Proteomic Characterization of Selenite Resistance in a strain of *Enterobacter cloacae*

By

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Abstract

A multimetal resistant strain of *Enterobacter cloacae* (*E. cloacae*) grew in the presence of 40 mM selenite and demonstrated the ability to transform it into a red precipitate. Established growth curve studies in M-9 minimal salts medium showed that the *E. cloacae* strain grew in the presence of selenite only when the medium was supplemented with L-cysteine. During early log-phase, selenite or water was added. One hour later, cells were harvested for protein analysis by two dimensional gel electrophoresis (2DGE). Protein profiles of cultures grown under the resistant (cysteine) and sensitive (no cysteine) conditions were compared and differentially expressed polypeptides spots were excised and sent to Ohio State University for identification by mass spectrometry. The identified proteins were classified under three possible functions: 1) oxidative stress response, 2) selenium processing and 3) selenite efflux. None of the identified proteins indicated a possible role for cysteine in selenite resistance.

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LIST OF SYMBOLS AND ABBREVIATIONS

EDTA……..Ethylene Diamine Tetra Acetic Acid

HCl……...Hydrochloric Acid

sec…….Seconds

µL……...Microliter

µM……...Micromolar

NaCl……Sodium Chloride

$MgCl_2$……...Magnesium Chloride

BSA…….Bovine Serum Albumin

DTT……...Dithioththreitol

mL ……millilter

Tris……Tris(hydroxymethyl)aminomethane

µg…….Microgram

mM……millimolar

mg……...milligram

M……...molar

$MgSO_4$ ……Magnesium sulfate

mL……..…milliliters

min…….minutes

rRNA……...ribosomal ribonucleic acid

SeO_3^{2-}……..Selenite

SeO_4^{2}-……..Selenate

Se……....Selenium

Zn……zinc

Cd……cadmium

Cu……copper

pMol…….....picomoles

DMSe……...Dimethyl Selenide

DMDSe…….Dimethyl Diselenide

E. coli……...*Escherichia coli*

FDH…….Formate dehydrogenase

tRNA……transfer RNA

γ ……..…gamma

β……...beta

ATP……..Adenosine triphosphate

H_2O_2 ……...Hydrogen peroxide

O_2^- …….....Superoxide

GSH …….....Glutathione

DNA……..deoxy ribonucleic acid

RNA …….....ribonucleic acid

% …….....percentage

V…….....volts

Kb…….kilobase

bp ……basepairs

CHAPTER I

Introduction

Statement of the problem

During the Second World War, the Y-12 Plant in Oak Ridge, TN processed uranium to make the first nuclear bomb, which was dropped on Hiroshima, Japan (Oak Ridge Environmental Peace Alliance website). Then during the height of the Cold War, to prevent Soviet Union aggression, large amounts of mercury were used to process lithium for hydrogen bomb production. This process required large amounts of mercury which was not contained and nearly 920,000 kg were spilled into the surrounding environment (Rouse Campbell et al., 1998). Disposal of other wastes was done in different places in the area. Four S-3 ponds, located near the origins of the Bear Creek and East Fork Poplar Creek, held various acidic liquid wastes, uranium nitrite, and other heavy metals (Oak Ridge Environmental Peace Alliance website).These ponds lacked coverings and linings to allow liquid wastes to evaporate or become decontaminated as they passed through the soil. Instead, these wastes simply leaked into the two creeks, causing the surrounding sediment to become highly contaminated (Widner et al., 1996).

Heavy metal remediation has been a long-term challenge in such industries such as metal plating, leather, photography, battery, automobile and refrigeration that generate large quantities of metal waste. In spite of many governments' regulations, large amounts of such waste are being dumped in heavily populated areas (Bar, C.2007).

Found in group VIA of the periodic table, selenium is a naturally occurring element and is classified as a metalloid having characteristics of a metal and a non-metal. Selenium is found in four inorganic oxidative states: selenate, SeO_4^{2-} (VI), selenite, SeO_3^{2-}(IV), selenide, Se^{2-}(II), and elemental selenium, Se^0 (0). Selenate and selenite are readily soluble in water and are toxic to living organisms (Turner, R.J. 1998). Elemental selenium is not soluble in water and is non-toxic. Selenide, which is highly toxic and reactive, is usually not available because it is oxidized to elemental selenium under aerobic conditions.

Selenium is normally very essential for humans and animals. However, it is very toxic at higher concentrations. In some regions of the world, part of the daily food is artificially enriched with selenium for health reasons, while in others; it is polluted with selenium (Laeuchli 1993). Selenium naturally occurs in elevated levels in the soils of some areas, and contamination is generated by agricultural irrigation, fossil fuel combustion, petroleum refining, and mining operations (Haygarth et al., 1991). Selenium contamination is a common occurrence in the western United States and became a major issue at the Keterson National Wildlife Refuge, in the San Joaquin Valley in California (Saiki, M.K.1987;Frankenkenberger, W.T., Jr. 2001). In this particular area, concentrated selenium in agricultural drainage water and soil exceeded normal levels killing or causing birth defects in aquatic animals and birds. Since then, considerable research has been expanded to quantify the ecological and ecotoxicological effects of selenium and its chemical and biochemical interactions in the environment (Swift 2002).

Some microorganisms have been found to be resistant to high concentrations of selenium. Some bacteria developed a variety of resistance mechanisms, for example, the reduction, oxidation or methylation of organic and inorganic selenium species (Stoltz, J.F. 2002). Other bacteria can use selenate as a terminal electron acceptor (Schroder, I. 1997). Every mechanism involved in the detoxification of selenium leads to a modification of the species of this element (Sarret, G. 2005).

The bacterial strain of *Stenotrophomonas maltophilia* ORO2 (*S. maltophilia* SO2) was isolated from East Fork Poplar Creek in Oak Ridge, TN. This strain was able to grow in the presence of toxic levels of selenium and demonstrated the ability to precipitate selenite. This bacterium and other prokaryotes have been found to reduce selenite to elemental selenium, which can be more easily removed from the environment (Dungan, R.S. 2003). While studying other bacterial isolates from this site, 16S rRNA and biochemical tests showed that we were actually working with a strain of *Enterobacter cloacae (E. cloacae),* instead of *S. maltophilia.* The reduction of selenite by other strains of *E. cloacea* has been observed in the periphery of the cell wall. Some prokaryotes also methylate selenium (Ranjard, L. 2003). Spontaneous selenium methylation can be observed in biologically active aqueous sewage sludge and soil extracts. In most of these environments, it is observed after addition of organic and inorganic selenium compounds, suggesting that a broadly distributed process is involved (Ranjard, L. 2003). Bacteria have been identified as the predominant Se-methylating organisms. These have been found mainly to belong to two groups, the *Protobacteria* and the *Cytophagales* (Chau et al., 1976; Doran, 1982; Dungan, 2000). When exposed to selenium oxyanions (selenate or selenite) or organic forms of selenium (selenocystine, selenocysteine and

selenomethionine), bacteria can generate dimethyl selenide (DMSe) or dimethyl diselenide (DMDSe) which leads to the volatilization, detoxification and removal of selenium from contaminated sites (Ranjard, L.2003). The volatile forms are five to seven hundred times less toxic than other derivatives, and this prevents them from entering the food chain. This process may be used for the bioremediation of selenium oxyanions.

Precipitation is a second bioremediation strategy for removing selenium from contaminated environments. Since selenium in contaminated sites generally exists as the oxyanions, selenite or selenate, the ability of bacteria to reduce these oxyanions to insoluble elemental selenium may be a useful tool for eliminating dissolved selenium. The dissolved selenium that was reduced to elemental selenium can then be removed by filtration (Frankenberger, W.T., Jr 2001). However, little progress has been made in treating selenium contaminated waters due to the cost associated with treating large volumes of water and due to the high salt content (Dungan et al., 2003).

Selenium Uptake and Transport

Selenate is believed to enter the cell through the sulfate permease system which comprises of *cys*A, *cys*U and *cys*W genes (Kredich 1996; LaRossa 1996). Mutations in these genes give rise to selenate resistance (Turner et al., 1998). However, selenate as well as sulfate uptake is inhibited in the presence of cysteine (Brown and Shrift 1982). Selenite can also enter the cell through this sulfate transporter. Nonetheless, a different yet uncharacterized carrier likely exists because repression of sulfate permease expression does not totally inhibit selenite uptake (Turner et al., 1998). In addition, selenite transport is not shut down by the presence of cysteine (Brown and Shift, 1982).

In *Escherichia coli* (*E. coli*), selenite can enter the cell via the sulfate transporter (Turner et al., 1998). However, in *Rhodobacter sphaeroides*, a polyol transporter has been suggested as the transporting agent of selenite into the cell (Bebien et al., 2001). Furthermore, in *E. coli*, a selenite inducible gene *gutS* has been shown to be upregulated in the presense of both tellurite and selenite. When database searches of the protein of this gene were done, it showed homology to membrane transport proteins (Guzzo et al., 2000). For this reason, it has been suggested that specific membrane transport proteins are expressed in some microorganisms when exposed to selenite. These proteins could be responsible for selenite transport into the cytoplasm.

L-cysteine in Selenite Resistance and Selenocysteine Biosynthesis

The bacterial strain of *E. cloacae* fails to grow when it is exposed to 40 mM sodium selenite during mid-log phase in M-9 minimal salts medium in the absence of cysteine. However, in the presence of cysteine, this bacterium is able to grow (fig. 3.1). It is evident that once inside the cell; there is a reduction of selenite to selenide (Turner et al., 1998). Selenide is further oxidized to elemental selenium (Dungan et al., 2003) and finally incorporated into amino acids cysteine and methionine as selenocysteine and selenomethionine (Turner et al., 1998).

Muller et al. (1997) suggested that two pathways of selenium incorporation exist: direct and indirect. In the direct pathway, selenite enters the cell through a selenium specific pathway and is incorporated into selenocysteine. There is a chemical similarity between selenium and sulfur because they are in the same group in the periodic table. Meanwhile in the indirect pathway, it enters the cell through the sulfate transport system and is unintentionally incorporated, producing selenocysteine instead of cysteine. Hence,

the enzymes involved in the biosynthetic pathway of cysteine can generate free selenocysteine (Lacourciere et al., 2002).

Selenocysteine (Sec), the 21st amino acid is cotranslationally incorporated into several prokaryotic and eukaryotic selenoproteins at inframe UGA stop codons (Ogasawara et al., 2004) and provides an important active site residue in select redox proteins such as *E. coli* formate dehydrogenase. It is now known that UGA serves as both a termination codon and a Sec codon. The ways by which UGA serves as a Sec codon and how Sec is biosynthesized and incorporated into protein have been studied in detail with eubacteria (Bock 2001). Four genes, *SelA, SelB, SelC* and *SelD*, are required for selenocysteine codon recognition and translation (Zizoni et al., 1986; Leinfelder et al., 1988; Bock and Stadtman 1998). According to these processes, selenium is derived from an activated selenium donor, selenophosphate, generated by *SelD* gene product, selenophosphate synthetase (Leinfelder et al., 1990). However, the metabolic pathway that provides the selenium to selenophosphate synthetase (SPS) as a substrate for selenophosphate biosynthesis has not been identified (Ogasawara et al., 2004).

Selenide is incorporated into selenocysteine through a specific pathway (Bock,2001). The *SelD* gene which encodes SPS produces an activated form of selenium, selenophosphate. The selenium from this activated oxyanion is used for charging serine-tRNA^{Sec} with selenium by selenocysteine synthetase (*SelA*), thus generating selenocystyl-tRNA^{Sec}. Cysteine, unlike many other amino acids does participate in many redox pathways, for instance exchange and radical reactions, atom-, electron- and hydride-transfer reactions (Claus et al., 2003)

Selenium Toxicity

Spallholz and Hoffman (2002) elucidated various ways by which selenium may express its toxicity: the first mechanism involves formation of methyl selenide (CH_3Se^-) which either enters a redox cycle and generates oxidative stress, or forms free radicals which bind to and inhibit vital enzymes and proteins. Secondly, excess selenium as selenocysteine prevents selenium methylation metabolism. As a result, concentrations of hydrogen selenide, which is an intermediate metabolite accumulate in animals and are hepatotoxic, leading to other selenium- related adverse effects. There is a possibility that the presence of excess selenium analogues of sulfur-containing enzymes and structural proteins cause birth defects.

Selenate and selenite are the naturally occurring forms of selenium in the biosphere under aerobic conditions (Shamberger 1985). The two oxyanions, especially selenite, are toxic. Various redox reactions are produced by microorganisms in the biogeochemical cycle of selenium (Stolz et al., 1999). For instance in *E. coli*, the oxyanions are detoxified through their reduction into the elemental selenium or metabolized to hydrogen selenide (HSe^-), which can be incorporated into selenocysteine. Studies have shown that reduction of selenite involves reactions with sulfhydryl groups of thiol molecules such as glutathione which leads to the production of metabolites selenodiglutathione (GS-Se-SG), glutathioselenol (GS-SeH), HSe^- and finally to elemental selenium (Ganther 1968; Kice et al., 1980). Some reactions in this selenite pathway produce hydrogen peroxide (H_2O_2) and superoxide ($O_2.^-$) (Kramer and Ames 1988; Seko and Imura 1987). This causes damage to cell membranes and DNA (Seko and Imura 1997). However, selenate toxicity is expressed only after its reduction to selenite

or selenol (-SeOH) (Turner et al., 1998). Tolerance to selenite in microorganisms could be due to differences in non-specific uptake levels in the sulfate permease system. There is also a possibility that they posses similar selenite uptake systems due to selenite sensitivity (Weiss et al., 1965). It has been shown that selenium inhibits the growth of *E. coli* when present in higher amounts as selenite.

In this work, two dimensional (2D) gel electrophoresis was used to identify proteins induced by selenite in a multimetal resistant strain of *E. cloacae*. The identification of these proteins will not only provide new insights into the mechanism of selenium toxicity, but also the cellular protection against this compound under aerobic conditions.

Proteomics

Proteomics is a tool used to separate proteins by size and charge (Washburn and Yates 2000). This approach involves the use of two dimensional gel electrophoresis (2DGE) and mass spectrometry (MS) to identify the differentially expressed proteins under heavy metal stress. Similar work has been done at a different level using a pH gradient of 4-7 (Jasenec, 2007). Differentially expressed proteins were found which showed increased expression under both selenite sensitive and resistant conditions. However, all the proteins were not resolved. At pH 7, there was a large protein streak where proteins stopped migrating indicating that proteins with a PI above pH 7 exist. This proposed work was done in the pH range 5-8 to repeat the study and hopefully resolve many more proteins at a higher pH range and identify them as well.

Hypothesis

At high concentrations of selenite (40 mM), the *E. cloacae* strain is only able to survive when L-cysteine is present (Fig. 3.1). Increase in turbidity of the culture is indicative of sustained growth of bacteria when both L-cysteine and selenite are present. On the contrary, when L-cysteine is absent, the bacteria are unable to grow. Moreover, during the log phase of growth, harvested cells appear white and may be pumping selenite out of the cell. However, when the bacterial culture approaches the stationary phase, harvested cells appear red and may be reducing the selenite to elemental selenium.

Proteomics was used to test the hypothesis that certain proteins are produced by the *E. cloacae* strain in response to selenite, which enables the organism to be resistant. Protein profiles of the *E. cloacae* strain were generated under selenite sensitive and resistant conditions and the differentially expressed proteins were identified by tandem mass spectrometry. I predicted that many of these proteins may be involved in oxidative stress response, transporting selenite into and out of the cell and synthesizing cysteine, an amino acid responsible for maintaining an intracellular reducing environment under oxidative stress conditions (Carmel-Harel and Storz, 2000). In addition, GroEL, GroES, Superoxide dismutase (SOD) and other heat shock proteins which are normally produced in response to cellular stress, may also be produced in the absence of cysteine. By understanding the role of L-cysteine and proteins related to the process of selenite resistance, more would be understood about the conditions needed for bioremediation or selenite resistance by this organism.

Furthermore, studying ways in which bacteria maintain homeostasis in environments polluted with high concentrations of metals may lead to an understanding of how humans metabolize these metals and why high concentrations of these metals are toxic.

Specific Objectives:

Proteomics was used to identify the *E. cloacae* strain proteins involved in selenite-resistance/homeostasis by the following strategies:

1.) Establishment of growth curves for the *E. cloacae* in the presence and absence of selenite and L-cysteine to demonstrate selenite sensitivity and resistance. This was done by measuring the turbidity of growing cells at different time points.

2.) Creation of protein profiles of whole protein extracts from *E. cloacae* under selenite sensitive and resistant conditions to show differential protein expression. IPG strips used in this regard were of pH ranges 5-8.

3.) Excision of protein spots with increased or decreased expression in the presence and absence of selenite and cysteine and determination of their identities by mass spectrometry analysis.

CHAPTER II

Materials and Methods

Experimental Design

Initially, *Enterobacter cloacae* strain cells were cultured in M-9 media in the presence and absence of L-cysteine. Cells were grown for one and a half hours to early log phase and introduced to selenite or a water control. The cells were harvested one hour after adding selenite and lysed for protein extraction. Protein quantification was done using a Bradford Assay. Then two-dimensional gel electrophoresis (2DGE) was used to analyze proteins from each condition, in which proteins were separated by isoelectric point and molecular weight. Bio-Rad's PD Quest 2-D Image software was used to analyze differential protein expression whereby unique and over-expressed proteins which most probably play a role in selenite uptake and resistance were identified and excised from each gel. Identification of amino acid sequences of these peptides was done by Mass Spectrometry at The Ohio State University.

Bacterial Strain and Growth Medium

The *E. cloacae* strain is resistant to salts of mercury, cadmium, selenium, copper, zinc and silver. It is also resistant to ampicillin and streptomycin but sensitive to tetracycline, kanamycin and chloramphenicol. The M-9 minimal (Becton, Dickson and Co., Sparks MD, USA) medium contained 50 mM anhydrous disodium phosphate, 22mM monopotassium phosphate, 9 mM sodium chloride , 19 mM ammonium chloride , 1 mM $MgS0_4$ (Fisher Scientific, Fair Lawn, NJ.), 0.2% glucose (Amresco Inc. Solon,

OH) and 0.00005% thiamine (Fisher Scientific, NJ). When required, the medium was supplemented with 0.04 mg/ml L-cysteine (Fisher Scientific, NJ) and 40 mM selenite (MP Biomedicals, LLc, Solon, OH).

Growth Curve of the E. cloacae strain

Two 5 ml cultures, one grown in the presence of cysteine and the other grown in the absence of cysteine, were incubated at 37° C with shaking overnight and were then transferred to 50 ml of the corresponding media in sterile 250ml flasks and further incubated at 37° C with shaking overnight. These starter cultures were then transferred to the flasks containing 950 ml of M-9 media with and without cysteine, thereby creating two 1:20 dilutions. In addition, cultures were further divided into four flasks each having 500 ml of M-9 medium (two with L-cysteine and two without L-cysteine) and incubated at 37° C. Growth was followed by measuring turbidity using a Klett colorimeter every 0.5 hour.

After 1.5 hours of growth, 40 mM final concentration of sodium selenite (Na_2SeO_3) (MP Biomedicals, Aurora, OH) was added to two flasks, one containing and the second one lacking L-cysteine. Equal volumes of water were added to the controls lacking selenite. In total, there were four cultures with different conditions namely: No cysteine, No selenite (NCNS), No cysteine, selenite (NCS), Cysteine, no selenite (CNS) and Cysteine, selenite (CS). One hour after selenite exposure (NCS and CS), 300ml of cells from each culture were harvested by centrifugation at 5,000x g for 15 minutes. The cells were washed with 1X M-9 salts twice to remove excess medium and selenite. The supernatant was removed and the cell pellets were immediately frozen at -80°C for future protein analysis by two-dimensional gel electrophoresis (2DGE).

Lysing of Cells and Protein Extraction

The cells were resuspended in 1 ml of lysing buffer containing 8 M urea, 2 M thiourea, 2% CHAPS, 2% SB 3-10, 40 mM Tris, and 0.2% ampholytes pH 3-10 (All from Amresco, Salon, OH). 10 µl/ml TBP (Bio Rad, Hercules, CA) and 10 µl/ml Halt Protease Inhibitor Cocktail (Pierce, Rockford, IL). 2ml conical tubes containing 1ml of 0.1mm glass beads (Biospec Products Inc, Bartlesville, OK) were filled with the cells. Lysing buffer was added to fill any remaining spaces. Cells were lysed by homogenization using a Mini Bead Beater 8 (Biospec, Bartlesville, OK) for a total of five minutes, in one minute intervals. Samples were placed on ice for at least 30 seconds between each treatment. RNase (Amresco Solon, OH) and DNAse (Pierce, Rockford, IL) were added to concentrations of 0.2µg/ml and 0.2 U/ml, respectively. After 10 minutes when the lysates lost viscosity, they were centrifuged for 10 minutes at 16,000 x g, and transferred to 1.5 ml microcentrifuge tubes. If protein samples were not used immediately, they were frozen at –80°C until further analysis.

The Bio Rad's 2D clean-up kit (Bio Rad, Hercules, CA) was used to remove excess RNA, DNA, polysaccharides, lipids and other unwanted materials from the whole protein extracts. This procedure was performed on three 150 µl portions of cell lysate and combined later. Each 150 µl portion of cell lysate was mixed with 300 µl of precipitating agent 1, vortexed and incubated on ice for 15 minutes. Samples were centrifuged at 16000 x g for 5 minutes forming a tight pellet. The supernatant was discarded and pellets were centrifuged again at 16,000 x g for 30 seconds after which the pellets were washed

in 40 µl of wash reagent 1 and centrifuged at 16,000 x g for 5 minutes. The supernatant was discarded followed by addition of 25 µl of proteomic grade water. This mixture was vortexed for 20 seconds and 1 ml of pre-chilled wash reagent 2 and 5 µl of wash additive 2 were added to each sample. The samples were then vortexed for 1 minute and incubated at 20^0C for 30 minutes during which period samples were vortexed for 30 seconds after every 10 minutes. Samples were then centrifuged at 16,000 X g for 5 minutes to form a tight pellet and the supernatant was discarded followed by a brief 30 second centrifugation. Pellets were then air-dried at room temperature for 5 minutes and resuspended in a total of 300µl of Rehydration Buffer containing 8M urea, 1% CHAPS, 15 mM dithiothreitol (DTT), trace bromophenol blue (all from Amresco, Salon, OH); and 0.2% Bio-Lyte 3/10 Ampholyte (Bio Rad, Hercules, CA).

Modified Bradford Assay

Determination of the concentration of protein extracted from cells of the *E. cloacae* strain was done using the modified Bradford Assay described in Bio Rad's ReadyPrep™ Sequential Extraction Kit Instruction Manual (Bradford, M.M. 1976; Ramagli and Rodriguez, 1985). Standards were prepared using bovine serum albumin (BSA) in amounts of 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 µg /µl dissolved in rehydration buffer containing 8 M urea, 1% CHAPS, 15 mM DTT, 0.2% ampholytes pH 3-10 and trace Bromophenol Blue. The assays were done by mixing 80 µl of 0.12 N HCl with 20 µl of protein sample or standard accordingly, followed by 3.5 ml of Bradford Dye.

Absorbances were read using an EL 311 microplate reader (Bio-Tek instruments Inc, Winooski, VT) at 595 nm. The mean absorbance value for the blank was subtracted from the mean value of each standard or sample. The absorbances vs standard protein

amounts were plotted to generate a linear graph that was used to interpolate the protein concentrations in the lysates.

Two-Dimensional Gel Electrophoresis (2DGE)

Isoelectric focusing (IEF) was carried out using a Bio Rad Protean IEF cell (Bio Rad, Hercules, CA) (O'Farrell 1974). The protein (150 µg) was loaded onto an 11cm IPG Ready strip (Bio-Rad Hercules, CA) with a fixed pH range of 5-8. Protein samples were mixed with Rehydration Buffer containing 8 M urea, 1% CHAPS, 15 mM DTT, 0.2% ampholytes pH 3-10 and trace Bromophenol Blue, to increase the sample volume to 200µl. An appropriate amount of sample was placed in a lane of the focusing tray between the electrodes. The IPG strip was placed over the sample with the gel side down. The IPG strip was then overlaid with mineral oil (Bio Rad, Hercules, CA) and placed in the IEF cell. Active rehydration was programmed at 50V and 20°C for 12 hours.

After completion of active rehydration, isoelectric focusing began immediately. The voltage started at 0 V and ended at 8,000 V for a total of 40,000 volt hours with a temperature setting of 20°C. The strips were held at 500 V after isoelectric focusing until used for the 2nd dimension.

The IPG Ready Strips were then removed and prepared for the 2nd dimension by SDS-PAGE. The strips were blotted on filter paper to remove mineral oil. Lanes in an equilibration tray were filled with Equilibration Buffer I containing 6M urea, 2% SDS, 0.375 Tris-HCl pH 8.8, 20% glycerol and 130mM DTT. The IPG strips were placed gel side facing up into the lanes containing Equilibration Buffer I. The IPG strips were completely submerged in Equilibration Buffer I and shaken on low power for 10 minutes. The empty lanes in the equilibration tray were filled with Equilibration Buffer II

containing 6 M urea, 2% SDS, 0.375M Tris-HCl pH 8.8, 20% glycerol, and 135 mM iodoacteamide. The IPG strips were placed in these lanes and shaken for 10 minutes.

The IPG strips were then removed from the equilibration tray and briefly submerged in 1 X Tris Glycine SDS (TGS) buffer containing 0.192 M Glycine, 0.025M Tris base, and 0.1% SDS (Amresco Solon, OH). The strips were then placed into the spacer plate, making contact with the 10.5%-14% Criterion Precast gel (Bio Rad Hercules, CA).

The gel side of the IPG strip faced forward and the positive end was on the left side of the precast gel. Overlay agarose containing 0.5% agarose and trace bromophenol blue in 1 XTGS , was melted and placed on top of the IPG strip while ensuring that there were no air bubbles as the overlay agarose solidified. A gel box was assembled and filled with 1 x TGS buffer and the proteins were separated at 150 V until bromphenol blue reached the bottom of the gel.

SYPRO® Ruby Protein stain (Berggrein et al., 2001) was used to stain gels. At first, gels were submerged in fixing solution containing 35% methanol (AAPER alcohol & chemical Shelbyville, KY), 10% Glacial acetic acid (Fisher Scientific, Fair Lawn, NJ), and 35% H_2O for one hour. Gels were then removed from the fixing solution and immersed in SYPRO® Ruby stain (Bio Rad, Hercules, CA) with shaking on an orbital shaker overnight. The SYPRO® Ruby stain was then removed and the gels rinsed in water for 15 minutes after which they were imaged under UV light or stored in 10% acetic acid.

2DGE Analysis

Gels were analyzed for protein patterns as digital images using the Bio-Rad Gel Chemidoc™ XRS Gel Documentation System (Bio-Rad, Hercules, CA). The gels were visualized using an ultraviolet transillumination. The digital images were saved to a computer for analysis. Bio-Rad's PD Quest 2-D Image Analysis (Bio-Rad, Hercules, CA) was used to analyze the gels. Protein extracts were analyzed using 2DGE in triplicate samples, creating a matchset for each condition being observed.

Matchsets serve to demonstrate the accuracy of the three profiles. Matchsets were generated by designating one 2D gel as a master and comparing it with the other 2D gels in a specific matchset. Protein spots were matched through an automated detection and matching process. About six to eight landmarks were designated on each of the gels in a matchset to improve the alignment of the three gels for comparison. After matching spots as accurately as possible, match rates were calculated for each gel in comparison to the master gel.

In addition, matchsets for each condition were compared by creating higher-level matchsets, which served to compare the number of matched and unmatched spots in each matchset generated. Identification of landmarks was also done in each matchset and compared with the landmarks on other gels so as to improve alignment of the images. These matchsets were of critical use in locating unique and over-expressed proteins.

Protein Spot Excision and Identification

When 2DGE analysis was completed, protein spots of interest were located for excision. Therefore, a 70% increase in protein concentration was needed. Protein concentrations were increased from 150 μg to 255 μg in a volume of 200 μl for isoelectric focusing. Protein was loaded onto 11 cm IPG strips with a pH range of 5-8 (Bio-Rad, Hercules, CA) and isoelectric focusing was carried out, followed by SDS-PAGE. The same procedure was carried out as previously described.

The excised protein samples were digested with trypsin and analyzed by multidimensional liquid chromatography (LC/LC) followed by tandem mass spectrometry (Ms/Ms) (Aebersold and Mann, 2003). Liquid chromatography was used to separate the digested peptides and mass spectrometry to identify the amino acid sequence of the trypsin digested peptide fragments by separating them by size and charge (Aebersold and Mann, 2003). Over-expressed as well as under-expressed and unique proteins were identified and their roles in selenite uptake and resistance were predicted.

Chapter III

Results

Results

Growth curves were performed to define the selenite resistance phenotype in the presence of cysteine. In this regard, four overnight cultures were diluted 1/50 in M-9 medium with two cultures containing cysteine and the other two lacking it. Optical density was measured every 30 minutes as the cultures grew at 37 ^{0}C (Table 3.1). After 1.5 hrs of growth during early log phase, selenite was added to a final concentration of 40mM selenite to two flasks, one containing cysteine and one lacking it. Equal volumes of water were added to the other two flasks as a control. Thus the cells were grown under four conditions; NCNS, NCS, CNS and CS. All the cells growing under the NCNS, CNS and CS conditions demonstrated an increase in turbidity (Fig3.1). After 8 hrs, the average turbidity of the NCNS, CNS and CS cultures were 182, 215 and 348 Klett Units respectively where as the turbidity of the NCS culture was 49. Thus, cysteine appears to be required for cell growth in the presence of selenite.

One hour after adding selenite, cells were harvested from each culture for 2DGE analysis and lysed using a mini-bead beater. After treating the protein extract using a Bio Rad's 2D Clean Up Kit, protein concentrations were determined using a modified Bradford assay.

Growth Curve of *E. cloacae* strain

Table 3.1: Average Klett Unit Measurements of the *E. cloacae* strain growth.

TIME	NCNS	NCS	CNS	CS
0	13	13	15	15
0.5	15	14	19	19
1.0	17	17	24	25
1.5	24	24	35	35
2.0	28	28	39	40
2.5	34	35	50	58
3.0	40	39	65	70
3.5	50	42	72	84
4.0	55	45	80	95
4.5	62	48	96	115
5.0	78	48	120	132
5.5	89	49	145	165
6.0	95	49	156	195
6.5	115	49	168	237
7.0	128	49	180	286
7.5	159	49	196	320
8.0	182	49	215	348

Key:

NCNS= no cysteine, no selenite; **NCS**= no cysteine, selenite

CNS= cysteine, no selenite; **CS**= cysteine, selenite

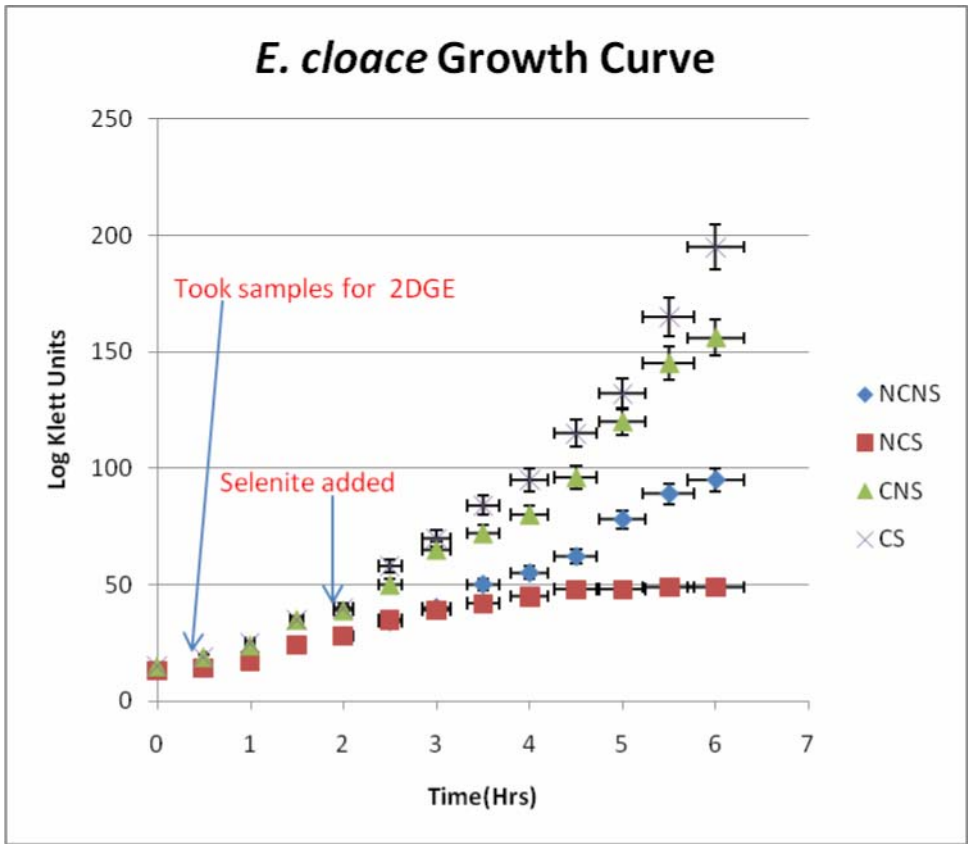


Figure 3.1: Growth Curve of the *E.cloacae* strain in M9 minimal media. Samples were grown in the presence and absence of cysteine and selenite. Cells exposed to both cysteine and selenite show exponential growth. However, cells lacking cysteine failed to grow after addition of selenite.

NCNS = No Cysteine, No Selenite

NCS = No Cysteine, Selenite

CNS = Cysteine, No Selenite

CS = Cysteine, Selenite

Bradford Assay is a colorimetric assay for measuring total protein concentration in a given solution. It involves the binding of the dye Coomassie Brilliant Blue to protein in an acidic solution, and its concomitant absorbance shift from 465 nm to 595 nm. Raw data from the modified Bradford assay are shown in Table 3.2 and Figure 3.2. Different concentrations of bovine serum albumin (BSA) were mixed with Bradford reagent, and the absorbances of the mixtures were measured at 595 nm. A plot of the absorbance vs BSA concentration resulted in a linear curve with an R^2 value of 0.9876 (Figure 3.2). Extrapolation from this curve showed that the total protein in the four samples ranged from 1.74µg/µl to 3.71 µg/µl. The equation Y= 0.182X +0.0403 was used for substituting the average absorbance for Y and solving for X to determine the proteins concentrations. The NCNS total protein concentration was 1.7µg/ml, the NCS total protein concentration was 2.97µg/ml, the CNS total protein concentration was 3.71µg/ml and the CS total protein concentration was 2.57µg/ml

150µl of each protein sample were separated by two dimensional gel electrophoresis (2DGE) using an 11 cm, pH 5-8 IPG strip for isoelectric focusing and a 10.5-14% polyacrylamide gradient gel for SDS PAGE. The gel images were saved and three trials were performed in order to give a more accurate representation of the protein spot distribution. Figure 3.3 shows raw 2D images of protein from the four conditions. By comparing the size and intensity of similar spots in each gel, differences in protein expression were detected. For instance, if a spot in the CS gel appeared larger and brighter than it is in the CNS or NCNS gels, then the expression of that polypeptide was thought to be over- expressed or up-regulated in the presence of selenite and may play a

role in selenite resistance. If the opposite happens then that spot is considered under-expressed or down- regulated as a result of selenite addition.

In addition, if a spot in NCS condition is smaller than that observed in the CS condition, then cysteine is considered to have played a role in selenite resistance (CS) thus making the spot be highly expressed under selenite resistant conditions.

As a way of increasing the accuracy of comparing spots, overlapping transparencies and inverted images of the raw 2D images were used in addition to primary and higher level matchsets. In this way, spots were identified as being unique, downregulated or upregulated hence differential expression (Figs. 3.4). These spots were selected for excision.

PD Quest 2-D Image Analysis Software (Bio-Rad, Hercules, CA) was used to create matchsets (Fig.3.5) from the triplicate gels. The matchsets demonstrated accuracy of the three protein profiles for all the four conditions and are normally used to identify protein spots with different intensities. In each set of three gels for each condition, the gel that had many spots and the least streaking was selected as the master. The master was used to compare the number of matched and unmatched spots in each profile and this gave matchrates (Table 3.3).

Table 3.2: Modified Bradford Assay

The Modified Bradford assay was performed to determine the protein concentration in order to make gels for 2DGE analysis.

Table 3.2: **Absorbances from Modified Bradford Assay**

BSAμg/μl	Trial 1.	Trial 2.	Trial 3.	Average
B	0.374	0.392	0.381	0.382
0.5	0.493	0.468	0.487	0.100
1.0	0.672	0.639	0.614	0.259
1.5	0.695	0.688	0.720	0.319
2.0	0.723	0.835	0.782	0.398
2.5	0.814	0.910	0.867	0.481
3.0	0.843	0.883	0.887	0.489
3.5	1.003	1.032	0.917	0.602
4.0	0.942	1.170	1.038	0.668

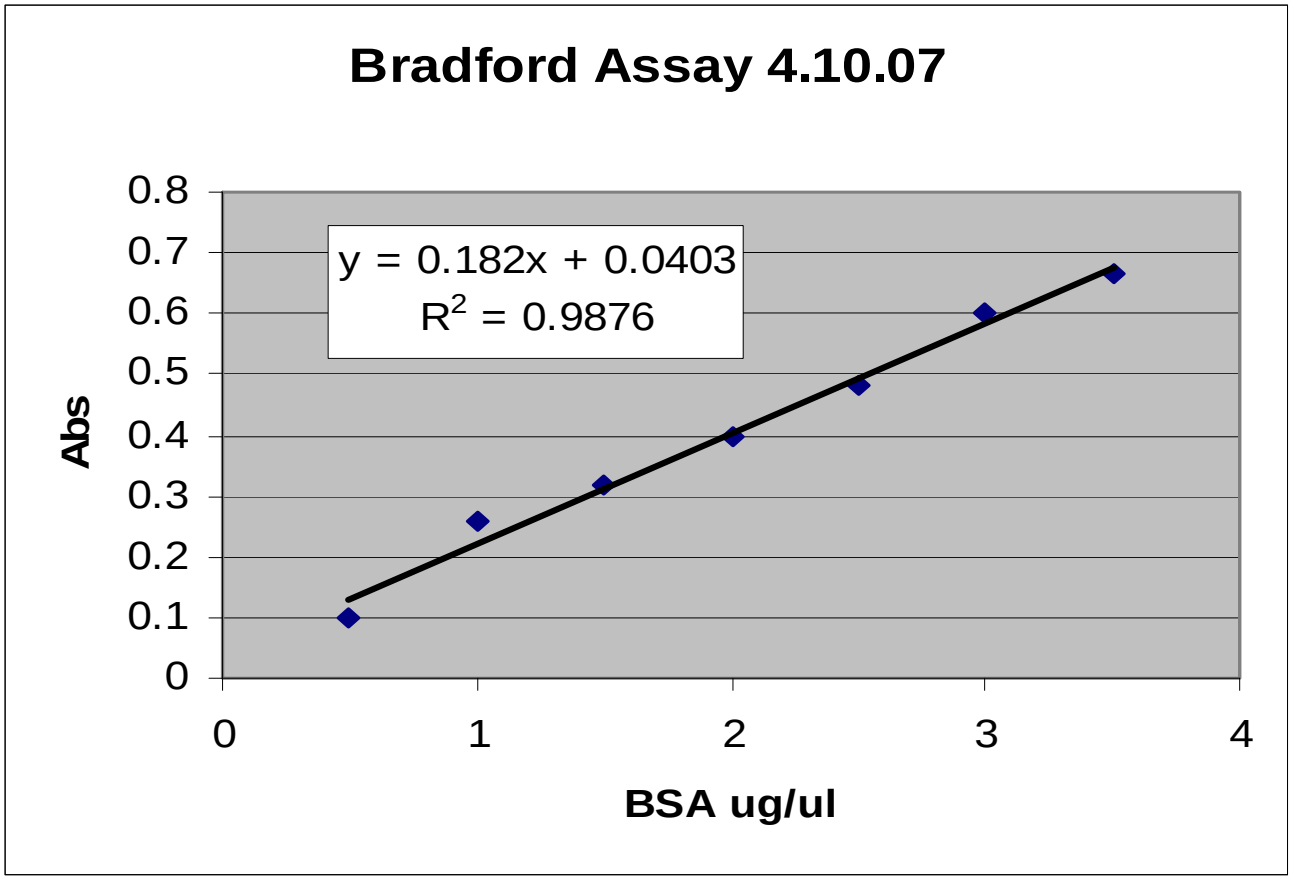


Figure 3.2: Standard curve used to determine the protein concentration.

Raw 2-D Gels

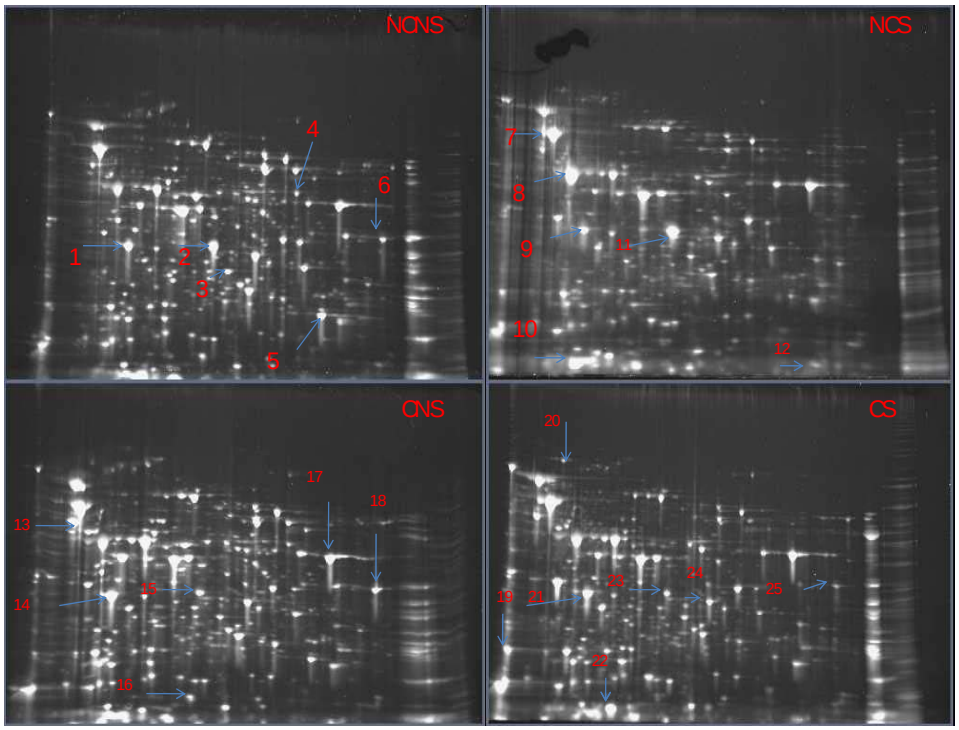


Fig.3.3: A set of raw 2D images of the four conditions using protein extracted from the *E.cloacae* strain NCNS, CNS, NCS & CS at 3.5 hrs, 37 ^{0}C. pH gradient 5-8, 11 cm strips. Arrows show differentially expressed proteins.

Matchsets

The created matchsets are composite images of 2D gels generated to show accuracy of the proteomes. Higher level matchsets serve to compare different conditions of the primary matchsets. They were compared using each gel as a master for comparison. The four conditions were: NCNS, NCS, CNS, and CS

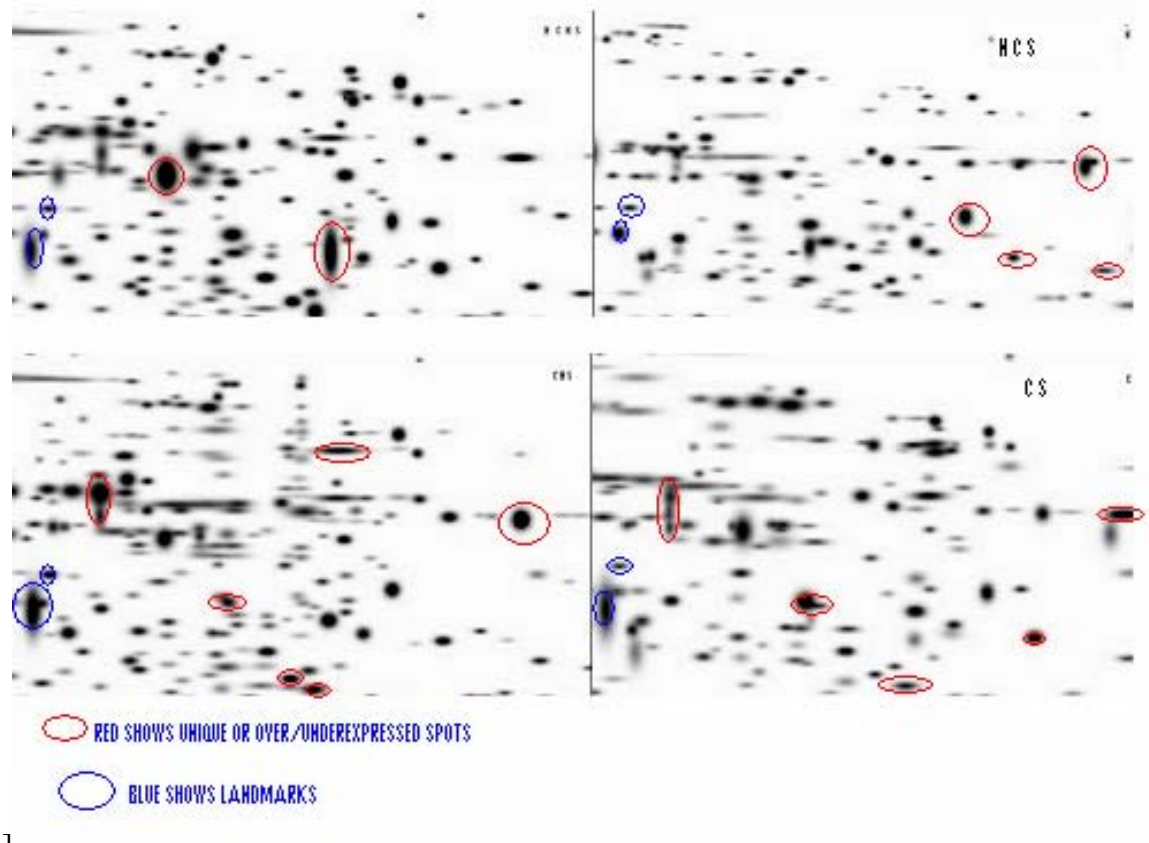
Table 3.3 Matchsets

Primary	**Higher Level matchsets**
NCNS 3.5 hrs at 37 ^{0}C	NCNS and NCS at 3.5hrs 37 ^{0}C
NCS 3.5 hrs at 37 ^{0}C	NCNS and CNS at 3.5 hrs 37 ^{0}C
CNS 3.5 hrs at 37 ^{0}C	CNS and CS at 3.5 hrs 37 ^{0}C
CS 3.5 hrs at 37 ^{0}C	NCS and CS at 3.5 hrs 37 ^{0}C

NCNS = no cysteine, no selenite, **NCS** = no cysteine, selenite, **CNS =** cysteine, no Selenite, **CS** =cysteine, selenite

Matchrates were determined by comparing the remaining two gels in a matchset to the master. Through this, numbers of matched and unmatched spots in each profile were identified. When the master gel was compared to itself, the match rate was 100%. When the master was compared to the other two gels in a matchset, the match rates were always lower. For instance, the NCNS master gel demonstrated a match rate of 100% when compared to it, but only a 70% and 72% match rate when it was compared to the other two NCNS gels. The 100% match rate for the master gels shows that the program is recognizing each spot successfully. The NCS master gel demonstrated a higher similarity to other NCS gels with match rates of 88% and 80%. The CNS had a much lower rate of

67% and 69% when compared to other CNS gels in a matchset. CS gels had rates of 78% and 76%. (Table 3.3).



]

Figure 3.4: Matchsets

The images in Figure 3.4 demonstrate a comparison of matchsets from three 2D gels of protein extract from all four conditions. Circled spots show differentially expressed proteins which were excised and identified.

The matchsets created for each set of 3 gels were called lower level matchsets. Higher level matchsets were created by comparing protein profiles of the lower level matchsets. Composite protein profiles for each lower level matchset were constructed and

compared to other composite protein profiles. For example, a composite protein profile for the NCNS gels was constructed and compared to a composite protein profile of the NCS gels (Figure 3.5 A and B). Comparisons of other composite gels, A and B, are shown in figures 3.6-3.8, and the matchrates for the higher level matchsets are shown in Table 3.5. NCNS and NCS have a matchrate of 72%, NCS and NCNS have a matchrate of 76%, CNS and CS is 63%, CS and CNS is 66%, NCNS and CNS is 68%, CNS and NCNS is 78%, NCS and CS is 74% and CS and NCS is 59%.

Matchrates from Primary Matchsets

Table 3.4: Matchrates from primary Matchsets

Condition	Gel	Spot Count	Matched Spots	Unmatched Spots	Match Rates
NCNS	Master	425	425	0	100%
	Gel 2	460	331	129	72%
	Gel 3	450	315	135	70%
NCS	Master	385	385	0	100%
	Gel 2	320	282	38	88%
	Gel 3	296	237	59	80%
CNS	Master	348	348	0	100%
	Gel 2	383	257	126	67%
	Gel 3	390	269	121	69%
CS	Master	402	402	0	100%
	Gel 2	482	376	106	78%
	Gel 3	475	361	114	76%

NCNS = no cysteine, no selenite; NCS = no cysteine, selenite; CNS = cysteine, no Selenite; CS = Cysteine, selenite

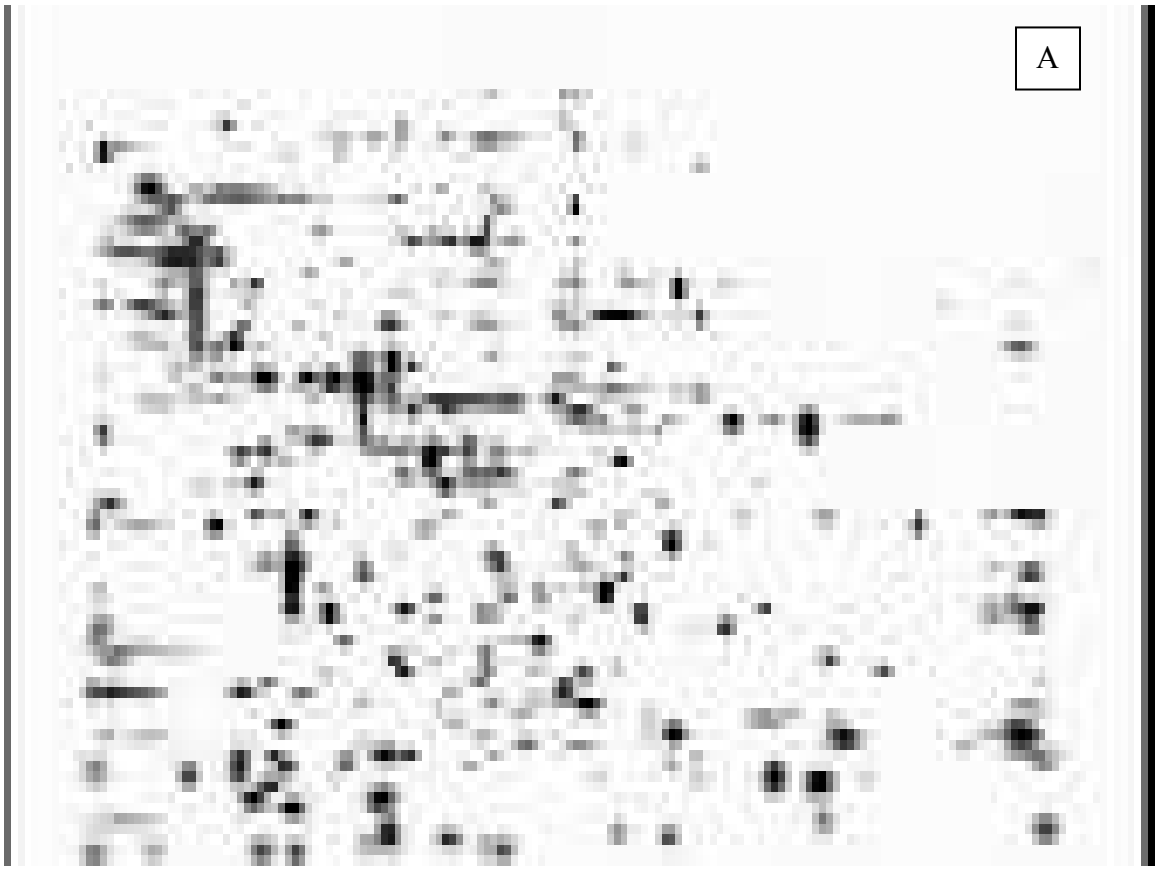
Figure 3.5: Higher Level Matchsets for NCNS and NCS

These figures show a comparison of NCNS and NCS HL Matchsets. They demonstrate differences in protein expression in the absence of selenite (NCNS) and presence of selenite (NCS) when cysteine is absent. Part A compares the NCNS Matchset to the NCS Matchset. Part B compares the NCS Matchset to NCNS Matchset. pH range is from 5-8 and the molecular weight ranges from 250kDa-10 kDa.


A


B

Figure 3.6: Higher Level Matchsets for CNS and CS
The figures show a comparison of CNS and CS HL matchsets. The figures demonstrate differences in protein expression in the absence selenite (CNS), when cysteine is present in both conditions. Part A compares CNS Matchset to CS Matchset. Part B compares the CS Matchset to the CNS Matchset.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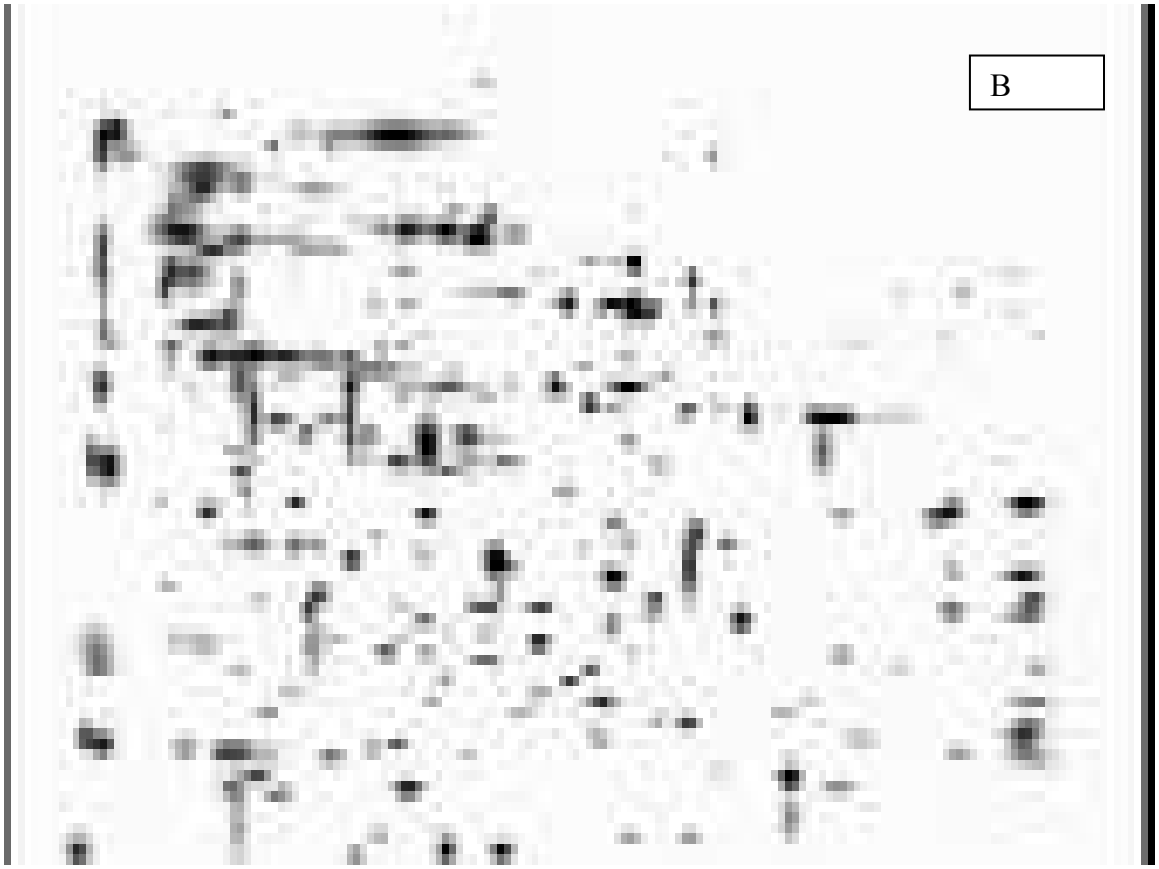

B

Figure 3.7: Higher Level Matchsets for NCS and CS
The figures show a comparison of NCS and CS HL matchsets. The images demonstrate differences in protein expression after the addition of selenite in the absence and presence of cysteine. Part A compares the NCS Machset to CS Matchset. Part B compares the CS Matchset to the CNS Matchset.


A

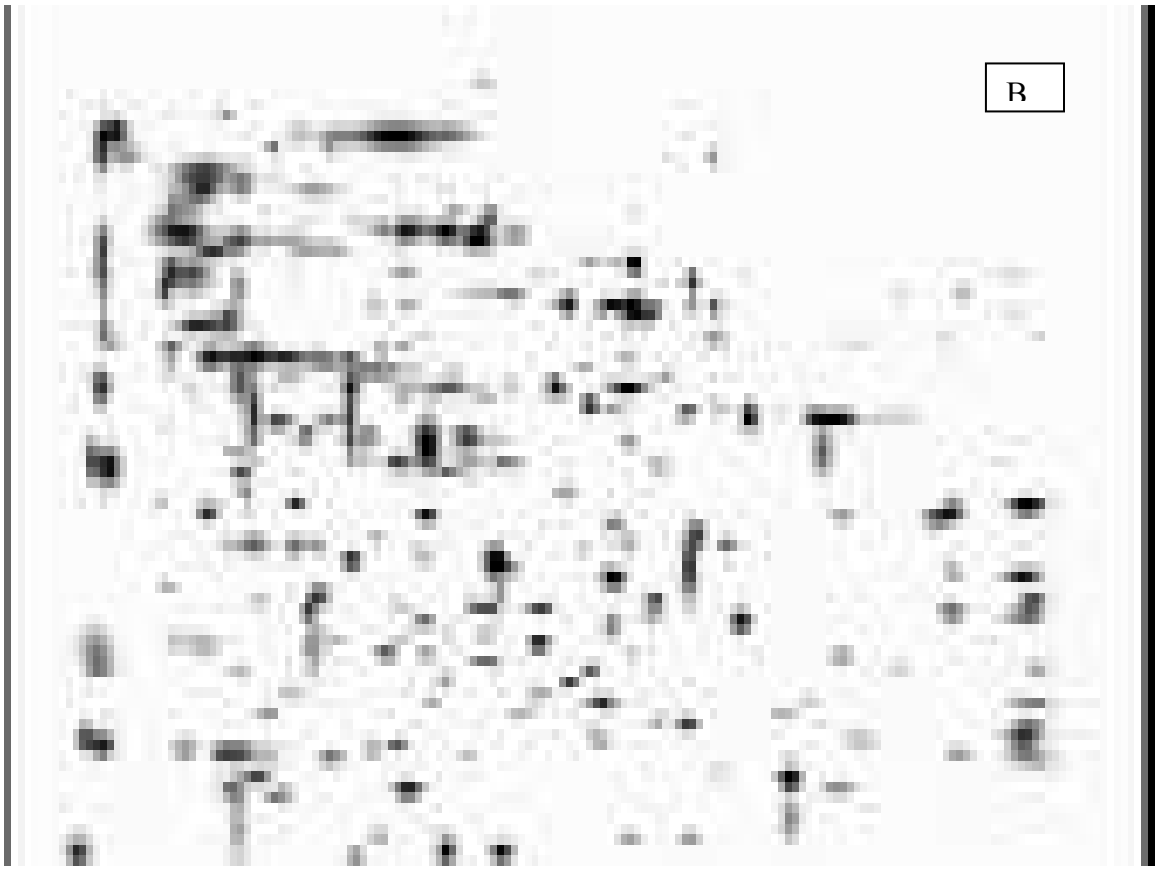
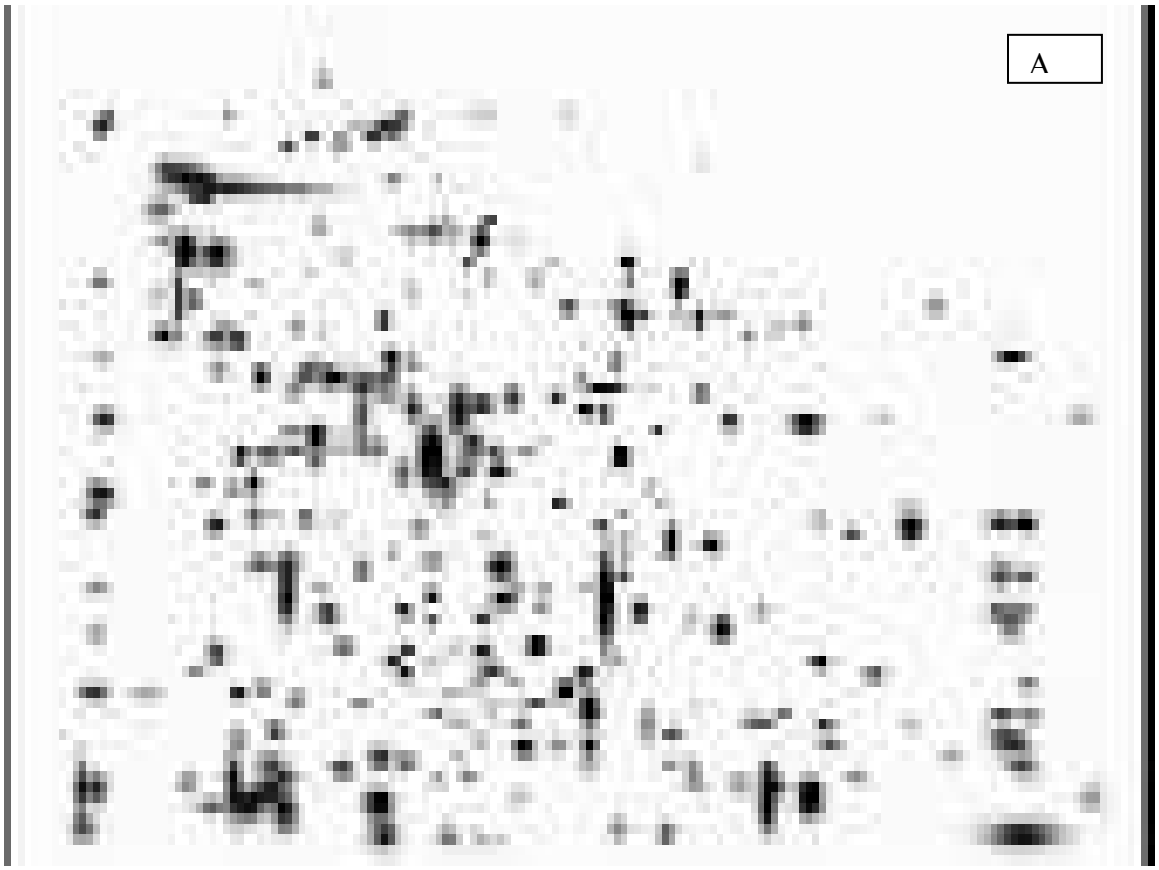

B

Figure 3.8: Higher level Matchsets for NCNS and CNS
The figures show differences in protein expression with and without cysteine, when selenite is absent. Part A compares the NCNS Matchset to the CNS Matchset. Part B compares the CNS Matchset to the NCNS Matchset.


A

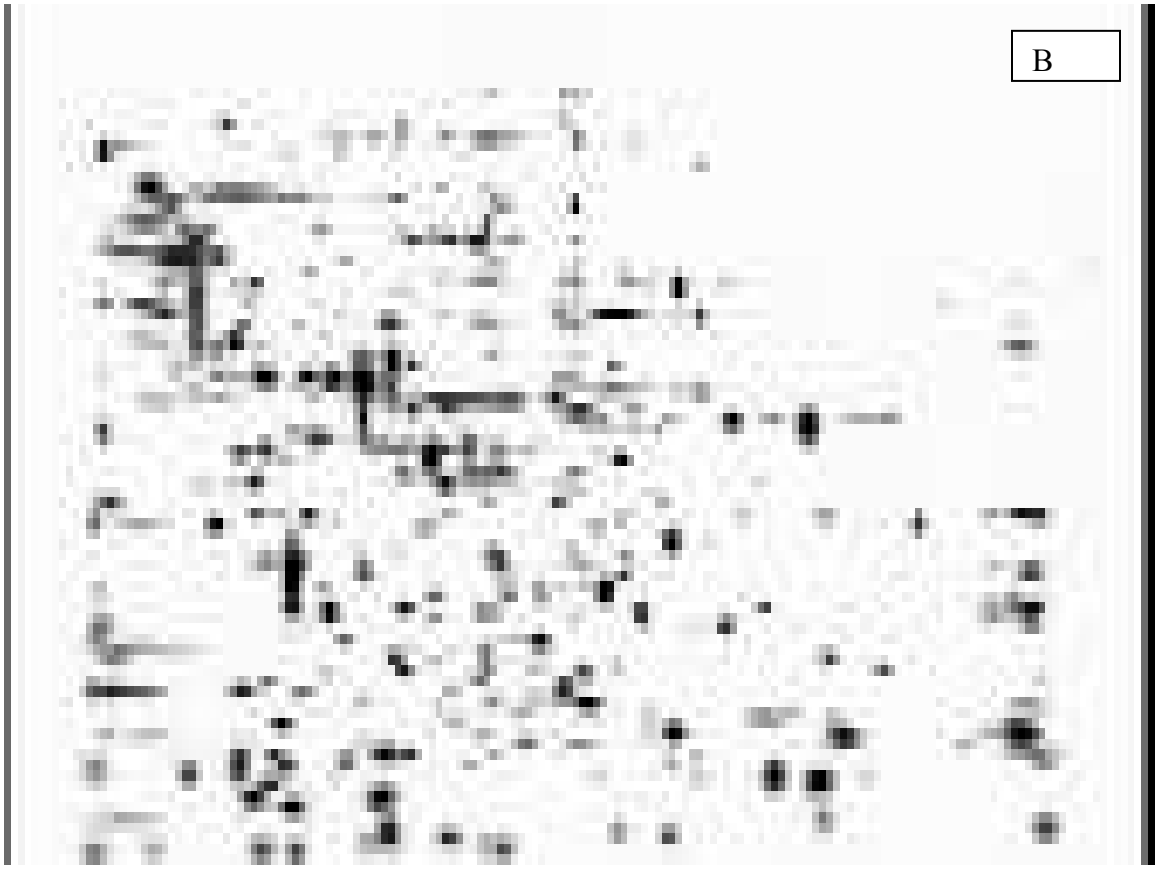

B

Table 3.5: Match Rates from Higher Level Matchsets

Matchset	Total Spots	Matched Spots	Unmatched Spots	Match Rate
NCNS and NCS	410	295	115	72%
NCS and NCNS	425	323	102	76%
CNS and CS	300	189	111	63%
CS and CNS	366	242	124	66%
NCNS and CNS	381	259	122	68%
CNS and NCNS	416	325	91	78%
NCS and CS	393	291	102	74%
CS and NCS	375	221	154	59%

NCNS = no cysteine, no selenite; **NCS** = no cysteine, selenite; **CNS** =cysteine, no selenite; **CS** = cysteine, selenite.

For each gel, two spots that appeared at the same position in each gel were identified as landmarks. In addition, there were spots that were unique, overexpressed or underexpressed in each gel. There were many spots that showed differences and similarities when the gels were compared. Therefore, differences in protein spot intensities were identified by analyzing raw 2D images (overlapping transparencies of the raw 2D gels were also used to confirm accuracy of all three trials), primary matchsets and higher level matchsets. After analyzing all the gels, spots that were overexpressed, underexpressed and unique in a set of gels for a specific condition were excised and sent to The Ohio State University Proteomics Facility for digestion with trypsin and analysis by mass spectrometry. Amino acid residue sequences of short sequences generated by the trypsin digests were identified and compared with those in the MASCOT database which matched them to known proteins. Landmarks demonstrated the accuracy of the method and were identical. Isoelectric points and molecular weights of each spot were estimated before they were analyzed by mass spectrometry.

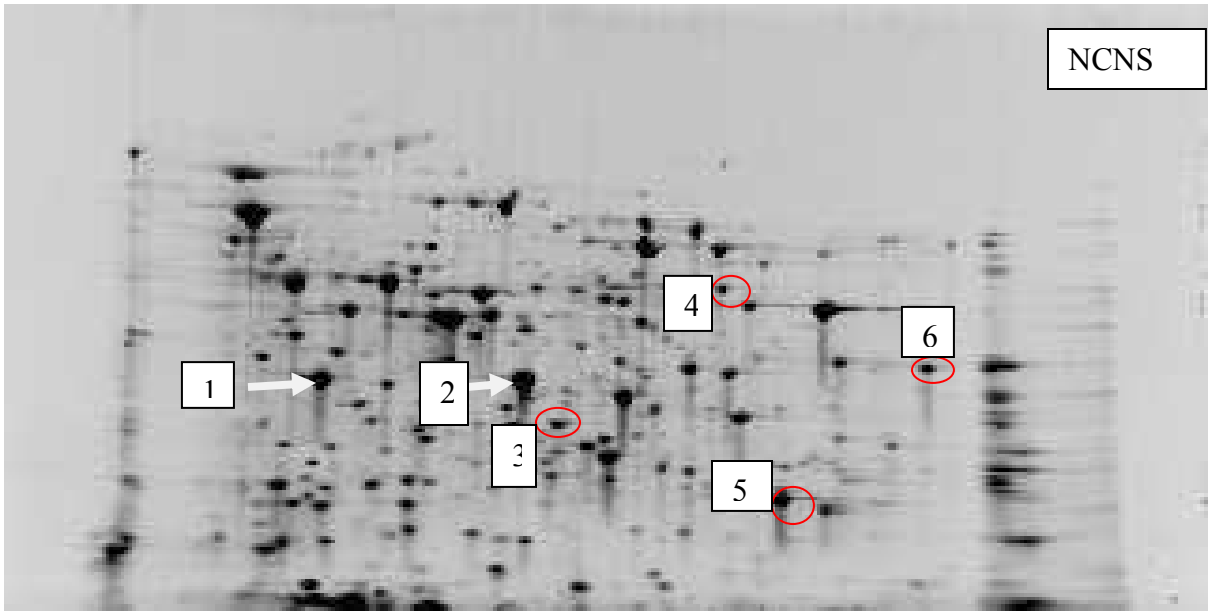
Twenty five spots were excised from all the four gels and sent for mass spectrometry analysis (Figure 3.10). The samples showed significant matches in the MASCOT database. Landmark 1 (Spots 1, 9, 14, and 21) was shown to be similar to *Enterobacter cloacae* protein translation elongation factor EF-Ts. Sequence coverage was also similar and ranged from 36% to 56% (Figure 3.11). Landmark 2 (Spots 2, 11, 15 and 23) was shown to be similar to *Enterobacter sp.* NLPA Lipoprotein.

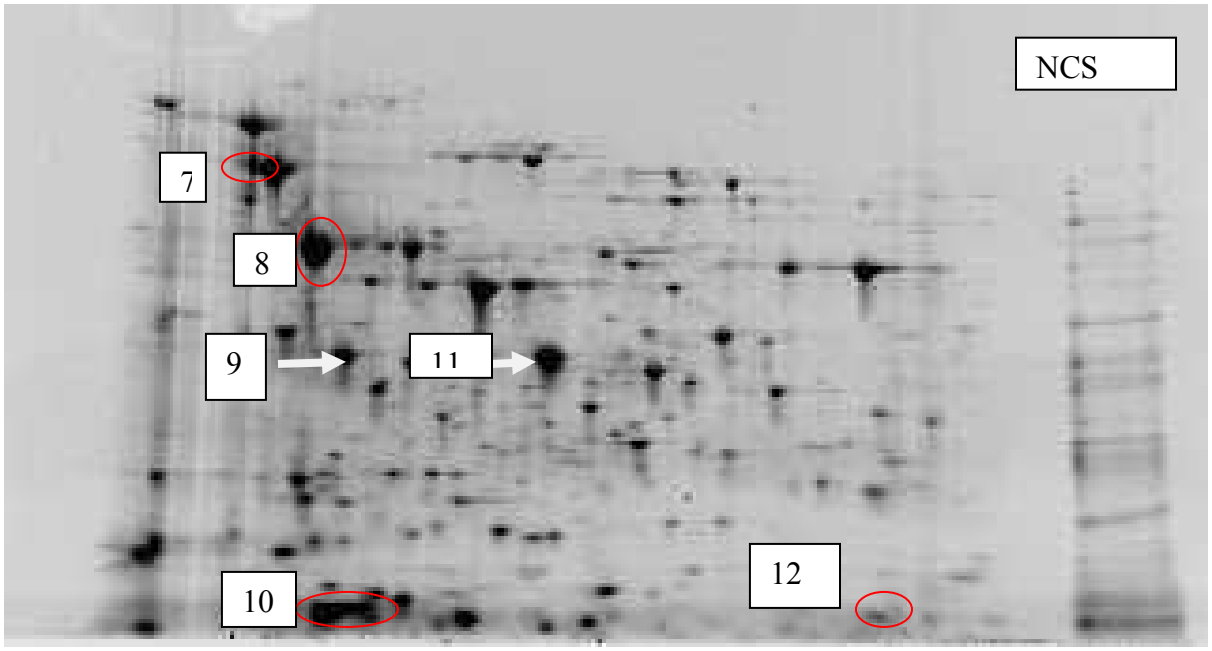
In the no cysteine, no selenite condition (NCNS), spots 3 showed similarity to Klebsiella *pneumonia's* periplasmic putrescine permease protein with sequence coverage of 16% . Spot number 4 showed similarity to *Enterobacter sp*. glycine

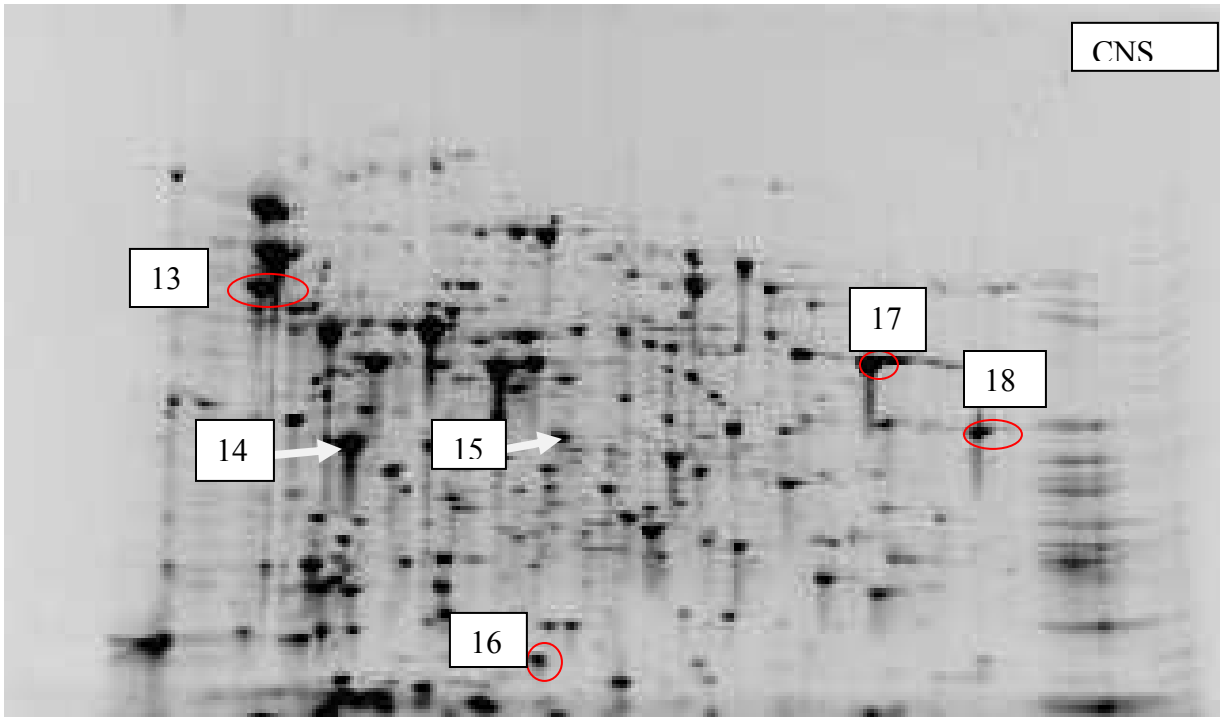
hydroxymethyltransferase with sequence coverage of 10%. Spot 5 had polypeptides that matched 29% of *Enterobacter sp.* superoxide dismutase. Unique spot 6 was matched a *Salmonella enterica sp*. protein named Ionositol-5-monophosphate dehydrogenase and had sequence coverage of 16%. Under the NCS condition which was the selenite sensitive condition, unique protein spot 7 contained polypeptides that matched 53% of *E. coli* GroEL protein. Spot 8 did not match any known protein. Spot 10 was overexpressed and contained polypeptides that showed similarity to heat shock protein- hsp 20 from *Enterobacter sp*. 638. Spot 12 showed similarity to *Enterobacter sp*. ribosomal protein with sequence coverage of 55%

Under the CNS condition unique spot 13 which showed similarity to *E coli*'s ATP synthase sub-unit B and demonstrated sequence coverage of 53%. Spot 16 was unique and showed 16% similarity to a Fur protein from *E. cloacae*. Spot 17 was unique and had 3% similarity to OTcase from *E. coli*. Spot 18 was same as spot number 5, a superoxide dismutase and spot 19 was similar to a surface antigen (D15) from *Enterobacter sp*.with sequence coverage of 5%. Under the CS condition spot 20 showed 11% similarity to TolC protein from *E. cloacae*. Spot 22 showed 21% similarity to outer membrane protein x (OmpX) from *Salmonella enterica* sp. Spot 24 was a putative actin. Overexpressed spot 25 showed 24% similarity to glyceraldehydes 3-phosphate-dehydrogenase from *Enterobacter sakazaki.*

Figure 3.9: Excised protein Spots from 2D gels
The following figure shows landmarks, unique and overexpressed protein spots which were excised from NCNS, NCS, CNS and CS 2D gels. Landmarks are shown by arrows while other proteins of interest are shown by red circles.


NCNS
1
2
3
4
5
6


NCS
7
8
9
11
10
12


CNS
13
17
18
14
15
16

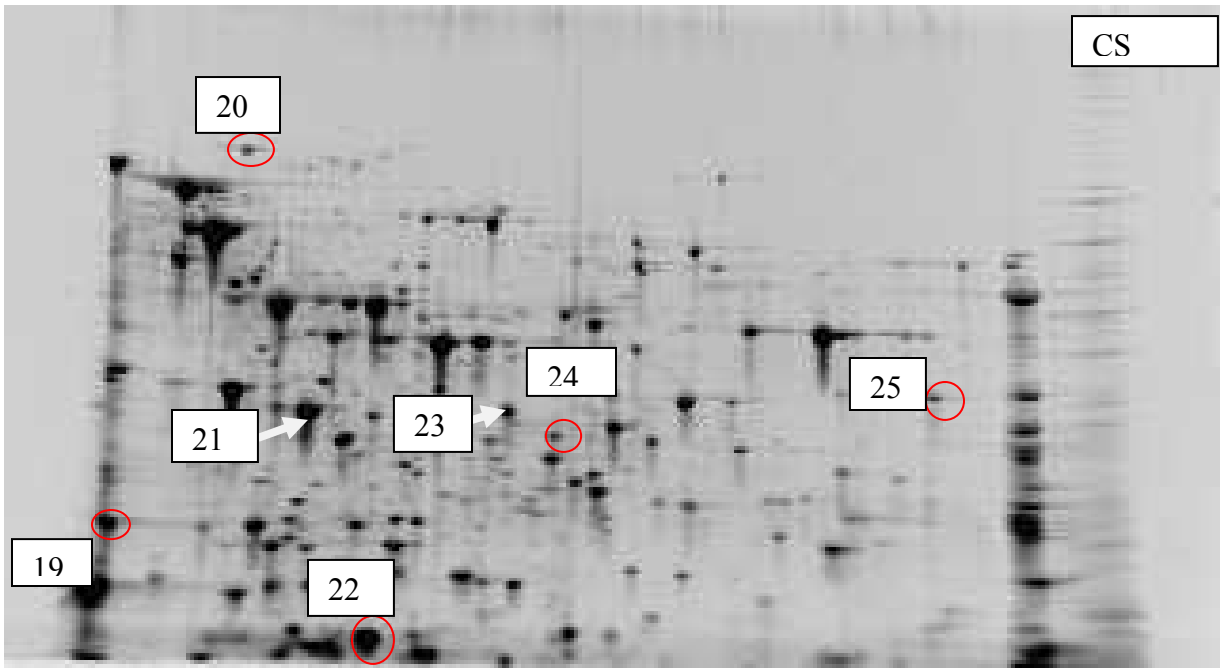

CS
20
24
25
21
23
19
22

Table 3.6: Excised Protein Spots
Identification of these spots was based on the highest similarity to the proteins in the MASCOT database.

Spot #	Protein Name	NCBI Accession	pI(observed)	Species	Mascot Score	SC (%)	Expression
1	Translation elongation factor Ts	gi|146310372	5.12	*Enterobacter cloacae*	595	37%	NCNS Landmark 1
2	NLPA Lipoprotein	gi|146311429	5.68	*Enterobacter sp.*	333	20%	NCNS Landmark 2
3	Periplasmic putrescine	gi|152969442	6.4	*Klebsiella pneumonia*	179	16%	NCNS Overexpressed
4	Glycine hydroxymethyltransferase	gi|146312677	5.98	*Enterobacter sp.*	171	10%	NCNS Overexpressed
5	Superoxide dismutase	gi|146313695	6	*Enterobacter sp.*	218	29%	NCNS -overexpressed
6	Ionositol-5-monophosphate dehydrogenase	gi|16761428	6.18	*Salmonella enterica sp.*	281	16%	NCNS-overexpressed
7	GroEL protein	gi|16225120	4.81	*E.coli*	1306	53%	NCS Unique
8	Unnamed protein product	gi|12843046	8.22	*Mus musculus*	69	4%	NCS Overexpressed
9	Translation elongation factor Ts	gi|16759207	5.12	*Salmonella enteric sp.*	501	36%	NCS Landmark1
10	Heat shock protein HSP 20	gi|146309681	5.6	Enterobacter sp.	110	13%	NCS Overexpressed
11	NLPA Lipoprotein	gi|146311429	5.68	*Enterobacter sp.*	127	7%	NCS Landmark2
12	Ribosomal protein	gi|146310039	5.7	Enterobacter sp.	398	55%	NCS Overexpressed
13	ATP synthetase subunit B	gi|15804332	4.9	*E.coli*	968	53%	NCS Overexpressed
14	Translation elongation factor Ts	gi/146310372	5.12	*Enterobacter cloacae*	674	56%	CNS Landmarkk1
15	NLPA Lipoprotein	gi /146311429	5.68	*Enterobacter cloacae*	127	7%	CNS Landmark 21

16	Fur protein	gi|28172774	5.8	*Enterobacter cloacae*	144	16%	CNS overexpressed
17	OTcase	gi|40960	5.6	*E. coli*	83	3%	CNS Overexpressed
18	Superoxide dismutase	gi|14631369	6.0	*Enterobacter sp.*	188	16%	CNS Overexpressed
19	Surface antigen (D15)	gi|14631038	5.16	*Enterobacter sp.*	209	5%	CS Overexpressed
20	TOL C Protein	gi|11218285	5.2	*Enterobacter cloacae*	224	11%	CS unique
21	Translation elongation factor Ts	gi|16759207	5.13	*Salmonella enteric sp.*	743	44%	CS Landmark1
22	Outer membrane protein x	gi|16759751	5.7	*Salmonella enteric sp*	264	36%	CS Overexpressed
23	NLPA Lipoprotein	gi|14631142	5.68	*Enterobacter sp.*	240	16%	CS Landmark 2
24	Putative actin	gi|15297186	6.92	Klebsiella pneumoniae	90	9%	CS Overexpressed
25	Glyceraldehyde 3-phosphate dehydrogenase	gi|146552329	6.67	*Arabidopsis thaliana*	71	7%	CS Overexpressed

Fig. 3.10: Sequence Coverage of selected Proteins of Interest
The sequences on the subsequent two pages were found to be significant matches in the MASCOT database for the excised protein spots. Amino acids matching these known sequences are shown in red. The number of matched peptides was compared to the total number of peptides to determine the sequence coverage (SC %).

NCNS GEL-Spot 1- Landmark 1

Translation Elongation Factor-Ts; SC = 37%

```
   1 MAEITASLVK ELRERTGAGM MDCKKALVEA NGDIELAIEN MRKSGAIKAA
  51 KKAGNVAADG VILTKIDGTY GIILEVNCQT DFVAKDGGFQ AFANKVLDAA
 101 IAGKITDVDV LKAQFEEERV ALVAKIGENI NIRRVASIEG DVLGSYQHGA
 151 RIGVLVAAKG ADEELVKQLA MHIAASKPEF VKPEDVSAEV VEKEYQVQLD
 201 IAMQSGKPKE IAEKMVEGRM KKFTGEVSLT GQPFVMDPTK SVAQLLKEHN
 251 ADVTGFIRFE VGEGIEKVET DFAAEVAAMS KQS
```

NCNS GEL- Spot 2 Landmark 2

NLPA Protein; SC = 20%

```
   1 MKKTLTLIAA ATLSALSFAS WADTLTVGAS NTPHAEILEQ AKPILAKQGI
  51 DLEIKPFQDY ILPNTALAGR DIDANYFQHI PYLNSVLKDH AGDKDYDFVS
 101 AGAIHIEPIG IYSKKYKSLK DLPEGGKIIM RDAVSEEGRI LSIFEKEGVI
 151 KLKPGIEKVT ARISDIVENP KKLKFTPNVE AALLPQMYNN DEGDAVVINA
 201 NYAIDAGLDP VHDPIAVESG ENNPYANIIT VHRGDEKKKD IVALVNVLHS
 251 KEIQDWIRTK YKGAVIPVNN
```

NCNS GEL- Spot 3

Periplasmic putrescine-binding permease protein; SC = 16%

```
   1 MTAFGKKWLT GLVTGALMAV SAGSLAAEQK TLHVYNWSDY IAPDTVANFE
  51 KETGIKVVYD VFDSNEVLEG KLMAGSTGFD LVVPSASFLE RQLAAGVFQP
 101 LDKSKLPNWK NLDPEVLKLV AKHDPENKYA MPYLWATTGI GYNVDKVKAV
 151 LGKDAPVDSW DLVLKPENLE KLKSCGVSFL DAPEEIFATV LNYLGKDPNS
 201 SKADDYTGPA TDLLLKLRPN IRYFHSSQYI NDLANGDICV AIGWAGDVWQ
 251 AANRAKEAKN GVNVSYFIPK EGALAFFDVF AMPADAKNKD EAYQFLNYLM
 301 RPDVIAKISD QVFYANGNKA STPLVSETIR NNPAIYPPAD VFAKLFTLKV
 351 QDPKIDRVRT RAWTKVKSGK
```

NCNS GEL –Spot 4

Glycine hyroxymethyltransferase; SC = 10%

```
   1 MLKREMNIAD YDAELWQAME QEKVRQEEHI ELIASENYTS PRVMQAQGSQ
  51 LTNKYAEGYP GKRYYGGCEY VDIVEQLAID RAKELFGADY ANVQPHSGSQ
 101 ANFAVYTALL QPGDTVLGMN LAQGGHLTHG SPVNFSGKLY NIIPYGIDES
 151 GKIDYEDMAK QAETHKPKMI IGGFSAYSGV VDWAKMREIA DSIGAYLFVD
 201 MAHVAGLIAA GVYPNPVPHA HVVTTTTHKT LAGPRGGLIL AHGGNEELYK
 251 KLNSAVFPSA QGGPLMHVIA AKAVALKEAM EPEFKVYQQQ VAKNAKAMVE
 301 VFLNRGYKVV SGGTENHLFL LDLVDKNLTG KEADAALGRA NITVNKNSVP
 351 NDPKSPFVTS GIRIGSPAVT RRGFKEAEVK ELAGWMCDVL DNINDEAVIE
 401 RIKGKVLDIC ARFPVYA
```

NCNS GEL-Spot 5

Superoxide dismutase;SC = 29%

```
   1 MSYTLPSLPY AYDALEPHFD KQTMEIHHTK HHQAYVNNAN AALESLPEFA
  51 ELSVEELITK LDQLPADKKT VLRNNAGGHA NHSLFWKGLK TGTTLQGDLK
 101 AAIERDFGSV DAFKAEFEKA AATRFGSGWA WLVLKGDKLA VVSTANQDSP
 151 LMGEAVSGVS GFPIVGLDVW EHAYYLKFQN RRPDYIKAFW DVVNWDEAAA
 201 RFAAKK
```

NCS GEL Spot 6

Ionisitol-5-monophosphate dehydrogenase; SC = 16%

```
   1 MPMLRIAKEA LTFDDVLLVP AHSTVLPNTA DLSTQLTKTI RLNIPMLSAA
  51 MDTVTEARLA IALAQEGGIG FIHKNMSIER QAEEVRRVKK HESGVVTDPQ
 101 TVLPTTTLHE VKALTERNGF AGYPVVTEDN ELVGIITGRD VRFVTDLNQP
 151 VSVYMTPKER LVTVREGEAR EVVLAKMHEK RVEKALVVDD NFHLLGMITV
 201 KDFQKAERKP NSCKDEQGRL RVGAAVGAGA GNEERVDALV AAGVDVLLID
 251 SSHGHSEGVL QRIRETRAKY PDLQIIGGNV ATGAGARALA EAGCSAVKVG
 301 IGPGSICTTR IVTGVGVPQI TAVSDAVEAL EGTGIPVIAD GGIRFSGDIA
 351 KAIAAGASAV MVGSMLAGTE ESPGEIELYQ GRSYKSYRGM GSLGAMSKGS
 401 SDRYFQSDNA ADKLVPEGIE GRVAYKGRLK EIIHQQMGGL RSCMGLTGCA
 451 TIDELRTKAE FVRISGAGIQ ESHVHDVTIT KESPNYRLGS
```

NCS GEL- Spot 7

GroEL protein; SC = 53%

```
  1 MAAKDVKFGN DARVKMLRGV NVLADAVKVT LGPKGRNVVL DKSFGAPTIT
 51 KDGVSVAREI ELEDKFENMG AQMVKEVASK ANDAAGDGTT TATVLAQAII
101 TEGLKAVAAG MNPMDLKRGI DKAVTAAVEE LKALSVPCSD SKAIAQVGTI
151 SANSDETVGK LIAEAMDKVG KEGVITVEDG TGLQDELDVV EGMQFDRGYL
201 SPYFINKPET GAVELESPFI LLADKKISNI REMLPVLEAV AKAGKPLLII
251 AEDVEGEALA TLVVNTMRGI VKVAAVKAPG FGDRRKAMLQ DIATLTGGTV
301 ISEEIGMELE KATLEDLGQA KRVVINEDTT TIIDGVGEEA AIQGRVAQIR
351 QQIEEATSDY DREKLQERVA KLAGGVAVIK VGAATEVEMK EKKARVEDAL
401 HATRAAVEEG VVAGGGVALI RVASKLADLR GQNEDQNVGI KVALRAMEAP
```

NCS GEL-Spot 10

Heat shock protein; SC = 13%

```
  1 MRNHDLSPLL RQWIGFDKLA NALQSTAEHQ AFPPYNIEKS DDNHYRITLA
 51 LAGFRQDDLD IQLEGTRLTV KGTPEKPETE TKWLHQGLVI QPFSLSFTLA
101 DHMEVTGATF TNGLLHIDLT RNVPEALAPQ RIAISDRPAL NS
```

NCS GEL- Spot 12

Ribosomal Protein; SC = 55%

```
  1 MQVILLDKVA NLGSLGDQVN VKAGYARNFL VPQGKAVPAT KKNVEFFEAR
 51 RAELEAKLAD VLAAANARAE AINALGTVTI ASKSGDEGKL FGSIGTRDIA
101 DAVTAAGVDV AKSEVRMPNG VLRTTGEHEV DFQVHSEVFA KLVVNVVAE
```

NCS GEL –Spot 13

ATP Synthetase Subunit B; 53%

```
  1 MATGKIVQVI GAVVDVEFPQ DAVPRVYDAL EVQNGNERLV LEVQQQLGGG
 51 IVRTIAMGSS DGLRRGLDVK DLEHPIEVPV GKATLGRIMN VLGEPVDMKG
101 EIGEEERWAI HRAAPSYEEL SNSQELLETG IKVIDLMCPF AKGGKVGLFG
151 GAGVGKTVNM MELIRNIAIE HSGYSVFAGV GERTREGNDF YHEMTDSNVI
201 DKVSLVYGQM NEPPGNRLRV ALTGLTMAEK FRDEGRDVLL FVDNIYRYTL
251 AGTEVSALLG RMPSAVGYQP TLAEEMGVLQ ERITSTKTGS ITSVQAVYVP
301 ADDLTDPSPA TTFAHLDATV VLSRQIASLG IYPAVDPLDS TSRQLDPLVV
351 GQEHYDTARG VQSILQRYQE LKDIIAILGM DELSEEDKLV VARARKIQRF
401 LSQPFFVAEV FTGSPGKYVS LKDTIRGFKG IMEGEYDHLP EQAFYMVGSI
451 EEAVEKAKKL
```

CNS GEL- Spot 16

Fur protein; SC = 16%

```
   1 MTDNNTALKK AGLKVTLPRL KILEVLQGPD NHHVSAEDLY KRLIDMGEEI
  51 GLATVYRVLN QFDDAGIVTR HNFEGGKSVF ELTQQHHHDH LICLDCGKVI
 101 EFSDDSIESR QREIAARHGI RLTNHSLYLY GHCAEGDCRE DDHAHDAK
```

CNS GEL-Spot 17

OTcase; SC = 3%

```
   1 MSDLYKKHFL KLLDFTPAQF TSLLTLAAQL KADKKNGKEV QKLTGKNIAL
  51 IFEKDSTRTR CSFEVAAFDQ GARVTYLGPS GSQIGHKESI KDTARVLGRM
 101 YDGIQYRGHG QEVVETLAQY PGVPVWNGLT NEFHPTQLLA DLMTMQEHLP
 151 GKAFNEMTLV YAGDARNNMG NSMLEAAALT GLDLRLLAPK ACWPEESLVA
 201 ECSALAEKHG GKITLTEDVA AGVKGADFIY TDVWVSMGEA KEKWAERIAL
 251 LRGYQVNAQM MALTDNPNVK FLHCLPAFHD DQTTLGKQMA KEFDLHGGME
```

CNS Spot 18

Surface antigen (D15); SC = 5%

```
   1 MAMKKLLIAS LLFSSATVYG AEGFVVKDIH FEGLQRVAVG AALLSMPVRQ
  51 GDTVNDEDIS NTIRALFATG NFEDVRVLRD GDTLLVQVKE RPTIASITFS
 101 GNKSVKDDML KQNLEASGVR VGESLDRTTL SDIEKGLEDF YYSVGKYSAS
 151 VKAVVTPLPR NRVDLKLVFQ EGVSAKIQQI NIVGNHAFTT DELISTFQLR
 201 DEVPWWNVVG DRKYQKQKLA GDLETLRSYY LDRGYARFNI DSTQVSLTPD
 251 KKGIYITVNI TEGDQYKLSG VEVSGNLAGH SAEIESLTKL QPGELYSGAK
 301 VTKMEDGIKK LLGRYGYAYP RVQTQPEIND TDKTVKLRVN VDAGNRFYVR
 351 KIRFEGNDTS KDSVLRREMR QMEGAWLGSD LVDQGKERLN RLGYFETVDT
 401 DTQRVAGSPD QVDVVYKVKE RNTGSFNFGV GYGTESGVSF QVGVQQDNWL
 451 GTGYSVGING TKNDYQTYSE FSVTNPYFTV DGVSLGGRIF YNDFKADDAD
 501 LSSYTNKSYG LDGTLGFPIN EYNTLRAGLG YVHNDLSNMQ PQVAMWRYLD
 551 SIGQAASKSS DNNGFAADDF TFNYGWTYNR LDRGFFPTEG SRVNLNGKVT
 601 VPGSDNEFYK LTLDTASYFP IDDDHKWVVL GRTRWGYGDG LGGKELPFYE
 651 NFYAGGSSTV RGFQSNNIGP KAVYYGGNDK DNCNKTSSSE VCSSDDAVGG
 701 NAMGVASLEI ITPTPFISDK YANSVRTSFF WDAGTVWDTN WENTAQMKAA
 751 GVPDYSDPSN IRMSAGIALQ WMSPLGPLVF SYAQPFKKYD GDKAEQFQFN
 801 IGKTW
```

CS GEL-Spot 20

TOL C Protein; SC = 11%

```
  1 MQMKKLLPIL IGLSLTGFSA MSQAENLLQV YQQARLGNPD LRKSAADRDA
 51 AFEKINEARS PLLPQLGLGA DYTYTNGFRD NNGIDSNATS ASLQLTQTLF
101 DMSKWRELSL QEKSAGIQDV TYQTDQQTLI LNTATAYFQV LSAIDALSYT
151 EAQKQAIYRQ LDQTTQRFNV GLVAITDVQN ARSQYDTVLA NEVTARNNLD
201 NALEALRQVT GNYYPELASL NVDSFKTDKP QAVNALLKEA ENRNLTLLQA
251 RLSQDLAREQ IRLAQDGHLP TLSLSASTGV SDTSYSGSKT TSQAYDDSNV
301 GQNKVGLSFS LPLYQGGMVN SQVKQAQYNF VGASEQLESA HRNVVQTVRS
351 SFNNVNASIS SINAYKQAVV SAQSSLDAME AGYSVGTRTI VDVLDATTTL
401 YNAKQQLSSA RYQYLINQLN IKQALGTLNE QDLQMLNSTL GKPVSTSPDS
451 VAPENPQQDA AVDNFTANSS APVAQPAAAR STAPASSGTN PFRH
```

CS GEL-Spot 22

Outer membrane protein x; SC= 36%

```
  1 MKKIACLSAL AAVLAVSAGT AVAATSTVTG GYAQSDMQGV MNKTNGFNLK
 51 YRYEQDNNPL GVIGSFTYTE KDRTENGSYN KGQYYGITAG PAYRLNDWAS
101 IYGVVGVGYG KFQQTENQGL NRTASNSDYG FSYGAGMQFN PIENVALDFS
151 YEQSRIRNVD VGTWIAGVGY RF
```

CS Spot 24

Putative actin; SC = 9%

```
  1 MKLKVLALAA TLGLTTMAAQ ASELPDGPHI VTSGTASVAA VPDIATLAIE
 51 VNVSAKDAAS AKKQADDRVA QYLSFLEKSG IAKKDINSAN LRTQPDYDYQ
101 NGKSILKGYR AVRTVEVTLR QLDKLNGLLD GALKAGLNEI RSVSLGVAQP
151 DAYKDKARKA AIDDAVHQAQ ELAAGFHSKL GPVYSVRYHV SNYQPSPMVR
201 MMKAADAAPV SAQETYEQAT IQFDDQVDVV FELQPAQAAA PATPAKPAET
251 PKPAO
```

CS GEL – Spot 25

Glyceraldehyde 3-phosphate dehydrogenase; SC = 7%

```
  1 MADKKIRIGI NGFGRIGRLV ARVVLQRDDV ELVAVNDPFI TTEYMTYMFK
 51 YDSVHGQWKH HELKVKDDKT LLFGEKPVTV FGIRNPEDIP WGEAGADFVV
101 ESTGVFTDKD KAAAHLKGGA KKVVISAPSK DAPMFVVGVN EHEYKSDLDI
151 VSNASCTTNC LAPLAKVIND RFGIVEGLMT TVHSITATQK TVDGPSMKDW
201 RGGRAASFNI IPSSTGAAKA VGKVLPSLNG KLTGMSFRVP TVDVSVVDLT
251 VRLEKAATYD EIKKAIKEES EGKMKGILGY TEDDVVSTDF VGDNRSSIFD
301 AKAGIALSDK FVKLVSWYDN EWGYSSRVVD LIVHMSKA
```

Chapter IV

Discussion

Discussion

The *E. cloacae* strain required to grow in the presence of 40mM selenite in M-9 minimal medium. It is postulated that the accumulation of red elemental selenium in the media (NCS and CS) following stationary phase resulted from cell lysis with the liberation of selenium granules (Tomei A.F. et al., 1995). Cell lysis could have occurred in the NCS condition as a result of addition of selenite and thus reduction in growth as compared to the CS condition where cysteine was present.

Furthermore, blockage of the indirect selenite transport pathway may have played a role in selenite sensitivity (Brown, T.A and Shrift, A.1982). There could have been a feedback inhibition caused by presence of cysteine and thus prevented selenite uptake through the sulfate permease system. In the absence of cysteine, selenite may have been mistaken for sulfur and entered into the cell.

Two-dimensional gel electrophoresis was used to identify 25 polypeptides that were thought to have played a role either by a reduced or increased level of expression in selenite resistance. These peptides were excised from the gels, sequenced and matches found in the MASCOT database. Eight of the protein spots were landmarks, two from each of the four conditions and the rest were either unique or overexpressed spots.

Proteins required for normal function were excised and identified as elongation factors (EF-Ts). These were represented by spots 1, 9, 14 and 21. EF-Ts functions during elongation stage of protein translation by forming a dimer, associating with EF-TU-GDP complex and promoting exchange of GDP to GTP resulting in regeneration of the active form of EF-Tu (Jiri et al., 1998). The elongation factors direct tRNAs to the ribosome for

protein synthesis. Their presence is an indication that despite the presence of selenite in the cell, there is normal functioning. This in some way contribute to selenite resistance.

The second landmark identified was NlpA lipoprotein. This protein was represented by spots 2, 11, 15 and 23. It is an amino acid ABC transporter. Recent studies in *E. coli* have shown that NlpA is not essential for growth but it does contribute to biogenesis of outer membrane vesicles (Bodero M.D. et al., 2007). This could be the reason why it appeared in all the four conditions. Spot 3 was Periplasmic putrescine-binding protein (Permease protein) with sequence coverage of 16% was unique and overexpressed. This protein is an ABC-family zinc binding protein and is known to transport a wide variety of substrates such as amino, peptides, sugars and vitamins. It is vital for maintaining ion homeostasis in several pathogenic and non-pathogenic bacteria (Beeram, R.C. et al., 2007). It was expressed in the NCNS condition and thus could be playing a role in the normal functioning of the bacterial cell.

Glycine hydroxymethyltransferase which was spot number 4 was expressed in the NCNS condition and had sequence coverage of 10%. This protein is plays a role in keeping levels of glycine to a minimum in a cell. Accumulation of the amino acid glycine is associated with strong growth inhibition or even death in bacteria (Eisenhut et al., 2007).

Spot 5 was another protein of interest found under the no cysteine and no selenite condition and was identified as superoxide dismutase (SOD). This protein catalyses the conversion of superoxide radicals to hydrogen peroxide and molecular oxygen. Thus it destroys free radicals. Free radicals are expressed in response to oxidative stress in a cell. Therefore, SOD is an important antioxidant defense mechanism in all cells exposed to

oxygen and selenite (Raimondi, S. et al., 2008). Since the cells were oxygenated by shaking, perhaps these cells produced large quantities of superoxide while they grew aerobically. Spot number 6 was Ionositol-5-monophosphate dehydrogenase (*Salmonella enteric sp*) with sequence coverage of 16%. It is an essential enzyme that catalyses the first step unique to GTP synthesis.

Under the sensitive condition (NCS), a protein of interest found there was GroEL which was spot 7 and had sequence coverage of 53%. This heat shock protein plays a role in cellular stressful conditions (Neidhardt et al., 1985). It was overexpressed as a result of the toxic effects of selenite. GroEL serves as a molecular chaperone by protecting newly synthesized proteins and proteins under stressful conditions. GroEL functions by binding to its cochaperone GroES thus forming a complex that protects proteins from aggregating by encapsulating the polypeptides individually. It plays an important function in protein-protein interactions such as folding and assisting in the establishment of proper protein conformation and prevention of unwanted protein aggregation due to stress. GroEL is known to be overexpressed during varied stress conditions and has been shown to be vital during heat stress and hence named heat shock protein. Furthermore, GroEL is essential for growth at all temperatures (Fayet et al., 1989).

Spot 10 contained polypeptides that significantly matched heat shock protein HSP 20. The sequence coverage was 36%. Heat shock proteins are a group of proteins whose expression is increased when the cells are exposed to elevated temperatures or stress (Fayet et al., 1989). In this case it is selenite which is causing the stress. The increase in expression is transcriptionally regulated. HSPs process old proteins for recycling and help newly synthesized proteins fold properly. These activities are part of a cell's own repair

system called the cellular stress response or heat shock response (Graham, A. Pockley., 2001).

Ribososmal protein (spot 12) was another protein found in the sensitive condition (NCS) and had sequence coverage of 55%. Ribosomal proteins hold a unique position in biology because their function is closely related to the large rRNAs of the ribosomes. They basically play many roles in translation (Ditlev, E.B. and Poul Nissen., 2005). ATP synthase sub-unit B is another protein found in the sensitive condition. It is known to produce ATP from ADP in the presence of a proton gradient across the membrane. It plays a role in the initial biosynthetic assembly of an intact H+ channel (Erwin, S. and Karlheinz, A. 1984).

Under the CNS condition unique spot 13 which showed similarity to *E coli*'s ATP synthase sub-unit B and demonstrated sequence coverage of 53%. It produces ATP from ADP in the presence of a proton gradient across the membrane.

Fur protein (spot 16) was found in the CNS condition and had sequence coverage of 16%. Fur (ferric uptake regulator) is an iron-sensing repressor that controls the expression of genes for siderophore biosynthesis and iron transport (Jin-Won Lee and John, D.H. 2006).Its role here (CNS) is not well understood. It could have been that, the iron content in the minimal medium was low hence its (Fur protein) expression.

Ornithine transcarbamylase (OTcase)-spot 17 was a protein found in the CNS condition and had sequence coverage of 3%. OTcase catalyses an essential step in arginine biosynthesis and also plays a catabolic role (Justin, O.N. and Robert, L. Switzer 1983).It is involved in post-translational processing. Its role here is basically catabolic. Surface antigen (D15) is another protein identified having sequence coverage of 5% and

found in the CS condition. It is understood to play a role in outer membrane protein biosynthesis and also in the assembly of outer membrane proteins. Glyceraldehyde 3-phosphate-dehydrogenase with sequence coverage of 7% was the last spot which was found in the resistant condition. It is a C- terminal domain and a tetrameric NAD-binding enzyme involved in glycolysis and glyconeogenesis.

Another protein of great interest was found in the resistant condition (CS). Spot number 20 demonstrated 11% sequence coverage of TOL C. This protein plays a role in export of chemically diverse molecules ranging from large protein toxins to small toxic compounds such as antibiotics. It confers pathogenic bacteria with both virulence and multidrug resistance (Muriel et al., 2007). It is a member of the efflux/transport pump concentration and may have been overexpressed in response to the high selenite (Sharff et al., 2001) and to export selenite out of the cell thus conferring resistance to the *E .cloacae* strain.

Spot 22 was outer membrane protein x with sequence coverage of 21%. This protein is one of the many proteins found in the membrane of *E. coli* (Chen et al., 1980). It plays a role in the transportation of molecules into and out of the cell. It may have been also overexpressed for selenite transport.

The protein matches found in the MASCOT database were not 100%. However, two peptide matches to a database protein is sufficient for determining the identity of a spot. The conserved regions of the identified polypeptides can be back-translated to design degenerate primers which can be used in polymerase chain reactions (PCR) with to amplify short segments of the gene. The PCR products can then be used to probe genomic DNA digests of the *E. cloacae* strain and identify larger DNA fragments which

encode them. This is followed by cloning and identification by southern hybridization. The sequence of the cloned gene can be used to show how the identified gene confers selenite resistance by using homologous recombination to knock it out. A loss of function indicates its involvement in selenite resistance. Knock-out results can then be confirmed by complementation experiments using the pBeloBac 11 plasmid, which can be transformed into our *E. cloacae* strain.

Besides this, transposon mutagenesis can also be used to identify genes involved in selenium homeostasis. 2DGE can used to confirm the transposon mutagenesis results and identify more proteins. pH range 5-8 may not have been successful in resolving all the proteins. At pH 8 there was a large protein streak where the proteins stopped migrating, which was an indication that proteins with a pI above 8 exist in the samples. Thus pH 7-10 may be used to repeat this study to identify more proteins. A larger gel size of about 96 cm can be used as well to carry out this exercise as 11 cm may not have resolved all the proteins.

Moreover, looking at the results of similar work done in my laboratory by a previous graduate student (A. Jasenec, 2007), there are many similarities and differences in protein identity and expression that were found. For instance, she worked with a pH of 4-7 and some of the proteins that were identified were similar or played similar roles as the ones that I identified. For example, the protein chain elongation factors which play a role in peptide elongation of many proteins by directing tRNAs to the ribosome for protein synthesis, small hsp proteins, GroES and outer membrane proteins. However, she did identify a protein named CysK in the NCS condition which I didn't find. This protein also known as cysteine synthase is thought to play a role in the biosynthesis of cysteine.

In my work, I did identify TolC which may be involved in pumping selenite out of the cell, superoxide dismutases and Fur protein. These are some of the proteins that were not identified in the pH range of 4-7. These kinds of differences in proteins identified necessitate the need to work with a higher and larger pH range (4-10) so as to identify many more proteins which may play a role in selenite homeostasis.

In conclusion, it is evident that selenite inhibits growth of *E .cloacae* strain when cysteine is absent, though no proteins were identified that suggested cysteine's role in selenite resistance. Furthermore, differential protein expression was observed under selenite resistant and sensitive conditions with some being overexpressed hence the need for these proteins in the presence of selenite. From the data obtained, several other proteins were identified using the 2D-gel electrophoresis and are thought to be involved in oxidative stress response and transporting of selenite into and out of the cell. These proteins may play a role in selenite homeostasis.

Chapter V

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