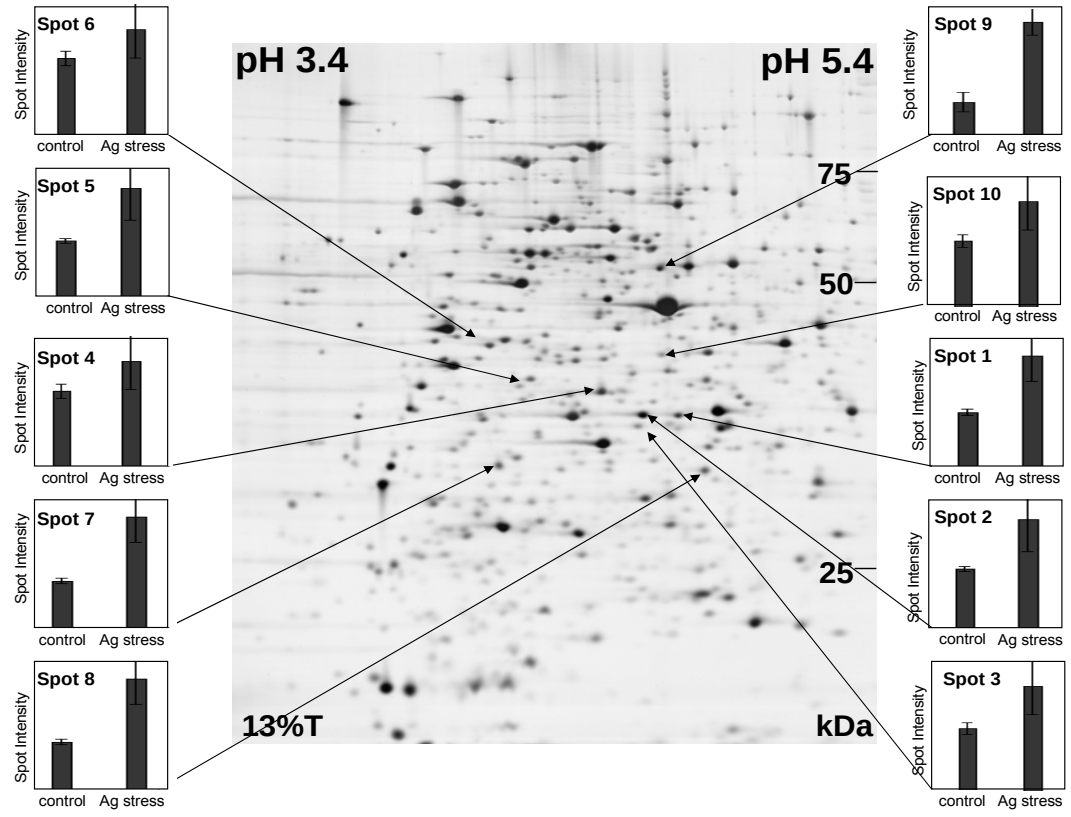
The important silver responsive up regulated proteins were sorted into three different functional classes: (i) Enzymes with antioxidant properties: This class includes only one enzyme NAD-dependent aldehyde dehydrogenases (ii) Proteins involved in repair of DNA: This class contains one protein Sugarekinase Ribokinase family (iii) Proteins involved in energy metabolism, this class contains three proteins 2-methylcitrate dehydratase 1, 2-methylcitrate synthase 2 and Succinyl-CoA ligase [ADP-forming] subunit alpha.

Three down-regulated proteins were identified under silver stress Catalase, Zn-dependent hydrolases, including glyoxylases and SAM-dependent methyltransferases.

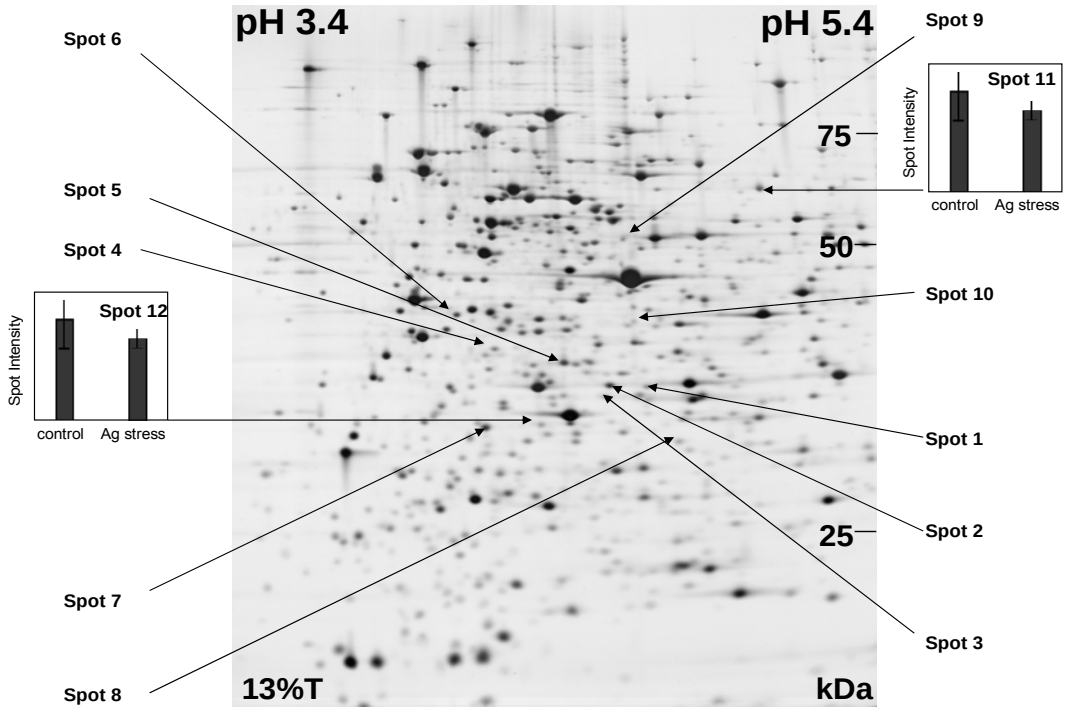
Table 9. List of identified *C. glutamicum* proteins whose expressions were stimulated by $AgNO_3$.

Spot	Protein information	Expectation	Induction	Repression	p*I*	Mass (kDa)
1	Hypothetical protein phosphate isomerase	0.00	2.2	_	4.6	35.3
2	phosphate isomerase	0.00	2.1	_	4.4	35.2
3	Hypothetical protein Cgl0161(Enzyme involved in inositol metabolism	0.00	1.9	_	4.4	34.9
4	Inositol-2 dehydrogenase	0.00	1.6	_	4.3	36.4
5	Sugarekinase Ribokinase family	0.001	2	_	4	37.2
6	Predicted dehydrogenases and related proteins (MYO-	0.00	1.6	_	3.9	38

	INOSITOL 2-DEHYDROGENASE)					
7	Putative uncharacterized protein	0.00	2.4	_	3.9	32
8	Sugar phosphate isomerases /epimerases	0.00	2.4	_	4.7	32.8
9	NAD-dependent aldehyde dehydrogenases	0.00	3.1	_	4.5	55.1
10	Predicted dehydrogenases and related proteins	0.00	1.8	_	4.6	36.79
11	Catalase	0.00	_	-1.6	5.2	57.2
12	Zn-dependent hydrolases, including glyoxylases	0.00	_	-1.6	4.2	30.1
13	Nitrate reductase beta chain	0.00	1.5	_	5.7	59.9
14	2-methylcitrate dehydratase 1	0.00	3.6	_	5.8	55
15	Glutamate-1-semialdehyde 2,1-aminomutase	0.00	1.7	_	5.6	46
16	Succinyl-CoA ligase [ADP-forming] subunit alpha	0.00	3.9	_	5.7	30.2
17	2-methylcitrate synthase 2	0.00	3	_	6	42.5
18	Glycine/D-amino acid oxidases	0.00	1.8	_	6.1	44.7
19	SAM-dependent methyltransferases	0.00	_	-1.5	5.3	30.7

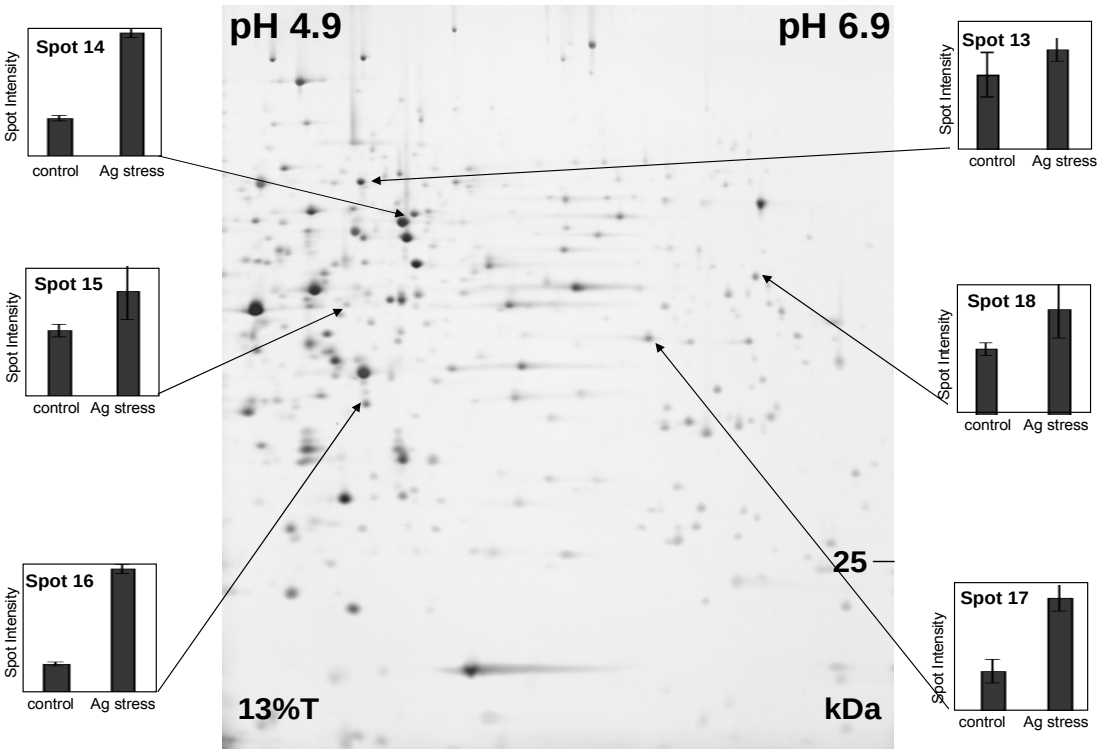


(A)

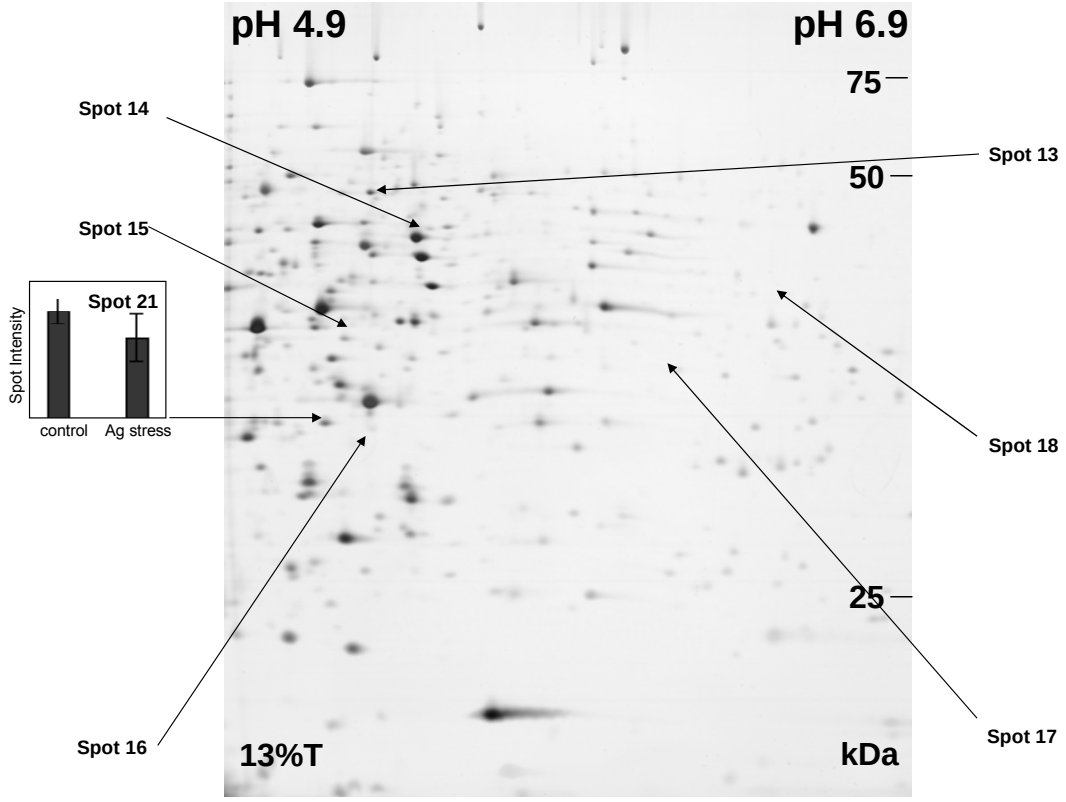


(B)

Figure 41. 2D gel images of *C. glutamicum* cells ranging from 3.4 to 5.4 treated with 120 µM $AgNO_3$ (A) or untreated (=control) (B). The stress proteins are numbered and their identities listed in Table 9.



(A)



(B)

Figure 42. 2D gel images of *C. glutamicum* cells ranging from 4.9 to 6.9 treated with 120 µM $AgNO_3$ (A) or untreated (=control) (B). The stress proteins are numbered and their identities listed in Table 9.

Silver-ion-mediated perturbation of the bacterial respiratory chain has raised the possibility of reactive oxygen species (ROS) generation (Park et al, 2008). The cell tried to minimize the toxic effect of ROS by up-regulating NAD- dependent aldehyde dehydrogenase (Figure 43).

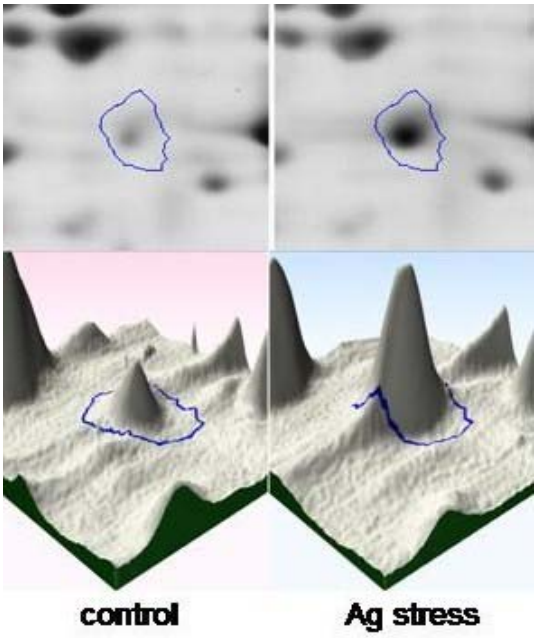


Figure 43. Overexpression of NAD- dependent aldehyde dehydrogenase (spot 9) after treatment with silver. "Zoom in" from IPG 3.4-5.4 (silver staining).

Many previous studies have reported that NAD- dependent aldehyde dehydrogenase has antioxidant properties, Lin et al (2006) have revealed that this enzyme attenuated significantly acetaldehyde-induced ROS generation in human cells, their results suggested that overexpression of NAD- dependent aldehyde dehydrogenase may effectively alleviate acetaldehyde-elicited cell injury.

In addition this enzyme protects the cell against cellular oxidative damage by preventing lipid peroxidation-induced protein adduction, functioning as an antioxidant, and maintaining intracellular redox balance (Lassen, 2006).

Under silver stress Sugarkinase Ribokinase family was 2 fold expressed (Figure 44), the enzyme catalyses the phosphorylation of ribose to ribose-5-phosphate using ATP.

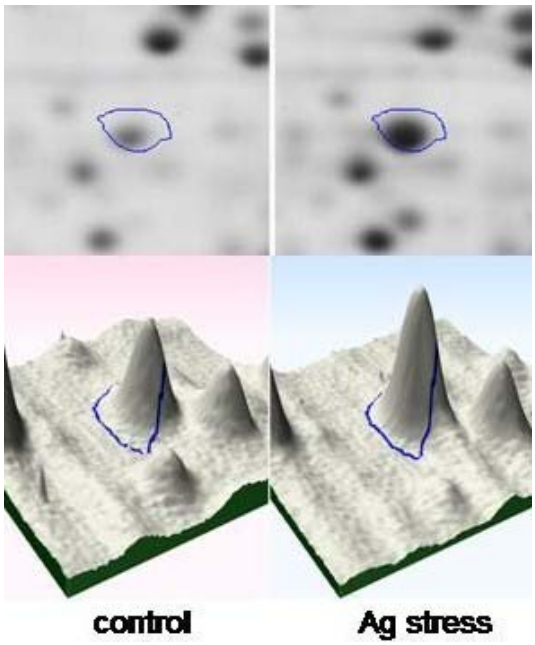


Figure 44. Overexpression of Sugarkinase Ribokinase family (spot 5) after treatment with silver. "Zoom in" from IPG 3.4-5.4 (silver staining).

The reaction is the first step in ribose metabolism and acts partly to trap ribose within the cell after uptake. Phosphorylation also prepares the sugar for use in the synthesis of nucleotides and DNA and for entry into the pentose phosphate pathway. protein probably involved in DNA repair in *E.coli* and yeast (Kulaeva et al., 1996).

2-methylcitrate dehydratase and 2-methylcitrate synthase were strongly overexpressed upon silver stress (3.6 – 3 fold respectively) (Figure 45). They participate to methylcitrate cycle, which is an important source of energy in the cell.

C. glutamicum is able to degrade propionate, and even use it as carbon source. Out of all the propionate degradation pathways, the 2-methylcitric acid cycle is the most widely distributed. In this pathway, the Cα methylene group of propionate is oxidized to a keto group yielding pyruvate, a common precursor for biosynthesis and energy production. In this microorganism the pathway begins with the activation of propionate to propionyl-CoA by propionate-CoA ligase, followed by the synthesis of (2*S*,3*S*)-2-methylcitrate from propionyl-CoA and oxaloacetate, catalyzed by 2-methylcitrate synthase. (2*S*,3*S*)-2-methylcitrate is then dehydrated to *cis*-2-methylaconitate by 2-methylcitrate dehydratase , followed by rehydration

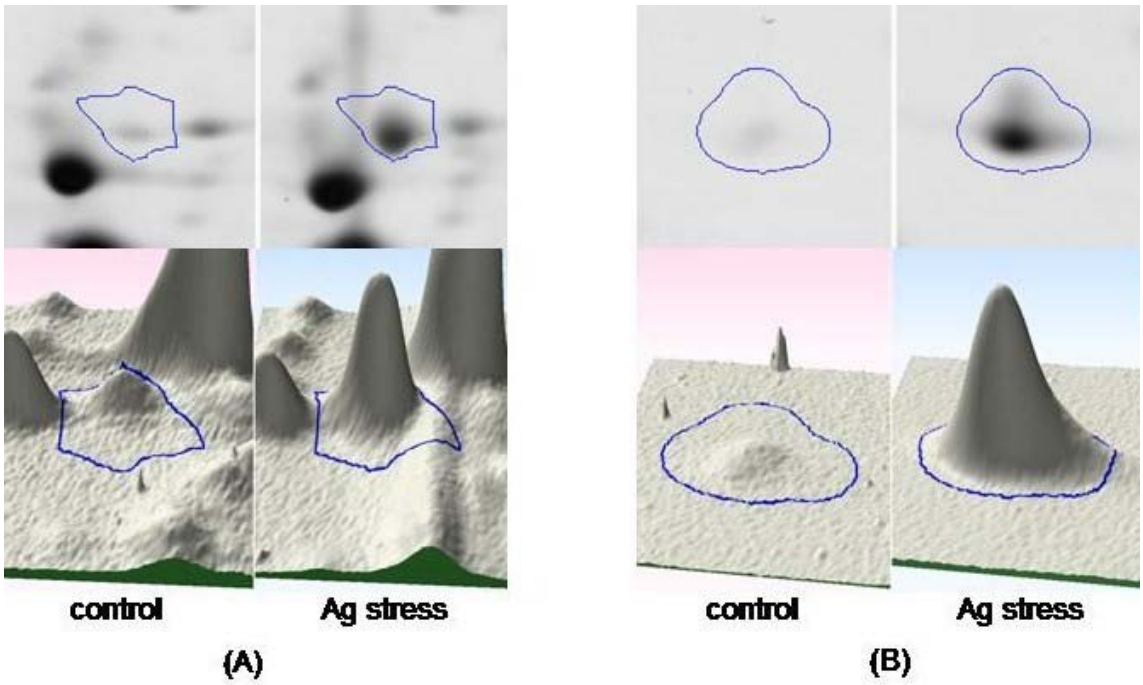


Figure 45. Overexpression of 2-methylcitrate dehydratase (spot 14) (A) and 2-methylcitrate synthase (spot 17) (B) after treatment with silver. "Zoom in" from IPG 4.9-6.9 (silver staining).

to methylisocitrate (catalyzed by aconitate hydratase 1 and aconitate hydratase 2) and cleavage of the later into pyruvate and succinate , performed by 2-methylisocitrate lyase subunit (Figure 46) (Muñoz-Elías et al., 2006).

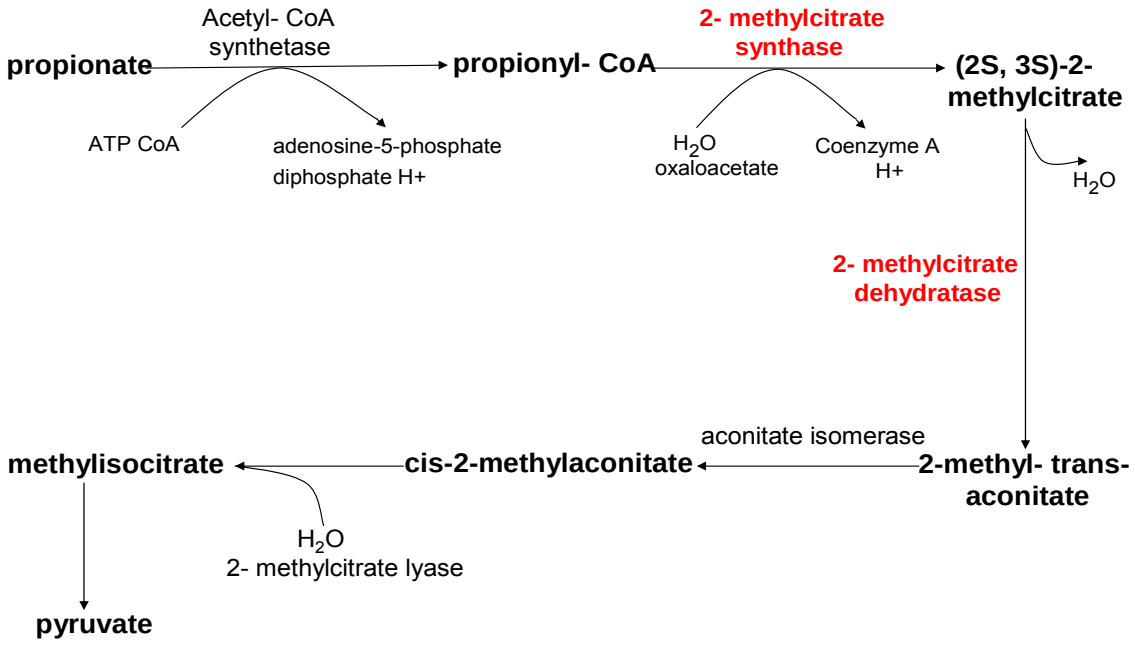


Figure 46. Production of pyruvate by methylcitrate cycle (Muñoz-Elías et al., 2006).

As mentioned above, pyruvate is a common precursor for biosynthesis and energy production. Oxaloacetate may be regenerated from succinate via the TCA cycle. The overexpression of these enzymes has not been reported under stress conditions, for this reason we could be considered them as biomarkers for silver stress.

Succinyl-CoA ligase was also strongly up-regulated under silver stress (3.9 fold) (Figure 47); the enzyme is a key enzyme in TCA cycle, which is very important energy source for the cell.

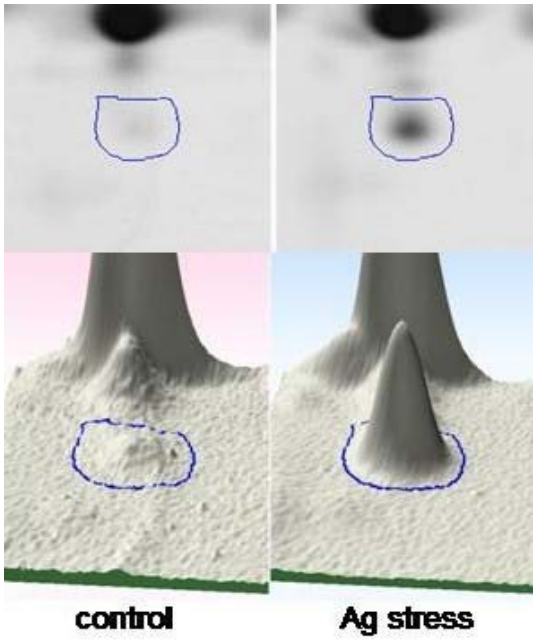


Figure 47. Overexpression of Succinyl-CoA ligase (spot 16) after treatment with silver. "Zoom in" from IPG 4.9-6.9 (silver staining).

Within the Krebs cycle, energy in the form of compound rich in energy, Adenosine triphosphate (ATP) is usually derived from the breakdown of glucose, although fats and proteins can also be utilized as energy sources. The cells appeared to exhibit an increased demand for energy to protect them selves from silver stress.

Interestingly the antioxidant enzyme catalase was down regulated (Figure 48). Catalase is one of the most efficient enzymes known. It is so efficient that it cannot be saturated by H2O2 at any concentration (Lledías et al., 1998). Catalase reacts with H2O2 to form water and molecular oxygen. It protects cells from hydrogen peroxide generated within them and plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response of cells (Hunt et al., 1998). In contract to our results this enzyme was strongly overexpressed upon heavy metal and oxidative stress (Vido et al., 2001; Godon et al., 1998), but Venkatesan et al (2007) supported our results, they have reported that catalase was downregulated in

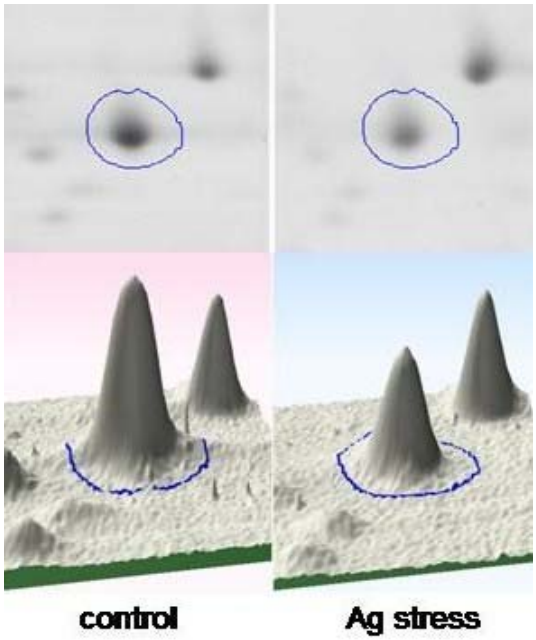


Figure 48. Downregulation of catalase (spot 11) after treatment with silver. "Zoom in" from IPG 3.4-5.4 (silver staining).

mesangial cells under oxidative stress. Isoir et al (2009) have the same observation when human cell line HaCaT was treated with 20Gy γ-radiation, the catalase activity was decreased. We suggest that the ability of silver nitrate to deplete the activity of catalase is an important feature of Ag^+ toxic mechanism.

Silver ions have been widely used as disinfectants that inhibit bacterial growth by interfering with the membrane permeability to protons and phosphate (Schreuers, 1982; Dibrov et al, 2002). In this study we could not find any overexpressed envelope proteins in spite Lock et al (2006) have reported that after the exposure of *E. coli* cells to silver nanoparticles the expression of many envelope proteins was increased.

Through proteomic analyses, we have identified a possible mode of the toxic mechanism of silver nitrate. Our results revealed that Ag^+ may target DNA and antioxidant enzyme (catalase), in the responses for the detoxification of Ag^+ the cells up-regulated proteins, which involved in reparation of damaged DNA, minimizing of toxic effect of ROS and energy generation. The induction of 2-methylcitrate dehydratase and 1, 2-methylcitrate synthase demonstrates the potential of these two enzymes as biomarkers of sub-lethal Ag^+ toxicity.

4.5 *A proteome analysis of C. glutamicum after exposure to lead*

4.5.1 Effect of lead on the growth of C. glutamicum cells

The *C. glutamicum* cells were cultivated in the presence of various concentrations of $Pb(NO_3)_2$. The measured optical densities of control and lead-treated cells over time are shown in Figure 49.

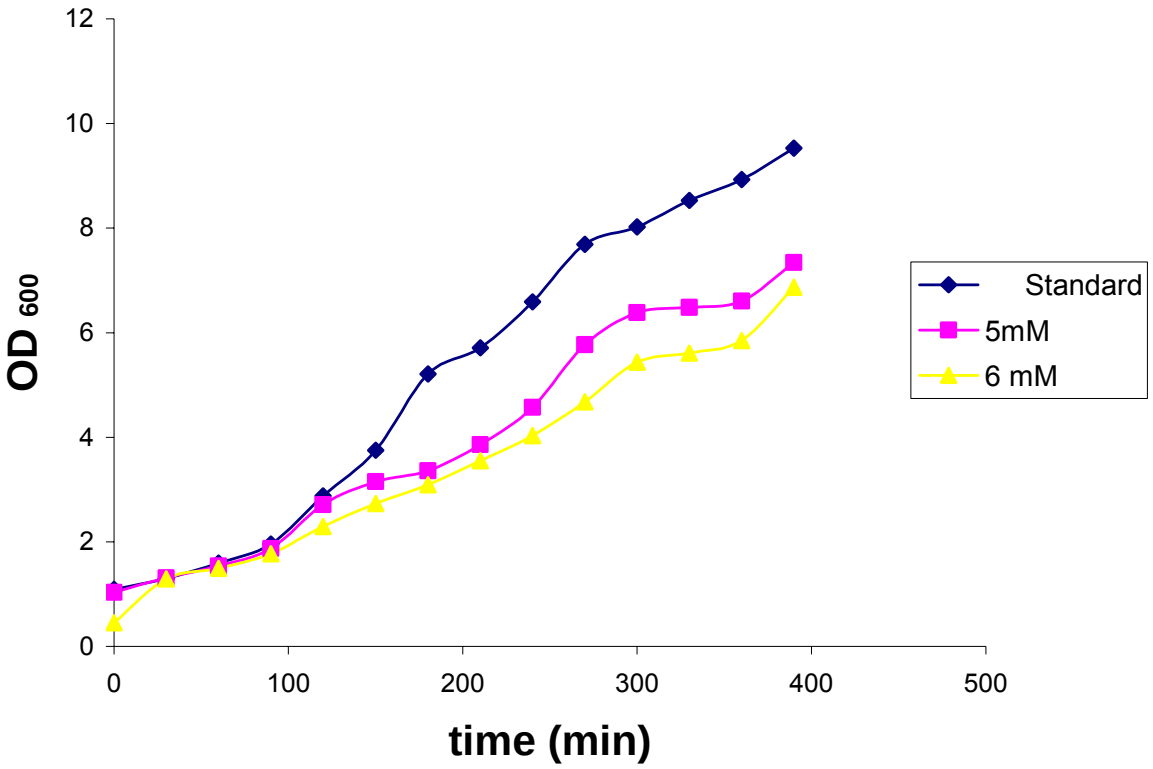


Figure 49. Effect of lead on the growth of wild-type *C. glutamicum* cells. *C. glutamicum* grown in BHI medium for about 5h at 30°C in BHI medium supplemented with different concentrations (5, 6 mM) $Pb(NO_3)_2$. Cells were harvested at OD = 6.5 for proteomic analysis.

There was a positive and a significant correlation between the concentration of lead and the inhabitation of the growth of *C. glutamicum* cells. Interestingly, *C. glutamicum* cells have more resistance to lead than many other bacteria such as *S. cerevisiae* (Chen and Wang 2007). In this study 6 mM $Pb(NO_3)_2$ was applied as sublethal concentration.

4.5.2 Differential protein expression

The exposure of *C. glutamicum* cells to 6 mM of lead reveals that lead is a strong inducer of many proteins. Extracts from untreated as well as treated cells were then subjected to

comparative 2-DE (Figures. 50, 51). The intensity of 23 spots changed after the exposure to lead, 13 proteins were induced and 10 proteins were repressed by lead. The identity of 7 proteins out of the 13 proteins induced and 7 proteins out of 10 repressed by lead is given in Table 10. Interestingly two new proteins were induced upon lead stress.

The important lead responsive up regulated proteins were sorted into three different functional classes: (i) Enzymes with antioxidant properties: This class includes two enzymes NADPH-quinone reductase and related Zn-dependent oxidoreductases and NAD-dependent aldehyde dehydrogenases (ii) Proteins involved in energy metabolism: This class contains two proteins ATP synthase and protein involved in propionate catabolism (iii) Proteins contains Fe^{3+} and involved in Fe^{3+} transportation: This class contains two proteins Hemoglobin-like flavoprotein (flavohemoprotein) and ABC-type Fe^{3+}-siderophores transport systems, periplasmic components.

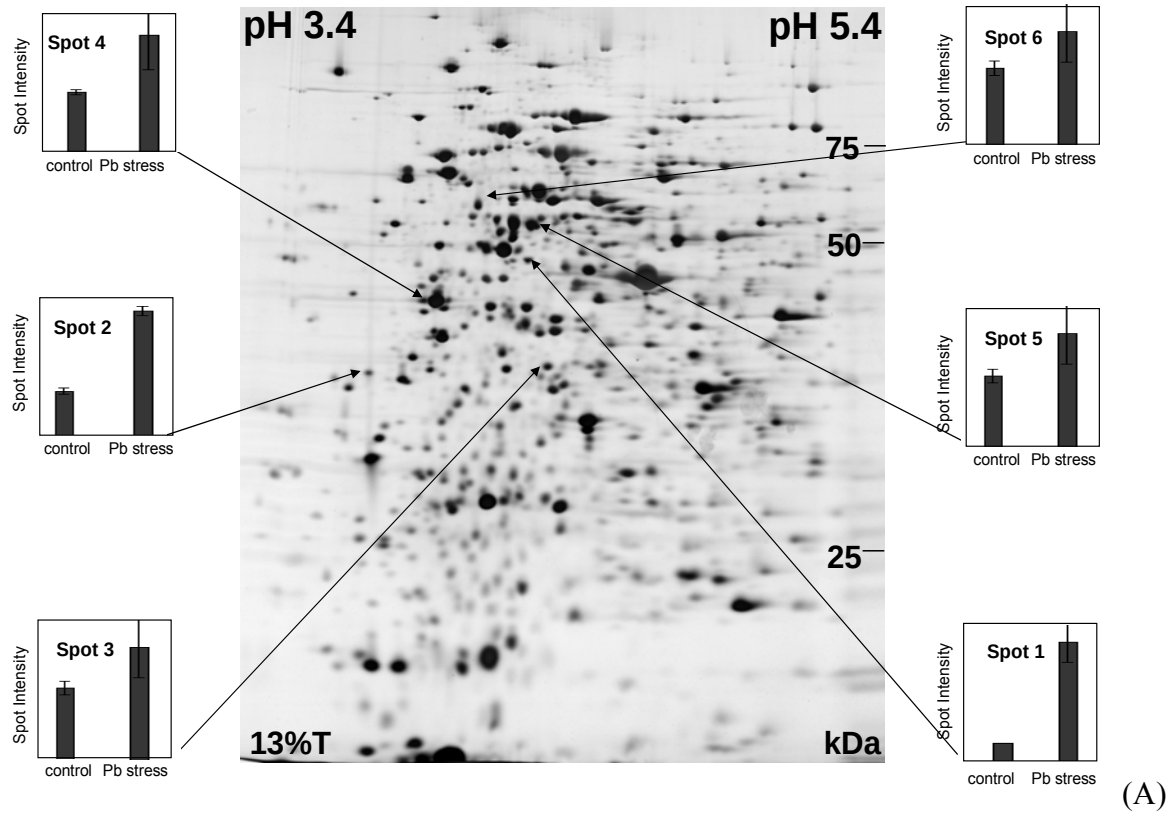
Ferritin-like protein, ABC-type sugar transport system, ATPase component, Succinate dehydrogenase/fumarate reductase Fe-S protein and Succinate dehydrogenase/fumarate reductase, flavoprotein subunits are the most important down-regulated proteins under lead stress.

Table10. List of identified *C. glutamicum* proteins whose expressions were stimulated by $Pb(NO_3)_2$.

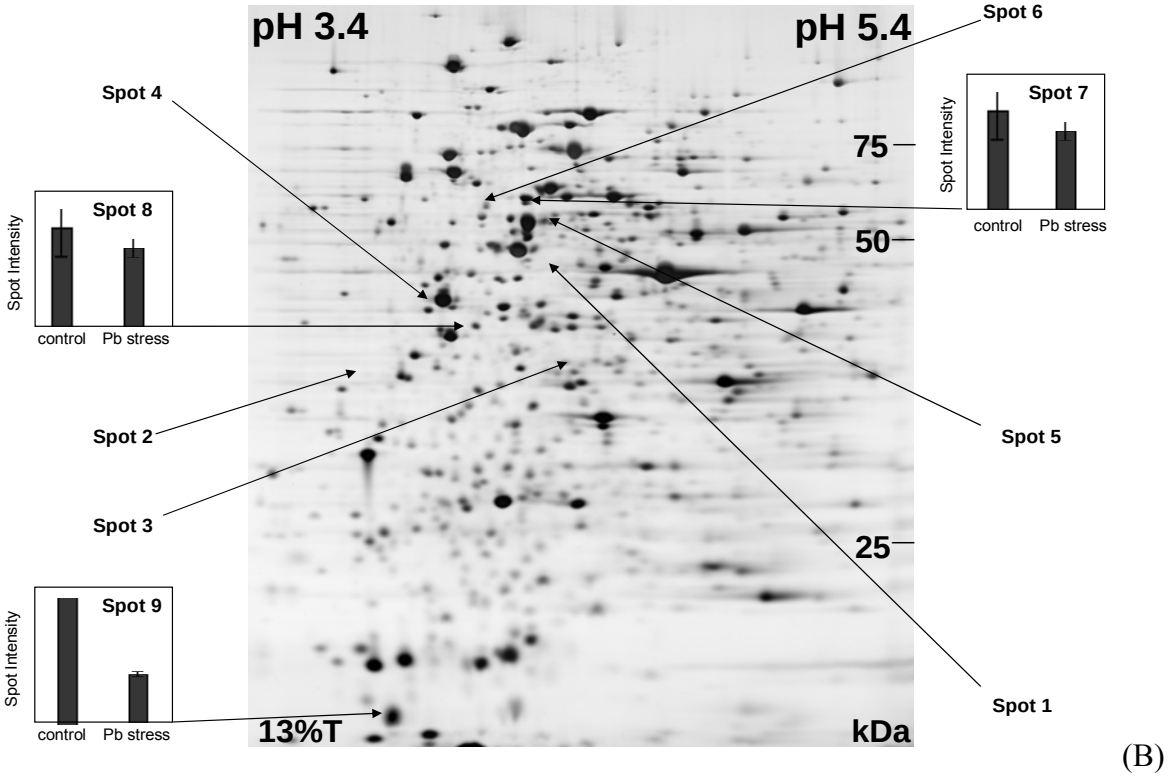
Spot	Protein information	Expectation	Induction	Repression	p*I*	Mass (kDa)
1	Uncharacterized protein involved in propionate catabolism (MMGE/PRPD family protein)	0.00	2.2	_	4.4	49.2
2	ABC-type Fe3+-siderophores transport systems, periplasmic components	0.00	2.1	_	3.9	33
3	NADPH:quinone reductase and related	0.00	1.9	_	4.8	39.6

	Zn-dependent oxidoreductases					
4	Hemoglobin-like flavoprotein (flavohemoprotein)	0.00	1.6	_	4.1	42
5	ATP synthase subunit beta	0.001	2	_	4.4	52.5
6	NAD-dependent aldehyde dehydrogenases	0.00	1.6	_	4.3	51.7
7	Phosphoglycerate dehydrogenase and related dehydrogenases or D-3-phosphoglycerate dehydrogenase (Phosphoglycerate Dehydrogenase	0.00	2.4	_	4.3	55.3
8	D-alanine--D-alanine ligase (down)	0.00	1.8	_	4.2	38.5
9	Ferritin-like protein	0.00	_	-2.9	4	18
10	Dehydrogenases with different specificities (Related to short-chain alcohol dehydrogenases) (putative oxidoreductase)	0.00	1.5	_	6.2	26.7
11	Succinate dehydrogenase/fumarate reductase Fe-S protein	0.00	_	-1.8	5.8	30.2

12	ABC-type transporter, ATPase component (ABC-type sugar transport system, ATPase component)	0.00	_	- 1.6	6	40.3
13	Succinate dehydrogenase/fumarate reductase, flavoprotein subunits	0.00	_	- 3	5.7	69.4
14	Nitrate reductase beta chain (probable respiratory nitrate reductase oxidoreduct)	0.00	_	-3.6	5.9	59.3

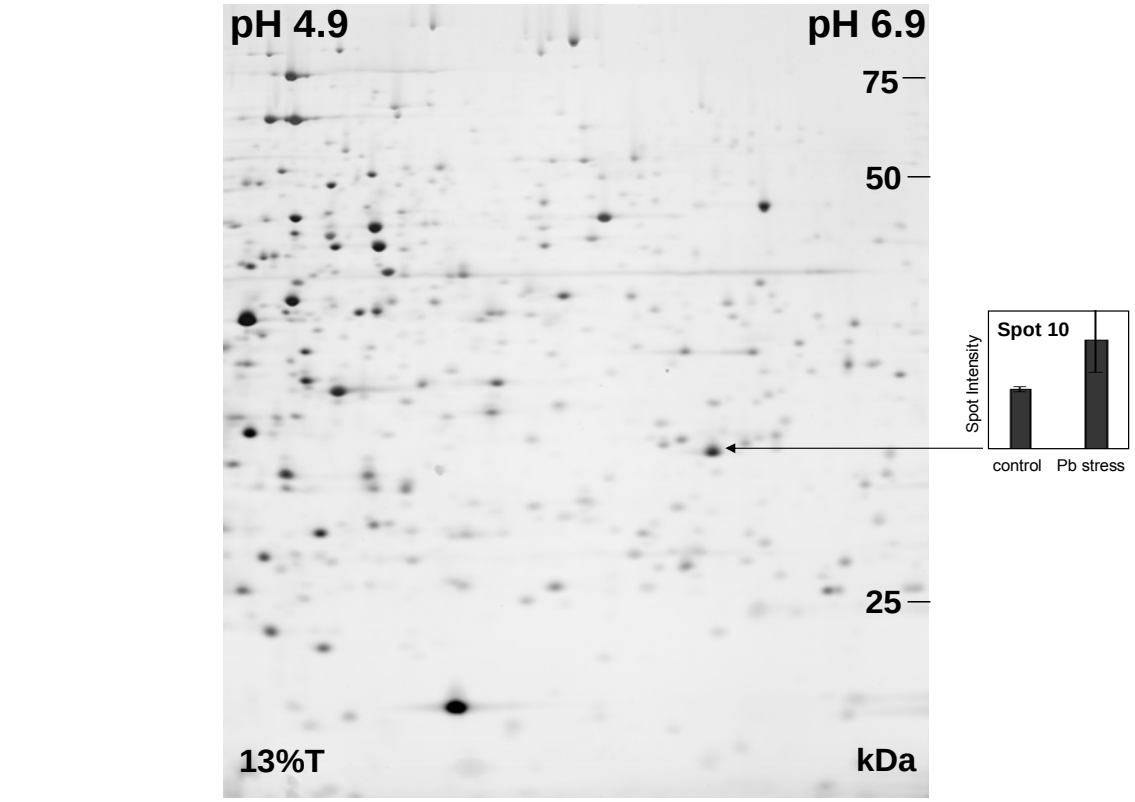


(A)

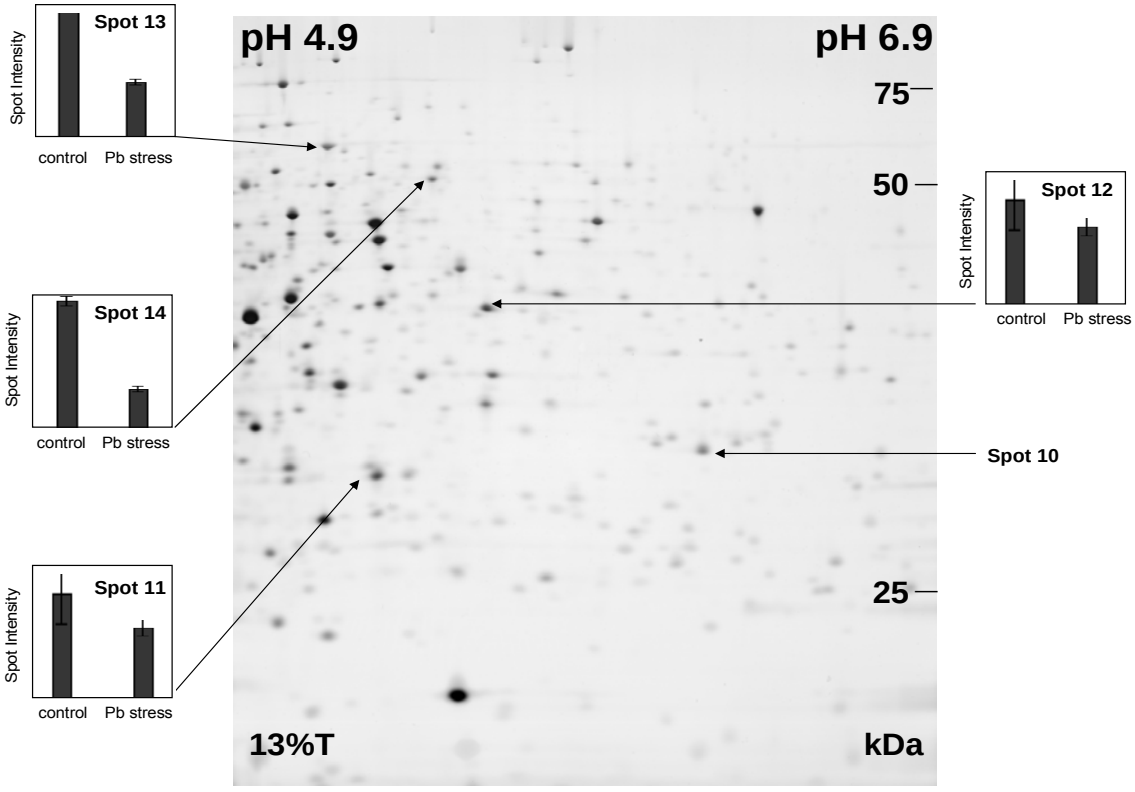


(B)

Figure 50. 2D gel images of *C. glutamicum* cells ranging from 3.4 to 5.4 treated with 6 mM $Pb(NO_3)_2$ (A) or untreated (=control) (B). The stress proteins are numbered and their identities listed in Table 10.



(A)



(B)

Figure 51. 2D gel images of *C. glutamicum* cells ranging from 4.9 to 6.9 treated with 6 mM $Pb(NO_3)_2$ (A) or untreated (=control) (B). The stress proteins are numbered and their identities listed in Table 10.

It has been reported that lead causes oxidative stress and increases production of reactive oxygen species (ROS) (Patrick 2006). In response to lead stress the cells overexpressed antioxidant enzyme NADPH:quinone reductase and NAD- dependent aldehyde dehydrogenase (Figure 52).

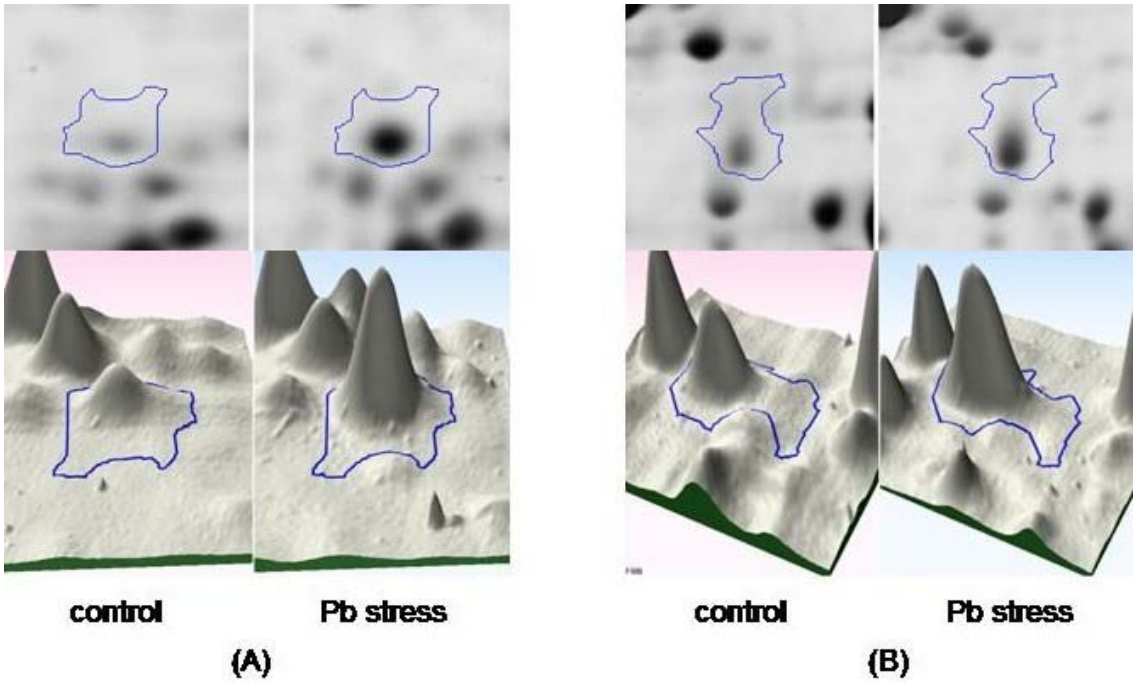


Figure 52. Overexpression of NADPH:quinone reductase and related Zn-dependent oxidoreductases (spot 3) (A) and NAD- dependent aldehyde dehydrogenase (spot 6) (B) after treatment with lead. "Zoom in" from IPG 3.4-5.4 (silver staining).

These enzymes can minimize the negative effects of free radicals and help the cell to adapt to the oxidative stress.

In this study we found that lead stress has a negative effect on succinate dehydrogenase/fumarate reductase Fe-S protein and Succinate dehydrogenase/fumarate reductase, flavoprotein subunits. These proteins are key enzymes in TCA cycle. They were repressed upon lead stress (Figure 53). In order to repair the damaged TCA cycle and to gain energy the cells up-regulated two enzymes involved in energy metabolism, protein involved in propionate catabolism and ATP synthase (Figure 54). By degradation of propionate and using it in methylcitrate cycle, the cell can produce pyruvate, which is an important substrate for TCA cycle. ATP synthase is an enzyme that can synthesize adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate by using some form of energy.

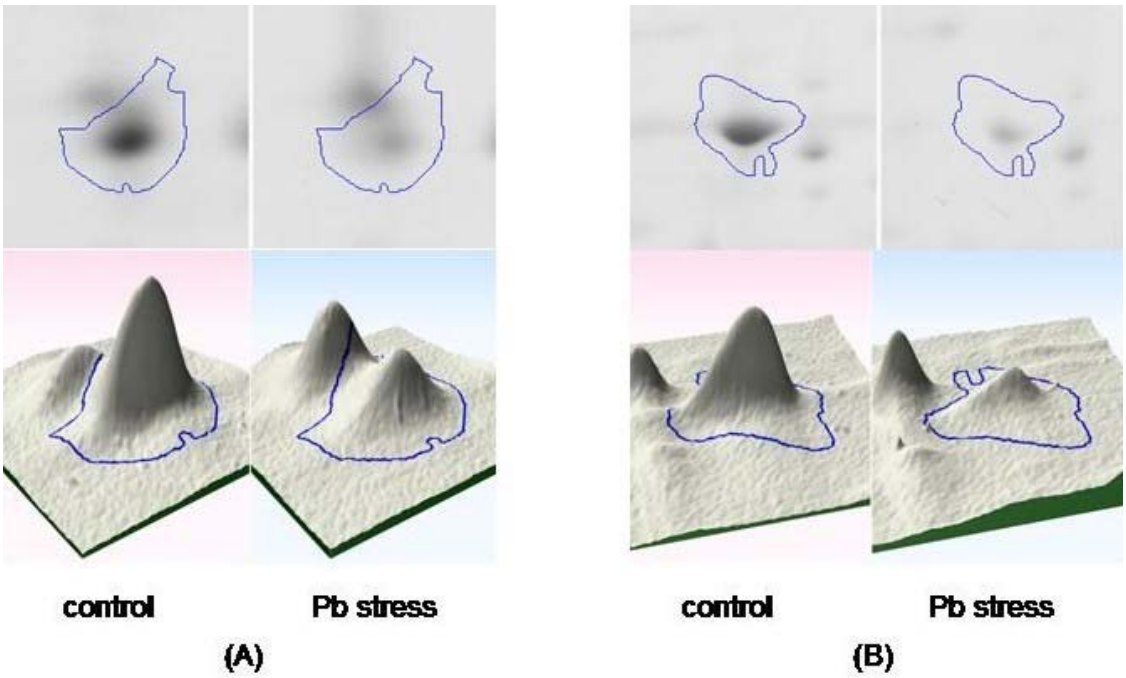


Figure 53. Downregulation of succinate dehydrogenase/fumarate reductase Fe-S protein (spot 11) (A) and Succinate dehydrogenase/fumarate reductase, flavoprotein subunits (spot 13) (B) after treatment with lead. "Zoom in" from IPG 4.9-6.9 (silver staining).

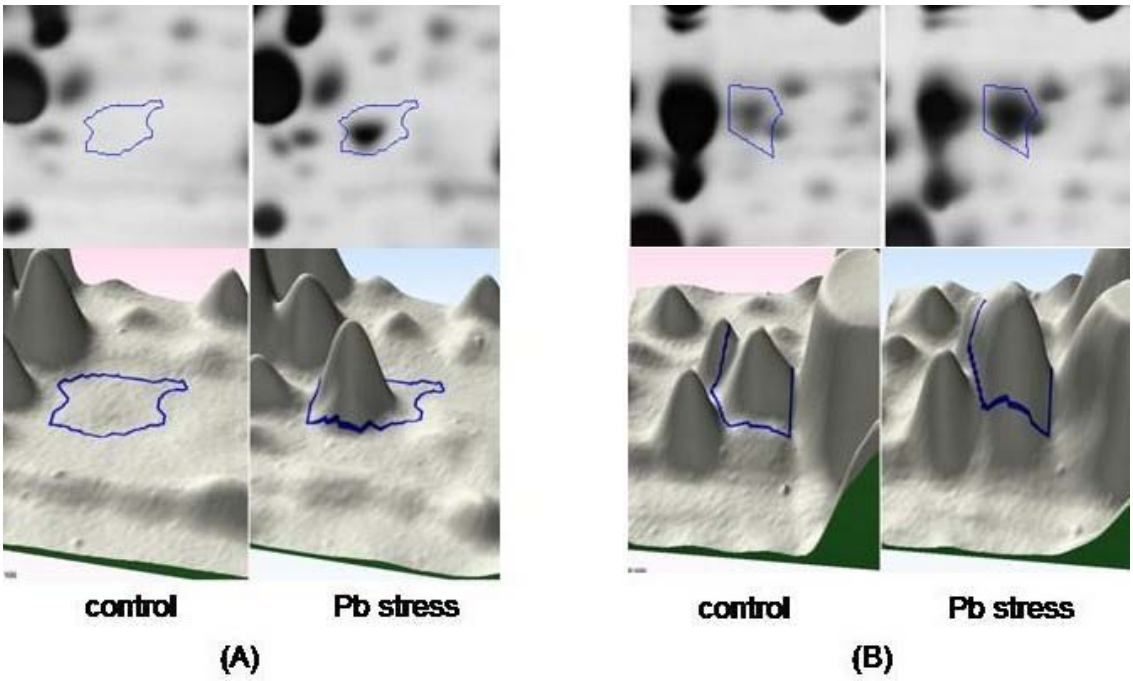


Figure 54. Overexpression of protein involved in propionate catabolism (spot 1) (A) and ATP synthase, subunit beta (spot 5) (B) after treatment with lead. "Zoom in" from IPG 3.4-5.4 (silver staining).

This enzyme is composed of fifteen subunits that work together to couple the flow of protons into the mitochondrial matrix to a rotating complex that catalyzes the ADP + P_i → ATP reaction (Elston et al., 1998). The structure of ATP synthase consists of two rotary motors, labeled F1 and Fo, that are connected by a flexible shaft. Under normal operation, the Fo motor uses the energy stored in a transmembrane ion gradient to drive the F1 motor in reverse so that ATP is synthesized from ADP and phosphate. In bacteria, anerobic conditions wipe out the ion gradient whereupon the F1 part becomes a motor, using the energy of ATP hydrolysis to turn the Fo part in reverse so that it functions as an ion pump (Oster and Wang, 2000).

Over-expression of this enzyme has been reported in yeast. The overexpression of this enzyme increased the resistance of yeast to salts, oxidative and cold stresses (Zhang et al., 2008). They suggest that induction of the ATP synthase plays a role in stress tolerance.

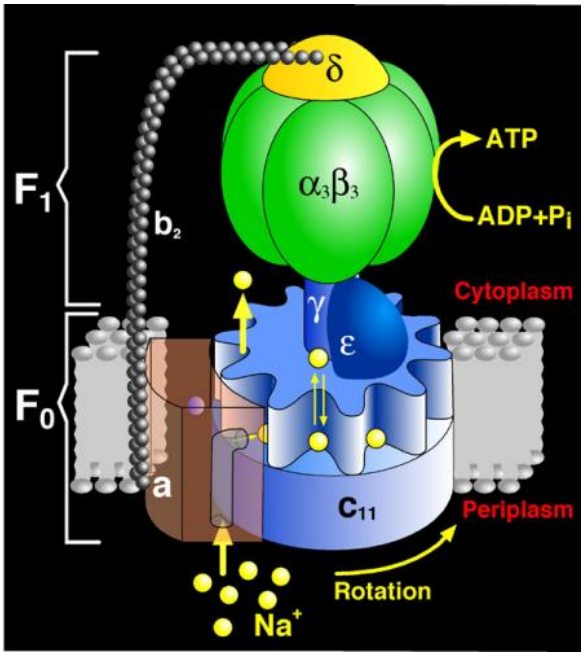


Figure. 55 The structure of ATP synthase, the universal protein that manufactures ATP (Oster and Wang, 2000)

Iron is essential for almost all life, essential for processes such as respiration and DNA synthesis. In response to increased demand for iron the cells up-regulated ABC-type Fe^{3+}-siderophores transport systems and Hemoglobin-like flavoprotein (flavohemoprotein) (Figure 56).

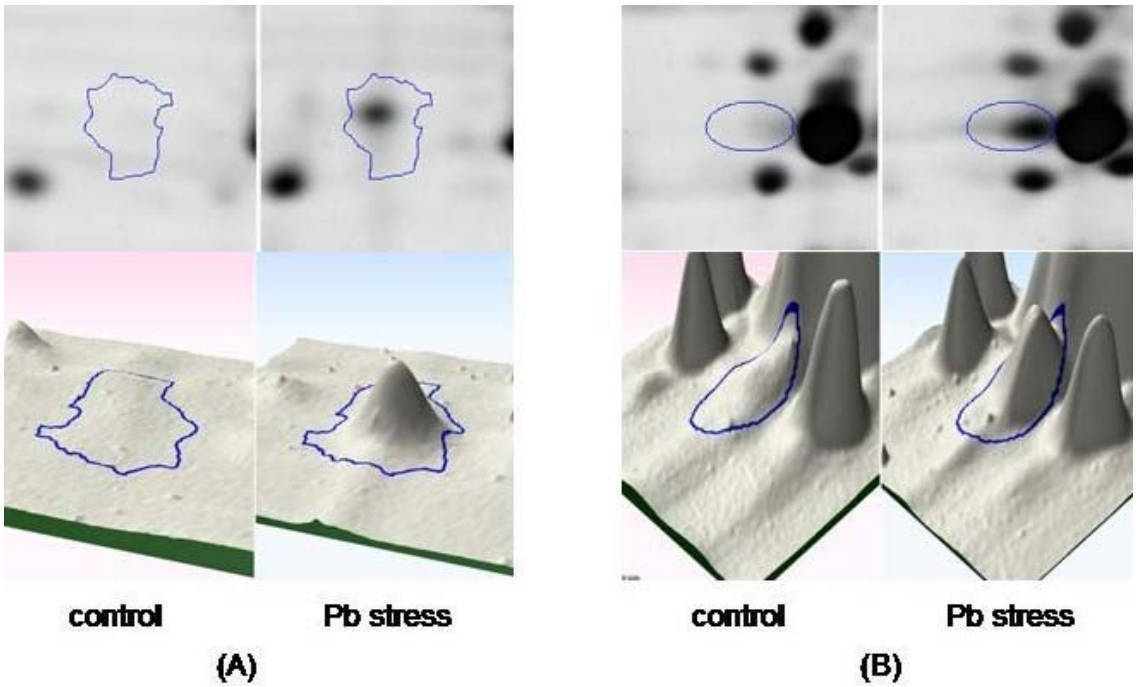


Figure 56. Overexpression of ABC-type Fe^{3+}-siderophores transport systems, periplasmic components (spot 2) (A) and Hemoglobin-like flavoprotein (flavohemoprotein) (spot 4) (B) after treatment with lead. "Zoom in" from IPG 3.4-5.4 (silver staining).

Siderophores are small, high-affinity iron chelating compounds secreted by microorganisms such as bacteria, fungi (Neilands, 1995). The cells release siderophores to scavenge iron from these mineral phases by formation of soluble Fe^{3+} complexes that can be taken up by active transport mechanisms (Figure 57).

In response to iron limitation in their environment, genes involved in microorganisms siderophore production and uptake are induced, leading to manufacture of siderophores and the appropriate uptake proteins. This is followed by excretion of the siderophore into the extracellular environment. Once outside the cell, the siderophore acts to sequester and solubilize the iron (Kraemer et al., 2000; Huyer and Page, 1988). Siderophores effectively bind with iron by forming an octahedral siderophore-iron complex. Siderophores are then recognized by cell specific receptors on the outer membrane of the cell (Olmo et al., 2003 ;Neilands, 1995). Following binding to these receptors they are transported across the cell membrane by a number of processes including but not limited to gating mechanisms and specific protein channels (Neilands, 1995; Roosenberg et al., 2000). The relatively weak complexation of Fe (II) affords an efficient pathway for iron release, via reduction of iron (III),

inside the cell. Siderophore decomposition or other biological mechanisms can also release iron (Roosenberg et al., 2000).

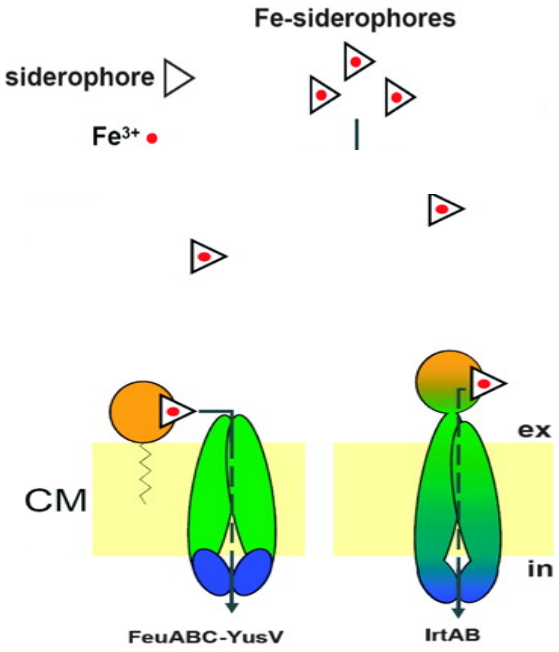


Figure 57. Gram-positive bacteria. Fe-siderophore uptake through the cell membrane (CM) by known ABC-type transporters (Miethke and Marahiel, 2007).

The iron gained from siderophores is necessary for the proper function of the enzymes that facilitate electron transport, oxygen transport, and other life-sustaining processes. For a steady supply of cells with iron, not only uptake but also storage of this metal is of critical importance. Furthermore, the means of non-toxic iron storage is important in protection against iron-mediated oxidative stress. On the other hand, oxidative stress resistance depends on iron because catalases require heme as a cofactor (Andrews et al., 2003; Arosio and Levi, 2002).

Flavohemoprotein plays a significant role in the detoxification of nitrosative stress and oxidative stress in *E. coli* (Frey et al., 2002). Similar functions or putative roles in nitrosative or oxidative stress responses have also been proposed or demonstrated for the flavohemoglobins from diverse microorganisms such as *Saccharomyces cerevisiae*, *Mycobacterium tuberculosis* (Zhao et al., 1996; Hu et al., 1999).

Interestingly our results show that Ferritin- like protein was strongly repressed under lead stress (Figure 58). This protein has a special structure, which enables it to store iron and keep it in a soluble and non-toxic form.

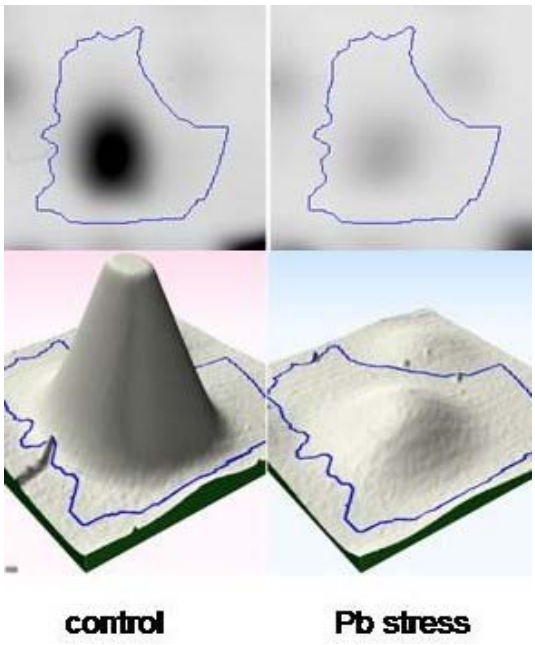


Figure 58. Downregulation of ferritin- like protein (spot 9) after treatment with lead. "Zoom in" from IPG 3.4-5.4 (silver staining).

Ferritin has the shape of a hollow sphere. Inside the sphere, iron is stored in the Fe(III) oxidation state. It is incorporated in the mineral ferrihydrite, $[FeO(OH)]_8[FeO(H_2PO_4)]$, which is attached to the inner wall of the sphere. To release iron when the cell needs it, the iron must be changed from the Fe (III) to the Fe (II) oxidation state. Then, the iron leaves through channels in the spherical structure. Thus, the structure of ferritin is extremely important for the protein's ability to store and release iron in the cell (Granier et al., 2003). We suggest that the degredation of ferritin contributes to expand the intracellular free iron pool, that is essential element for heme, which is important cofactor for many antioxidant enzymes.

C. glutamicum developed enzymatic mechanism to detoxify lead. In the presence of lead cells up-regulated proteins, which involved in detoxification of ROS, energy generation and iron transportation. Our data have clearly demonstrated the importance of iron for lead resistance.

The induction of stress proteins in *C. glutamicum* reveals the central role for these proteins in adaptation cell processes and provides the frameworke for understanding the toxic mechanism of lead and the defensev response of *C. glutamicum.*

4.6 Potential biomarkers for heavy metals

Measurement of the biological effects of pollutants has become of major importance for the assessment of the toxic effects of pollutants and the quality of the environment. Different

approaches may be applied to understand the toxic mechanism of pollutants and assess environmental quality, including ecological surveys and biomarkers. Biomarkers have important information about the environmental contaminants and their toxicity.

At a low concentration heavy metals can cause dramatic changes in protein synthesis of *C. glutamicum.* Cells respond to stress conditions by synthesizing general or specific stress proteins

The general stress proteins were induced by heavy metals stress in general, it means these proteins were also induced in other organisms under different kinds of stress (salt, pH and heat). In this study heavy metals induced many general stress proteins such as antioxidant enzymes and enzymes involved in transport system or energy metabolism (Table 11).

Table 11. General stress proteins induced by heavy metals stress.

General stress proteins	Mercury	Cadmium	Cobalt	Silver	Lead
Zn- dependent oxidoreductases	+	+	+	–	+
thioredoxin reductase	+	+	–	–	–
ABC-type transport system involved in Fe–S cluster assembly	–	+	+	–	–
NAD-dependent aldehyde dehydrogenases	–	–	–	+	+
Succinate dehydrogenase	–	+ Down	+ Up	-	+ Down

Succinate dehydrogenase is a general stress protein, which involved in energy metabolism. This enzyme was down-regulated upon cadmium and lead stress. We suggest that the stressed cell with cadmium and lead found another pathway for example pentose phosphate pathway to produce energy. Althouh general stress proteins play an central role in adaptation and protection cell processes, some proteins have special importance because they may be considered as potential biomarkers (Table 12).

Table 12. Potential biomarkers induced by heavy metals stress.

Potential Biomarkers	Mercury	Cadmium	Cobalt	Silver	Lead
lipoic acid synthase	–	+	–	–	–
2-methylcitrate dehydratase	–	–	–	+	–
1, 2-methylcitrate synthase	–	–	–	+	–
1,4-alpha-glucan branching enzyme	–	–	+	–	–
nucleoside-diphosphate-sugar epimerase)	–	–	+	–	–

For example lipoic acid synthase might be useful biomarker to detect the presence of cadmium in the living environmental organisms, the induction of 2-methylcitrate dehydratase and 1, 2-methylcitrate synthase seem to be sensitive biomarkers for exposure the cell to sub-lethal concentration of Ag^{+}, overexpression of proteins involved in cell wall biosynthesis (1,4-alpha-glucan branching enzyme and nucleoside-diphosphate-sugar epimerase) upon cobalt stress could be considered as biomarkers. To the best of our knowledge, these proteins have not been previously reported as stress proteins in bacteria after the exposure to heavy metals or another kind of stress. Biomarkers could be useful as a first indicator and alarm signal of the presence of such pollutant. In addition they may provide a useful tool for the simultaneous monitoring of heavy metals in the environment.

5 Conclusion

In this study, we described a proteomics investigation based on 2-DE to understand the toxic mechanisms of heavy metals (mercury, cadmium, cobalt, silver and lead) in *C. glutamicum.* We identified several proteins; most of these proteins were largely induced upon different kind of stresses (general stress proteins), but some proteins were specifically associated with the heavy metals-treated samples, providing evidences on their specific induction. All heavy metals except silver induced antioxidant enzymes such as manganese superoxide dismutase, Zn-dependent oxidoreductases and thioredoxin reductase, overexpression of antioxidant enzymes indicates that heavy metals can promote the production of free radicals and cause oxidative stress. We can not consider these proteins as specific stress proteins for heavy metals because they were up-regulated in many microorganisms under oxidative stress condition. Heat shock proteins (Hsp) have been reported as general stress proteins upon many stress conditions like heat, osmotic, salt pH and oxidative stress. Interestingly although many miroorganisms up-regulated heat shock proteins after exposure to heavy metals such as cadmium, lead and cobalt, *C. glutamicum* up-regulated only these proteins after treatment with cadmium.

Our results revealed that cobalt causes damage to cell membrane because two enzymes (1,4-alpha-glucan branching enzyme and nucleoside-diphosphate-sugar epimerase) involved in cell wall biosynthesis were up-regulated upon cobalt stress, they may participate to reparation of cell wall. We found that iron is very important for lead resistance. ABC-type Fe^{3+}-siderophores transport systems and Hemoglobin-like flavoprotein (flavohemoprotein) were strongly induced under lead stress. We are involved in the evaluation of stress proteins to find biosensors (biomarkers), which can be induced in the presence of low concentration of heavy metals. For example lipoic acid synthase may have the potential to serve as biomarker in order to detect the presence of cadmium in the living environmental organisms, the induction of 2-methylcitrate dehydratase and 1, 2-methylcitrate synthase demonstrates the potential of these two enzymes as biomarkers of sub-lethal Ag^{+} toxicity, overexpression of proteins involved in cell wall biosynthesis (1,4-alpha-glucan branching enzyme and nucleoside-diphosphate-sugar epimerase) upon cobalt stress could be considered as biomarkers. To the best of our knowledge, these proteins have not been previously reported as stress proteins in bacteria after the exposure to heavy metals or another kind of stress. These biomarkers may provide a useful tool for the simultaneous monitoring of toxic metals in the environment. In addition the stress proteins and their pathway should provide some insight into the molecular

mechanisms of heavy metals resistance in these soil bacteria and may demonstrate its utility in detecting environmental pollution by heavy metals. Finally *C. glutamicum* exhibited high resistance to heavy metals. It may develop probably highly specific defense mechanisms to cope with heavy metals exposure. This is in contrast to other pro- and eukaryotic model organisms, such as *Escherichia coli* and *S. cerevisiae*, which are naturally less exposed to heavy metal concentrations, and whose stress reaction is supposedly less specific.

6 Summary

Recent proteomic research has provided evidence that environmental chemicals induce the expression of characteristic stress-related proteins in living organisms. This opens the possibility of (at least in principle) using these protein expression profiles (*signatures*) for the detection of any kind of environmental chemical with the help of their highly specific „fingerprint". By developing an analytical assay based on these protein expression signatures, not only the identification and/or classification of the most important environmental chemicals, *e.g.,* in waste water, would be possible, but may also provide a better understanding of the damage and toxicity mechanisms of these chemicals which are not fully understood yet. In the long run, they should permit the development of fast and reliable assays for the most important environmental chemicals, either based on recombinant reporter organisms, or antibody arrays. Because of their toxic effects on cellular metabolism, contamination of soil and ground water with heavy metals is one of the major environmental and human health problems. Despite their well-known toxicity, the basis of their toxicity is not well understood. Humans are often exposed to heavy metals in various ways – mainly through the inhalation of metals in the workplace or polluted neighbourhoods, or through the ingestion of food (particularly seafood) that contains high levels of heavy metals or paint chips that contain lead. Heavy metals are associated with myriad adverse health effects, including allergic reactions, neurotoxicity, nephrotoxicity and cancer.

Corynebacterium glutamicum is gram-positive and non-pathogenic bacterium. It is used for the industrial production of the amino acids lysine and glutamine. *C. glutamicum*, a soil bacterium of the actinomycetes family which is abundant in contaminated soils, has probably developed highly specific defense mechanisms to cope with heavy metals exposure. *C. glutamicum* was also chosen based on the fact that besides of having a short generation time, the bacterium is safe to handle and its genome has been completely sequenced, and furthermore has been the subject of biological research for several decades.

One aim of the study was the analysis of response the heavy metals of *C. glutamicum* with advanced proteomic tools to identify stress proteins involved in the response to the heavy metals (mercury, cadmium, cobalt, silver and lead) , as some stress proteins could be considered as biomarkers for special heavy metals. These biomarkers can be used as an early warning system for heavy metals and protect the whole ecosystem from heavy metals.

Identification of these proteins and their mapping into specific cellular processes may help to gain deeper insight into the toxic mechanisms of heavy metals.

Analysis of the stress response of *C. glutamicum* to heavy metals was another aim of the study to identify stress proteins, which are involved in the adaptive and protective mechanism of *C. glutamicum.* This enables to gain a global understanding of the way in which *C. glutamicum* counteract the heavy-metals stress exposure.

Reproducible protein patterns are the basis for differential protein expression analysis. Hence, cell growth and stress conditions were standardized first, and a growth curve of stressed and unstressed bacterial cells was determined. Then, *C. glutamicum* cells of the exponential growth phase under stress-free conditions, considered as control, as well as cells exposed to heavy metals, considered as stressed sample, were harvested at an OD of 7, which corresponds to the mid-logarithmic phase. High resolution 2DE was applied for the separation of the proteins, permitting highly reproducible and sensitive detection of proteins. Proteins were separated by 2DE using two narrow overlapping immobilized pH gradients 3.4-5.4 and 4.9-6.9, as the majority of *C. glutamicum* proteins are located in this area. Proteins were visualized by staining with silver nitrate and/or Coomassie Brilliant blue. The computer based analysis of the 2-D gels for protein detection, spot matching between gels, and change of protein expression levels was performed with SameSpots software. Differentially expressed proteins were identified by MALDI-TOF-MS.

At a low concentration heavy metals can cause dramatic changes in protein synthesis of *C. glutamicum.* All heavy metals except silver induced antioxidant enzymes such as manganese superoxide dismutase, Zn- dependent oxidoreductases and thioredoxin reductase. The induction of antioxidant enzymes indicates that heavy metals can promote the production of free radicals and cause oxidative stress. These enzymes can minimize the negative effects of free radicals and help the cell to adapt to the oxidative stress. Our results show that the induction of proteins involved in energy metabolism upon heavy metals stress may reflect the increased need of the cell to generate enough energy to counteract the oxidative stress, which was caused by heavy metals. Cobalt and silver may target and damage DNA; in response to this damage ribonucleotide reductase and sugar kinase ribokinase were strongly overexpressed. They may be involved in DNA repair in *C. glutamicum.* During adaptation to cobalt stress, two enzymes involved in cell wall biosynthesis (nucleoside-diphosphate-sugar epimerase and 1,4-alpha-glucan branching enzyme) were overexpressed. These results indicate that cobalt probably damages the cell membrane and these enzymes involved in

strengthen or reparation of cell membrane. Our data have clearly demonstrated the importance of iron for lead resistance. In response to increased demand for iron the cells induced ABC-type Fe^{3+}-siderophores transport systems and Hemoglobin-like flavoprotein (flavohemoprotein), they are important for protection against oxidative stress.

Althouh all stress proteins play a central role in adaptation and protection cell processes, some proteins have special importance because they may be considered as potential biomarkers. For example lipoic acid synthase may have the potential to serve as biomarker in order to detect the presence of cadmium in the living environmental organisms, the induction of 2-methylcitrate dehydratase and 1, 2-methylcitrate synthase demonstrates the potential of these two enzymes as biomarkers of sub-lethal Ag^{+} toxicity, overexpression of proteins involved in cell wall biosynthesis (1,4-alpha-glucan branching enzyme and nucleoside-diphosphate-sugar epimerase) upon cobalt stress could be considered as biomarkers. To the best of our knowledge, these proteins have not been previously reported as stress proteins in bacteria after the exposure to heavy metals or another kind of stress. Biomarkers could be useful as a first indicator and alarm signal of the presence of such pollutant. In addition they may provide a useful tool for the simultaneous monitoring of heavy metals in the environment.

7 Zusammenfassung

Neue Forschungsarbeiten im Bereich der Proteomik haben gezeigt, dass Umweltchemikalien in lebenden Organismen die Expression von charakteristischen Stressproteinen induzieren. Dies eröffnet im Prinzip die Möglichkeit mit Hilfe eines für jede Chemikalienart, über entsprechend induzierte Expressionsmuster (Signaturen) typischen „Fingerabdrucks", die entsprechende Chemikalie in der Umwelt nachzuweisen. Durch die Entwicklung eines auf diesen Proteinexpressionssignaturen basierenden Testsystems wäre nicht nur eine schnelle und zuverlässige Identifizierung und Klassifizierung der wichtigsten Umweltchemikalien möglich, sondern es ließe sich über die Forschung der zugrunde liegenden adaptiven Netzwerke der Zelle auch ein besseres Verständnis der Wirkungsmechanismen und der Toxizität von bisher unzureichend untersuchten Umweltchemikalien erreichen. Darüber hinaus könnten Schnelltests für die wichtigsten Umweltchemikalien unter Verwendung rekombinanter Organismen entwickelt werden. Wegen ihrer toxischen Wirkungen auf den zellulären Stoffwechsel, ist die Kontamination der Erde und des Grundwassers eines der größten Umweltprobleme, die sich auch auf menschliche Gesundheit auswirkt. Trotz allem ist die Basis ihrer Toxizität nicht gut verstanden. Menschen sind oft Schwermetallen in verschiedener Weise ausgesetzt, hauptsächlich durch die Inhalation der Metalle am Arbeitsplatz, oder durch die Aufnahme von Lebensmitteln (besonders Meeresfrüchte), die hohe Konzentrationen von Schwermetallen enthalten. Schwermetalle sind verbunden mit unzähligen ungünstigen Gesundheitswirkungen und verursachen allergische Reaktionen, sind neurotoxisch, nephrotoxisch und Krebs erregend.

Als Modellorganismus eignet sich z.B. das nicht pathogene, genetisch kartierte Bodenbakterium *Corynebacterium glutamicum*, das vor allem zur Herstellung der Aminosäuren L-Glutamat und L-Lysin eingesetzt wird. *C. glutamicum* ist ein Bodenbakterium aus der Familie der Actinomyceten, das auch in kontaminierter Erde vorkommt. Das Bakterium hat wahrscheinlich spezifische Abwehrmechanismen entwickelt, um Schwermetall Stress zu bewältigen. *C. glutamicum* wurde auch ausgewählt, weil es eine kurze Generationszeit besitzt und das Bakterium sicher zu handhaben ist. Außerdem ist sein Genom vollständig sequenziert, und wurde als Testorganismus in der biologischen Forschung für mehrere Jahrzehnte verwendet.

Ein Ziel dieser Arbeit war die Analyse der Stressantwort von *C. glutamicum* nach der Behandlung mit Schwermetallen, mit Hilfe der differenziellen Proteomanalyse, um Stressproteine zu identifizieren, die durch Schwermetalle induziert werden. Manche

Stressproteine könnten als Biomarkers für spezifische Schwermetalle betrachtet werden. Diese Biomarker können als Frühwarnsystem für Schwermetalle benutzt werden um das Ökosystem vor Schwermetallen zu schützen. Die Identifikation dieser Proteine und ihrer Funktionen in der Zelle helfen einen tieferen Einblick in die toxischen Mechanismen der Schwermetalle zu gewinnen. Die Analyse der Stressantwort von *C. glutamicum* auf die Schwermetalle (Quecksilber, Cadmium, Kobalt, Silber and Blei) war ein weiteres Ziel dieser Arbeit um Stressproteine zu identifizieren, die an der Adaption und Schutzmechanismen von *C. glutamicum* beteiligt sind. Dies erlaubt, ein übergreifendes Verständnis des Mechanismus zu gewinnen, der *C. glutamicum* ermöglicht, den Schwermetall Stress zu bewältigen.

Reproduzierbare Proteinmuster sind die Basis der differentialen Proteinexpressionsanalyse. Daher wurden die experimentellen Konditionen für den Zellwachstum und der Stressexposition zunächst standardisiert und die jeweiligen Wachstumskurven der mit Schwermetall behandelten und unbehandelten Kultur bestimmt. Danach wurden die *C. glutamicum* Zellen der exponentiellen Wachstumsphase (stressfrei; betrachtet als Kontrolle) sowie der Schwermetall-exponierten Zellen (gestresste Probe) bei OD = 7 geerntet, welches der mid-logarithmischen Phase entspricht. Hochauflösende 2 DE Elektrophorese wurde angewandt, um die Proteine aufzutrennen, da sie eine hoch reproduzierbare und sensitive Detektion der Proteine erlaubte. Bei der 2 DE kamen zwei eng überlappende, immobilisierte pH Gradienten zur Anwendung, pH 3,4–5,4 und 4,9–6,9, weil in diesen Bereichen die größte Anzahl von Proteinen anwesend ist. Die Proteine wurden mittels Silbernitrat-Anfärbung und Coomassie-Brilliantblau Methode visualisiert. Mit Computer-gestützter Analyse (SameSpots Software) der 2D Gele konnten die Levels der Expressionen verglichen werden. Die Proteine wurden durch MALDI-TOF-MS identifiziert.

Bereits bei geringen Konzentrationen verursachen Schwermetalle dramatische Änderungen in der Proteinsynthese von *C. glutamicum* Zellen. Alle untersuchten Schwermetalle, mit Ausnahme von Silber, induzierten antioxidative Enzyme wie Mangan Superoxiddismutase, Zink-abhängige Oxidoreduktase und Thioredoxin Reduktase. Die Induktion antioxidativen Enzyme zeigt, dass Schwermetalle die Produktion von freien Radikalen verursachen, welche oxidativen Stress fördern. Diese Enzyme können den negativen Effekt der freien Radikalbildung minimieren und helfend für die Zelle wirken, sich auf den oxidativen Stress zu adaptieren. Die Resultate haben gezeigt, dass die Induktion von Proteinen,welche in dem Energiemetabolismus während der Stressexposition von Schwermetallen involviert sind, den erhöhten Bedarf der Zelle reflektieren mag, genügend Energie zu entwickeln, um sich gegen

den oxidativen Stress zur Wehr zu setzen. Kobalt und Silber zielen auf die DNA ab und schädigen diese, so dass als adaptive Antwort die Ribonukleotidreduktase sowie die sugar Kinase Ribokinase stark über-exprimiert werden. Diese Substanzen sind in der DNA Reparatur der *C. glutamicum* Zelle involviert. Während der Adaption an Kobaltstress, wurden zwei weitere Enzyme, die in der Zellwandbiosynthese involviert sind, über-exprimiert: *Nucleoside Diphosphate-sugar Epimerases* and 1,4-α-Glucan-verzweigende Enzym. Dieses Ergebnis deutet darauf hin, dass Kobalt die Zellmembran schädigt wobei diese Enzyme verstärkend auf die Reparatur der Membran wirken. Die Ergebnisse stellten dabei noch mal deutlich die Wichtigkeit von Eisen im Resistenz Verhalten der Zelle gegenüber Blei heraus. Als Stressantwort für den zunehmenden Bedarf an Eisen wurden ABS Type Fe3+-siderophores Transport System und (Flavohemoprotein) induziert; diese Enzyme sind für den protektiven Mechanismus der Zelle gegen oxidativen Stress von großer Wichtigkeit. Obwohl alle gefundenen Stressproteine eine zentrale Rolle in der Adaptions- und Protektionsphase der Zelle spielen, sind einige Proteine von besonderer Wichtigkeit, da sie das Potenzial haben, als Biomarker zu fungieren. So kann beispielsweise die α-Liponsäure Synthethase als Biomarker für die Detektion von Cadmium in lebenden Organismen dienen. Dasselbe gilt für die Induktion der Enzyme 2-Methylzitrate Dehydratase und 1,2-Methylzitrate Synthase, die ihr Potenzial demonstriert haben, als Biomarker für sub-letale Ag+ Toxizität zu fungieren. Die Überexprimierung unter Kobaltstress von Proteinen, die in der Zellwandbiosynthese involviert sind, (*Nucleoside Diphosphate-sugar Epimerases* and 1,4-α-Glucan-verzweigende Enzym), ist hierzu ein weiteres Beispiel.

Die gefunden Proteine wurden nach unserem Wissensstand als Stressproteine in Bakterien nach Exposition von Schwermetallen (oder jeglicher Form von Stress) bislang noch nicht beschrieben. Biomarker können sehr hilfreich als Erstindikatoren bzw. als Alarmsignal für die Präsenz von Schadstoffen sein. Außerdem können sie ein nützliches Instrument für die simultane Überwachung von verschiedenen Schwermetallen in der Umwelt darstellen.

8 Literature

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