Possible role of *E. coli* chromosomal arsenic resistant operon in selenite tolerance

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Possible role of *E. coli* chromosomal arsenic resistant operon in selenite tolerance

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// Abstract

Bioremediation is a method in which microorganisms are employed to clean up the polluted environment. In the present study we used a multimetal resistant bacterium *Enterobacter* sp. YSU, as our model of bioremediation. A 100 kb plasmid, pOR1, from *Enterobacter* sp. YSU conferred resistance to 40 mM selenite when it was transferred to *Escherichia coli* strain HB101 to create the new strain HB101(pOR1). Transposon mutagenesis of the pOR1 strain resulted a new strain selenite sensitive strain called G50 with the transposon inserted between 5' end of glutathione reductase and the 3' end of the arsenic resistance genes. The chromosomal arsenic resistance genes of E. coli confer a week level of arsenic resistance and require plasmid encoded genes to provide stronger levels of resistance. A knock out was carried out on pOR1 using pRed ET kit and a new strain, pRed Ars, was generated and minimal inhibitory concentration experiments (MIC) and growth curves were carried out on pOR1, G50 and pRed Ars strains using sodium arsenite and sodium selenite . From the MIC and RT PCR experiments it was concluded that *ars* operon may not be responsible for selenite resistance.

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I would like to dedicate my work to Youngstown State University.

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LIST OF SYMBOLS AND ABBREVIATIONS

DNA……………………………………………. Deoxyribo nucleic acid

PCR………………………………………Polymerase chain reaction

EDTA……………………………………………..Ethylene Diamine Tetra Acetic Acid

HCl…………………………………………………………...Hydrochloric Acid

Sec……………………………………………………………………….Seconds

µl……………………………………………………………………...Microliter

µM…………………………………………………………………...Micromolar

$CaCl_2$…………………………………………………………Calcium Chloride

UV…………………………………………………………………...Ultra Violet

NaCl…………………………………………………………Sodium Chloride

$MgCl_2$………………………………………………………...Magnesium Chloride

BSA……………………………………………………Bovine Serum Albumin

DTT………………………………………………………………...Dithiothreitol

ml ……………………………………………………………………milliliters

ssDNA………………………………………Single Stranded Deoxyribonucleic Acid

nm……………………………………………………………………..nanometer

dH_2O…………………………………………………………Deionized water

H_2O……………………………………………………………………...Water

Tris……………………………………………Tris(hydroxymethyl)aminomethane

dNTP……………………………………………...……deoxynucleotidetriphosphate

TBE……………………………………………………..………Tris-Borate-EDTA

Na_2-EDTA…………………………………………………….Sodium EDTA

µg……………………………………………………………………………….Microgram

µF……………………………………………………………………………….Microfarad

kV…………………………………………………………………………………kilovolts

_.…………………………………………………………………………………...Ohms

dG……………………………………………………………………..Free energy of oligo

% GC…………………………………………………………percentage of G and C in oligo

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

DMSe……………………………………………………………...Dimethyl Selenide

DMDSe………………………………………………………………Dimethyl Diselenide

E. coli*…………………………………………………………………...*Escherichia coli

FDH……………………………………………………………Formate dehydrogenase

tRNA………………………………………………………………………transfer RNA

GSH ……………………………………………………………...............Glutathione

DNA…………………………………………………………...deoxyribonucleic acid

RNA ……………………………………………………………........ribonucleic acid

NADP...nicotinamide adenine dinucleotide phosphate

NADH..nicotinamide adenine dinucleotide dehydrogenase

CH_3Hg…………………………………………………………… methyl mercury

CO_2 ……………………………………………………………Carbondioxide

CH_4 ………………………………………………………………......Methane

% ……………………………………………………………………......percentage

V………………………………………………………………………......volts

kb…………………………………………………………………..………kilobase

bp ………………………………………………………………………basepairs

ATCC………………………………………………......American Type Culture Collection

Chapter I: Introduction

Selenium:

Selenium is a non metal compound and is found in all materials of the earth. It was identified and isolated by a Swedish scientist, Jons Jacob Berzelius in 1817 when he was analyzing the red deposit on the wall of a lead chamber which was used to produce sulfuric acid (3). The name was derived from the Greek moon Goddess, Selene. It is a naturally occurring compound found in group VIA, has chemical properties similar to sulfur and tellurium and shares more chemical properties with sulfur than tellurium. Selenium occurs in four oxidation states. Elemental selenium, Se^0 (+0) is non toxic and is highly insoluble in water. Selenide, Se^{2-}(–II), is highly reactive and toxic but is readily oxidized to elemental selenium. The other two oxidation states of selenium Selenate, $SeO_4{}^{2-}$ (+VI), and selenite, $SeO_3{}^{2-}$ (IV), are highly soluble in water and are toxic to living systems at elevated concentrations (33).

Background behind selenite pollution:

A variety of physical, chemical and biological processes lead to distribution of selenium into the environment. The major source of environmental selenium is due to weathering of rocks. Usually sandstones and limestones contain lower amounts of selenium (38). Some soils naturally contain elevated levels of selenium, and anthropogenic activities such as coal mining, fossil fuel combustion and petroleum refining leads to selenium pollution in the soil. Water bodies generally contain less than 10 µg of selenium per one liter of water, whereas water polluted by agricultural activities contains 1400 µg of selenium per one liter. Normal soils contain 0.010mg to 2 mg of selenium per kg, and selenium contaminated soils contain 5 mg of selenium per one

kg soil. Contaminated soil contains two oxyanions of selenium, selenate and selenite, which are toxic and can accumulate in biological systems.(9).

Selenite toxicity:

Marco Polo first described selenium toxicity in horses as hoof rot disease that was caused by the ingestion of grain and forage which grew in selenium rich soils and contained 5–50 ppm Se (15) Selenium enters the food chain through plants, which take up selenium from the soils. Grazing from selenium rich soils causes alkali disease, which is characterized by loss of hair, emaciation in livestock along with dystrophic changes in hooves and concurrent loss of the tail switch. The subacute form of selenium toxicity was reported in the livestock that fed on plants growing in soils containing 10000 ppm selenium. This disease is characterized by anorexia, weight loss, blindness, ataxia, disorientation, and respiratory distress in livestock and may lead to blind staggers. A small dose of selenium intake, approximately 5 mg every day can kill many human beings (34).

Selenium toxicity in Kesterson reservoir:

Kesterson reservoir consists of a series of twelve ponds in Kesterson national wild life refuge. These ponds were used to drain subsurface water from agricultural field (26). Studies conducted on water fowl showed that the tissues and eggs of these birds contained levels of selenium high enough to cause adverse effects. Selenium concentrations in the food chain of the organisms that were eaten by water fowl showed high levels of this element. Selenium concentrations in other aquatic plants, insects, and mosquitofish at Kesterson averaged 50–150 µg/g, and were far higher than the threshold dietary levels associated with impaired reproduction for aquatic fowls.

Studies conducted in 1984 and 1985 showed that the aquatic birds (coots) failed to nest during nesting season, while some fowl were found dead. The livers of live as well as dead fowl contained high levels of selenium and the weights of the birds were 25% less than healthy birds.

Cause of selenium toxicity:

The main cause of selenium toxicity and other non metals like arsenic in biological systems is due to the generation of superoxide through interactions with thiols in mammals and birds ((30). In addition, the functionality of many sulfhydryl containing enzymes and proteins are altered. The sulfur in the amino acid methionine can be substituted with selenium leading to the formation of selenomethionine. This analogous form of methionine cannot be distinguished and selenomethionine will be incorporated into the proteins, leading to embryo malformations and toxicity. (30)

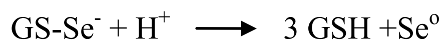
The toxicity of selenium species considerably varies due to the differences in physiochemical properties. The higher valence states of Se (IV) and Se (VI) are highly toxic to living organisms at elevated concentrations. (4). The toxicity of selenium can be attributed to replacement of sulfur or sulfur containing biomolecules in the proteins by selenium. In bacteria selenite toxicity might be the result of free radical formation when selenium reacts with cysteine or glutathione. Reactions with thiol groups results in the formation of selenotrisulfides.

$$4\ RSH + H_2SeO_3 \longrightarrow RS\text{-}Se\text{-}SR + RSSR + 3H_2O$$

The selenotrisulfide of glutathione is also known as selenodiglutathione which acts as a substrate for glutathione reductase.

$$GS\text{-}Se\text{-}SG + NADPH \longrightarrow GSH + GS\text{-}Se^- + NAD^-$$

The unstable selenopersulfide of glutathione ($GS\text{-}Se^-$) dismutases into elemental selenium (Se°)



$$GS\text{-}Se^- + H^+ \longrightarrow 3\ GSH + Se^o$$

The selenite might be reduced by glutathione in organisms having higher levels of glutathione (16).

Selenium as an essential micronutrient.

Selenium deficiency causes Keshan disease and Keshin Beck disease. Keshan disease is characterized by cardiopathy, a serious condition in which the heart muscle become inflamed and does not work properly (21). Keshin Beck disease is associated by endemic osteochondropathy disease (attributes to a disease of bone and cartilage) and found in Chinese mountainous regions where the selenium soil content is low. The human heart is especially vulnerability to low selenium levels and cardiomyopathy condition can be reversible by selenium supplementation. Other diseases are suspected with selenium deficiency in the diet. (29).

Studies have shown that low selenium and carotinoid blood serum levels are associated with cancer, cardiovascular disease and mortality among adults. Experiments on rodents showed that selenium supplementation will prevent the chemically induced carcinogenesis. Experiments that were conducted recently on prostate cancer patients have shown that, selenium and vitamin E can reduce the risk of prostate cancer in males (32). The decreased activity of the selenium containing enzyme, glutathione peroxidase was associated with coronary artery disease.

Selenium mediates arachidonic acid metabolism and its low serum concentrations are implicated with pathogenesis of atherosclerosis. Studies have shown that low serum selenium levels were an independent predictor of disease progression and mortality among HIV infected children and adults. (1).

Research conducted on people who live in regions that contain selenium deficient soils show that there is a possible link between selenium deficiency and a high incidence of HIV/ AIDS. Selenium is a potent inhibitor of HIV in vitro, and selenium levels progressively decline along with progressive loss of $CD4^+$ T cell in HIV infection. Studies conducted on HIV infected mice with low $CD4^+$ T cell count have shown activation and proliferation of $CD4^+$ T cell count with selenium supplementation (11) Thus, it appears to be as a crucial supplement for HIV infected patients. The mRNAs of several T cell genes have in frame UGA codons in their open reading frames suggest that these genes code for the enzymes such as selenoprotein P which contains most of the selenium found in human plasma (31). Selenium also appears to inhibit hepatitis B and C viruses and acts against the progression of liver cancer. Viruses may block the selenium supply to the host tissues and incorporate it into viral selenium proteins (27). In addition, rats suffering with liver necrosis can be cured by feeding them with torula yeast containing small amounts of selenium and vitamin E (30). Epidemiological studies have shown that selenium can reduce the toxic effects of methyl mercury (36).

It was proven that selenium supplementation along with other nutrients can prevent the recurrence of tuberculosis. (35).

Selenium sulfide is used as an effective inhibitor of fungal growth and is used in antidandruff shampoos. Selenium compounds can kill *Malassazia,* a fungus that lives on the scalps of human

beings and causes dandruff. Dermatomycosis furfuracea, a type of skin disease caused by different types of yeasts can be treated using lotions that have selenium sulfide as an active ingredient (23).

The affects of selenium are not all positive. A study conducted on type 2 diabetic individuals showed that there is a positive correlation between serum selenium levels and diabetes 2. Thus, diabetics have been discouraged from taking selenium supplements (6).

Selenium is an important co factor in many enzymes:

Selenium acts as an antioxidant that can reduce the risk of cancer. Studies have shown that selenium compounds act as anti-cancer agents in mouse models. Selenomethionine is the major component of dietary selenium and have intriguing antioxidant properties (8). The antioxidant effects of selenium can be seen in the extracellular space, cytosol and in gastrointestinal tract. It was proved that selenoproteins play a key role in regulating the reactive oxygen species in all tissues (11).

Glutathione peroxidases (GPx) reduce lipid hydroperoxidases to their corresponding alcohols and hydrogen peroxide and they further reduce hydrogen peroxide to water. It catalyzes certain reactions and can remove reactive oxygen species (ROS), preventing cell damage and reducing ageing. There are four kinds of glutathione peroxidases, glutathione peroxidase 1 and 3 are mostly found in the plasma of all mammalian species. Glutathione peroxidase 3 is an extracellular enzyme found in intestines. Glutathione peroxidase 4 is expressed by every mammalian cell at lower levels (24).

These four have antioxidant properties and remove hydrogen, lipid and phospholipid peroxides. In animals, glutathione peroxidase acts in cooperation with catalase in removal of hydrogen peroxide. Glutathione peroxidase, which contains selenium at the active site, uses the endogenous tripeptide glutathione as a specific co-factor. $2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$. GSSG is an oxidized form of glutathione that is reduced by the enzyme glutathione reductase and recycled again.

$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$

These antioxidant processes maintain membrane integrity, modify inflammation and prevent the propagation of oxidative damage to lipids, lipoprotein, DNA and other biological molecules. Mitochondrial selenoprotein is a form of GPx4 and safeguards developing sperm from oxidative damage and later maintains the stability or motility of the sperm by polymerizing into other structural proteins. Thus selenium plays a role in male fertility (27).

Thioredoxine reductases, which act as antioxidants, also contain selenium. Along with the antioxidant properties selenium in the form of selenoproteins perform two other activities in the cell: thyroid hormone metabolism and regulatulation of redox–active proteins. Selenium is also necessary for optimum immune responses and is involved in cell mediated immunity. Sufficient amounts of dietary selenium are important for the activity of all branches of immune response (5).

There are twenty five genes that code for selenoproteins in the human genome (42). These can be divided into two groups based on the location of selenocysteine in the primary amino acid residue structure. One group contains selenocysteine in the N terminal region, while the other

contains selenocystein in C terminal region. Selenoenzymes form families of thioredoxine reductases and iodothyronine deiodinases. SelenoproteinP is found in the plasma and is associated with endothelial cells and may protect the endothelial cells against damage from peroxynitrate. It is the only characterized selenoprotein that contains more than one selenocysteine amino acid residue per polypetide chain. Selenoprotein W is involved in muscle function (27). The functions of selenoproeins R, N, T, M and G are yet to be determined.

Selenocysteine:

Selenocysteine is a rare amino acid found in the selenoproteins (25) the majority of selenoproteins function as oxidation reduction enzymes, using selenocysteine in the active site. The chemical structure of selenocysteine is similar to cysteine, except selenium replaces the sulfur atom. Selenocysteine does not occur as a free aminoacid, and the codon, UGA, which is normally a stop codon for opal, also code for selenocysteine. (2). Selenocysteine tRNA is the key molecule in the expression of selenoproteins. The synthesis of selenocysteine occurs on its tRNA, a process which is different from other amino acids. Sec tRNA is aminoacylated with serine. Then, oxygen in the R group of serine is replaced with selenium to form selenocysteine. *cis* and *trans* acting factors help the cells to distinguish dual acting UGA as a stop and Sec codon. Selenocysteine insertion sequences (SECIS) and SECIS binding protein SBP2 act as *cis* acting factors. Selenocysteine insertion sequences are present in the 3' untranslated regions of all selenocysteine containing genes in eukaryotes. SECIS recruits SBP2 and these two bind to elongation factor (Efsec), which helps insert selenocysteine RNA into the ribosomal complex. Additional *trans* acting factor Sec synthase, which is implicated in the insertion of selenocysteine

is absent in eukaryotes selenoproteins. (10). The SECIS stem loop structures are found in all domains of life, from viruses to highly developed animals, including human beings (18) In *Escherichia coli* it has been demonstrated that at least four genes, *selA, selB, selC* and s*elD* are required for insertion of selenocysteine into selenium dependent formate dehydrogensase. The *selD* gene product, SELD protein catalyzes the ATP dependent formation of the diffusible selenium, a derivative from selenide. Selenocysteine synthase generates aminoacrylyl $tRNA^{sec}$ and 2, 3 double bonds to this are added by selenide and results in the formation of selenocysteyl tRNA.

In *E. coli* selD gene is located on the chromosome and a defect in this gene prevents insertion of selenium into formate dehydrogenases and into 2- selenouridine residues of tRNA. The SELD protein is composed of 347 amino acids. Among these, 7 are cysteine residues. At least one of these cysteine residues is required for the catalytic activity of SELD. Two cysteine residues are located at position 17 and 19 at the N terminal region of the protein sequence. When these two cysteine residues were replaced with serine residues, the enzyme SELD failed to insert selenocysteine into formate dehydrogenase. When the mutations were complemented with genes encoding cysteine at positions17 and 19, the ability to insert selenocysteine was restored. (17)

In *E .coli* the loaded serine will be converted to selenocysteine by a gene product called *selA*. In this process the *selA* gene product uses a selenium phosphate donor synthesized by another gene called *selD*. Translation of Sec –t RNA^{sec} is mediated by *selB* gene product. An analog of elongation factor TU (EF- Tu Sec) recognizes the Sec –t RNA^{sec} and SECIS, and inserts selenocysteine into the selenoproteins (28).

Import pathways of selenium in bacteria:

There are two import pathways of selenium into the cell: the specific pathway and the non-specific pathway. The specific pathway has not been resolved completely, but it involves the incorporation of selenocysteine into selenoproteins (22).The nonspecific pathway is mediated through sulfate carrier system. Studies have shown that microorganisms use same carrier system to transport selenate and sulfate. Once the selenate enters the cell it will be assimilated by the enzyme system that is responsible for sulfate metabolism. A kinetic examination on the selenate, selenite and sulfate conducted in a bacterium has shown that these three inorganic molecules were taken up by the same transporter system (19). After the uptake into the cell through the non-specific pathway, selenite diverges from the sulfate pathway and can be unintentionally assimilated into cysteine containing proteins (add Muller et al reference here)..

In *E. coli* selenate is thought to enter through sulfate permease system (*cysA, cysU and cysW*). Selenite also enters through this sulfate transporter; however there is an alternative and undetermined carrier also exists as the repression of sulfate permease expression does not affect selenite uptake completely.

Once the selenate enters the cell it is converted to selenite by nitrate reductases A and Z. The selenite is further converted into selenium Se^{o} which is incorporated into the proteins as selenomethionine or selenocysteine. (33).

Y12 Plant in Oak Ridge, TN:

The Y12 plant is a secret electromagnetic facility located in the town of Oak Ridge, which processed the uranium 238 to get large quantities of isotope uranium 235 in making first nuclear

bomb. During World War II the electromagnetic separation was on its culmination at Y12 plant. Along with uranium the facility also processed other radioisotopes such as berelium, bismuth, thorium, calcium, plutonium, americium, curium, nickel and samarium (20). From 1950 to 1963 lithium enrichment processes were carried out at Y 12 plant which resulted the release of mercury into soil, air and surface waters (37). About 330 metric tons of mercury was released into East Fork Poplar Creek and the surrounding area. A number of physical, chemical and biological processes resulted conversion of mercury into some organic and inorganic forms of mercury. The Department of Energy (DOE) has been working in and near East Fork Poplar Creek in identifying the ways to remediate mercury levels. (12)

***Enterobacter* sp. YSU:**

Enterobacter sp. YSU is a multimetal resistant aerobic, gram negative bacterium that was probably isolated from East Fork Poplar Creek (13). It has shown resistance to different metals like copper, mercury, gold, cadmium, silver and selenium salts. When total genomic DNA from *Enterobacter* sp. YSU was transformed into *E. coli* strain HB101*,* selenite resistant transformants contained a 100 kb plasmid which was named, pOR1. This new *E. coli* was named *E. coli* HB101 (pOR1) (41).

Transposon Mutagenesis of *E. coli* HB101 (pOR1):

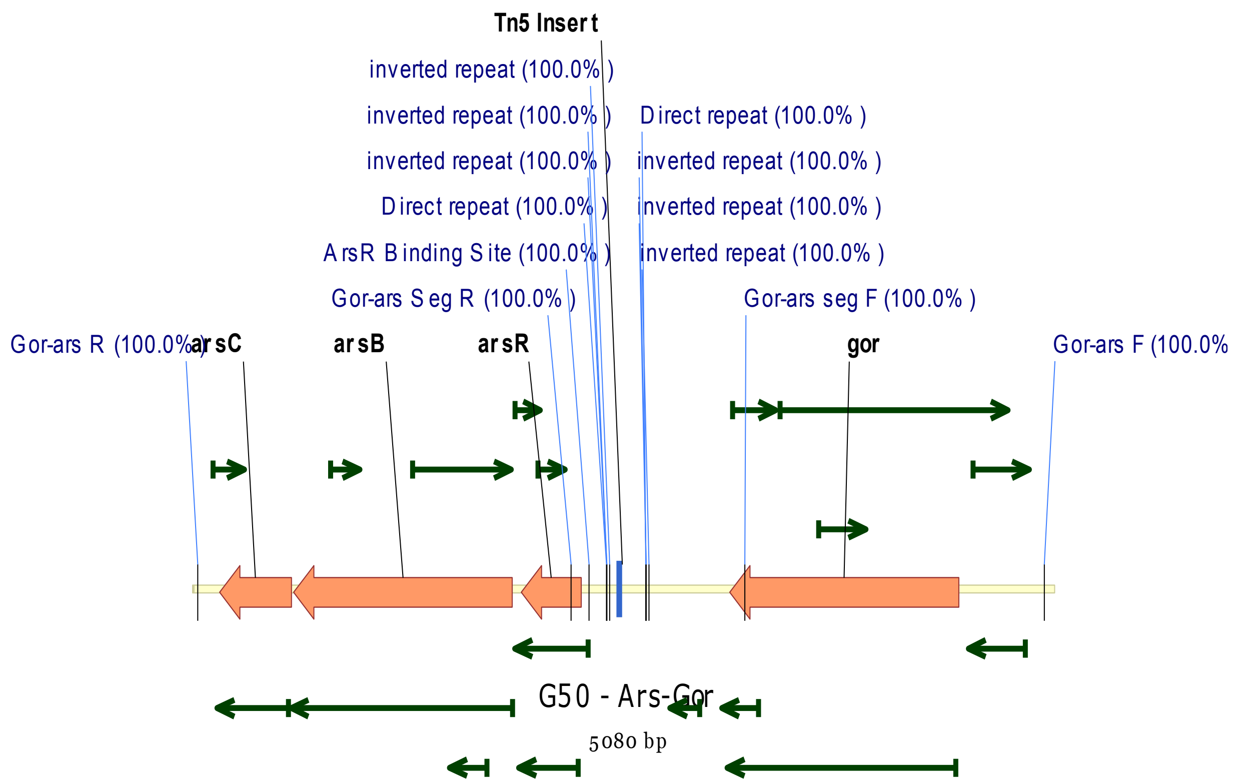
Transposon mutagenesis was performed on HB101 (pOR1) using the EZ-Tn*5* <R6K_*ori*/KAN-2>Tnp Transposome Kit from Embitech. The EZ-Tn*5* Transposome carrying a kanamycin resistance gene was electroporated into HB101 (pOR1) and plated on kanamycin plates.

Transformants were replica plated onto agar plates containing and lacking 40 mM selenite. Several of the interrupted genes from selenite sensitive mutants were rescued and sequenced. One of the mutants, G50, contained a chromosomal insert between a direct repeat DNA sequence that was located between the arsenic resistance (*ars*) and glutathione reductase (gor) genes (Fig 1).

Arsenic resistance operon:

Arsenic resistant genes are found in many microorganisms which include gram positive and gram negative bacteria. These are located either on the chromosome or on the plasmid. Many arsenic operons contain three genes which includes *arsR*, *arsB* and *arsC,* while some other operons contain 5 genes that include *arsRDABC*. The *arsR* gene encodes a regulatory protein which controls the level of arsenic operon expression. *arsD* gene codes for a protein called metallo chaperon and enhances the resistance. *arsA* gene codes for ArsA protein, which is an arsenite stimulated ATPase. *arsB* gene codes for ArsB protein which acts as transmembrane efflux pump and arsC codes for Ars C protein which reduces arsenate to arsenite and is pumped out of the cell (7).

Figure 1: Feature map of G50 strain. Green arrows represent open reading frames. Yellow line represents DNA sequence and the blue lines represent direct repeats and inverted repeats. Solid orange arrows represent the *ars* and *gor* genes. Within a direct repeat there are some inverted repeats.


Tn5 Insert
inverted repeat (100.0%)
inverted repeat (100.0%)
Direct repeat (100.0%)
inverted repeat (100.0%)
inverted repeat (100.0%)
Direct repeat (100.0%)
inverted repeat (100.0%)
ArsR Binding Site (100.0%)
inverted repeat (100.0%)
Gor-ars Seg R (100.0%)
Gor-ars seg F (100.0%)
Gor-ars R (100.0%)
arsC
arsB
arsR
gor
Gor-ars F (100.0%
G50 - Ars-Gor
5080 bp

Chapter II: Hypothesis

Hypothesis:

Based on the transposon mutagenesis experiment followed by sequencing conducted on HB101 (pOR1), it is hypothesized that the transposon insert in the G50 mutant may influence the expression of the *ars* operon or *gor*. The arsenic resistance operon could actually be a selenite resistance operon because the chromosomal *E. coli ars* operon is considered to be a weak arsenic resistance determinant (39).Other arsenic resistant strains of *E. coli* contain plasmids that also encode an additional *ars* operon. To determine if the chromosomal *ars* operon is involved in selenite resistance, and I constructed a knock out of the *ars* operon in HB101 (pOR1) and tested the knock out for resistance to selenite and arsenic resistance. On agar medium, I was able to restore selenite resistance by cloning the *ars* and *gor* genes back into the mutants. However, the results in liquid medium were ambiguous because the knock outs grew poorly even in the absence of selenite and arsenite.

Chapter II: Materials and Methods

Bacterial Strains Used:

Enterobactersp. YSU is a gram negative, facultatively anaerobic and rod shaped bacterium; it showed resistance to different metals such as cadmium, mercury, arsenic, copper and selenium (14). EC 100D p*ir* [F- *mcr*A Δ (*mrr-hsd*RMS-*mcr*BC) ø80d/acZ ΔM15 Δ/acX74 *rec*A1 *ara*D139 Δ (*ara, leu*) 7697 *ga*/U *ga*/K 7⁻ *rps*L *nup*G *pir*-116 (DHFR)} and EC 100D pir-116 [F- *mcr*A _ (*mrr-hsd*RMS-*mcr*BC) ø80d/acZ ΔM15 Δ/acX74 *rec*A1*end*A1 *ara*D139 Δ (*ara, leu*) 7697 *ga*/U *ga*/K 7- *rps*L *nup*G *pir*-116 (DHFR)} were used for gene rescue and were purchased from Epicentre Biotechnologies (Madison WI). A plasmid known as pOR1 was isolated from this bacterium and transferred to *E. coli* HB101 to become HB101 (pOR1). It also conferred resistance to selenite.

Media preparation:

Bacterial cells were grown at 37 ^{o}C in Luria Bertani (LB) medium (FisherScientific, Fair Lawn, New Jersey) which consisted of 10 grams of Bacto Tryptone, 5 grams of yeast extract and 5 grams of NaCl per liter of deionized water. When required, media were supplemented with 1.6% Agar (Amresco, Solon, and Ohio) and 50 µg/ml kanamycin sulfate (Amresco, Solon, Ohio).

M-9 Minimal medium:

Preperation of 5X M-9 Minimial salts:

Disodium posphate (anhydrous 33.9gms) monopotassium phosphate (15.0gms) sodium chloride (2.5 gms) ammonium chloride(5.0 gm) per liter was mixed in one liter of sterile water to get 5X M-9 salts and the M- 9 minimal medium was prepared by mixing 5X M-9 Salts from Difco®, 20% Glucose, 1 M $MgSO_4$, Thiamine, Cysteine in sterile water.

Methods

Transformation:

Transformation of competent *E. coli* cells was performed using a $CaCl_2$ technique(40). A single colony was inoculated into 5 ml LB medium and were grown overnight at 37 ^oC in a TC 8 roller drum (New Brunswick Scientific Edison, New Jersey). The overnight cultures were then transferred into fresh 100 ml LB broth to an optical density (600 nm) of 0.5. Cells were centrifuged at 3,000 X g again, resuspended in 10 ml of 0.15 M NaCl, centrifuged at 3,000 X g again, and resuspended in 1 ml of transformation buffer (15% glycerol, 0.1 M $CaCl_2$, 0.01 M Tris-HCl, pH 8.0 and 0.01 M Mg Cl_2). After incubating the cells overnight on ice in the refrigerator, the cells were frozen at -80°C. The cells were then thawed on ice, and 0.1 ml was gently mixed with the 0.03-0.5 µg of plasmid DNA. The transformation mixture was incubated on the ice for 30 min, the cells were heat shocked at 42 ^{o}Cfor 50 seconds and placed back on ice. LB media was added to a final volume of 1 ml, and the cells were incubated with shaking for 45 – 120 minutes. Volumes ranging from 10 to 1000 µl of cells were spread on LB-agar plates containing the appropriate antibiotic and incubated at 37 ºC overnight. The number of colonies that grew the next day were counted and recorded.

Electroporation

Electroporation used an electric shock to transform *E. coli* cells with DNA. Single colonies were inoculated into 3 ml of LB medium and grown overnight at 37 ^{o}C in a TC8 roller drum. The overnight cultures were then diluted 1:50 into 250 ml of fresh LB medium and grown at 37 ^{o}C with shaking in a C24 Incubator Shaker (New Brunswick Scientific, Edison, New Jersey) until the cells reached to an optical density between 0.4 and 0.6 at 600 nm (approximately 2 hours)as determined by an Eppendorf BioPhotometer spectrophotometer (Westbury, NY). The cells were then chilled to 4 ^{o}C and harvested by centrifuging at a speed of 8000 x g in an Eppendorf 5810 R centrifuge (Westbury, New York) at 4 ^{o}C. Cells were resuspended in equal volumes (250 ml) of ice cold water and centrifuged. The cold water washes were repeated twice followed by a wash in a 1:5 volume (50 ml) of ice cold 10% glycerol. The cells were resuspended in 0.5 ml of 10% ice cold glycerol and frozen at -80 ^{o}C . The electrocompetent *E. coli* cells (40 µl) were thawed on ice, mixed with 0.4-1 µl of approximately 1 µg DNA and electroporated in 2 mm cuvettes using a Bio-Rad Gene Pulser II (Bio-Rad, Hercules, CA) set at 25 µF, 2.5 kV and 200 **Ω**. The cells were immediately resuspended in 960 µl of SOC medium. After incubating them at 37 ^{o}C in an Isotemp Incubator (Fisher Scientific, Fair Lawn, NJ) for 45-120 minutes, 10 - 1000 µl of cells were spread on LB plates containing the appropriate antibiotic and incubated at 37 ^{o}C overnight. The number of colonies that appeared on the plates the next day were counted and recorded.

Transposon mutagenesis:

This was performed in *E. coli* HB101 pOR1 using EZ::TN5 Transposome from Epicentre (Madison, WI). The *E. coli* HB101 pOR1 cells were electroporated with 0.5 µl of EZ::TN*5* transposome. The cells were incubated in SOC medium (list the contents here) in a shaking

incubator for 1 hour the cells, spread on LB plates containing 50 µg/ml kanamycin and incubated overnight at 37°C.

Screening for Metal Sensitive Mutants by Replica Plating:

The colonies that grew on the LB-kanamycin plates above were spotted as a grid of 50 on fresh kanamycin plates and allowed to grow at 37 ^{o}C overnight. The cells were transferred to a Scienceware Velveteen Square (Bel-Art, Pequannock, NJ) that was mounted on a Scienceware Replica-Plating Tool (Bel-Art, Pequannock, NJ) solid cylinder. The cells were grown on M-9 agar and LB-agar plates with and without selenium. The plates were incubated overnight at 37 ^{o}C the colonies that grew on plates lacking selenium but failed to grow on plates containing selenium were selected for genomic preparations and gene rescue.

Purification of genomic DNA

The genomic DNA was purified using a Wizard Genomic DNA Purification Kit purchased from Promega (Madison, WI) and all the ingredients were supplied with this kit. The kit contains Cell Lysis Solution, Nuclei Lysis Solution, Protein Precipitation Solution, DNA Rehydration Solution and RNase Solution. An overnight culture of 1 ml was centrifuged at 13,000-16,000 x g for 2 minutes to pellet the cells. Next, the pellet was resuspended in 600 µl of Nuclei Lysis Solution by gently pipetting and incubated at 80 ^{o}C for 5 minutes to lyse the cells. The sample was cooled to room temperature and the tubes were inverted 2-5 times after adding 3 µl of RNase solution and incubated at 37 ^{o}C for 15-60 minutes. After the sample was cooled to room temperature, 200 µl of protein precipitation solution was added and the cell lysate was vortexed vigorously. After

incubating the preparation on ice for 5 minutes, the samples were centrifuged at 13,000-16,000 x g for 3 minutes. The supernatant was transferred to a clean 1.5 ml microcentrifuge tube containing 600 µl of room temperature isopropanol to precipitate the DNA. The DNA was pelleted by centrifugation at 13,000 - 16,000 x g and the supernatant was discarded. The pellet was washed with room temperature 70 % ethanol and centrifuged at 13,000 - 16,000 x g for 2 minutes. Again the supernatant was discarded and the pellet was air dried on clean absorbent paper and resuspended in 100 µl of DNA Rehydrating Solution. The DNA was stored at 4 ^{o}C.

Plasmid DNA Purification

Wizard® *Plus* SV Minipreps DNA purification System was used to purify DNA plasmids. The kit was supplied with Cell Resuspension Solution, Cell Lysis Solution, Alkaline Protease Solution, Neutralization Solution and Nuclease Free Water. The pellet obtained by centrifuging 3 ml of overnight culture in an Eppendorf 5415D at 12,000-16,000 x g for 20 seconds was resuspended in 250 µl of Cell Resuspension Solution by vortexing. Next, 250 µl of Cell Lysis Solution was added and the tube was inverted several times to lyse the cells. The lysate was incubated approximately 5 minutes and then mixed with 10 µl of Alkaline Protease Solution. The tube was inverted several times and incubated for 5 minutes at room temperature. Then, 350 µl of Neutralization Solution was added and the tube was inverted several times to mix the contents. The preparation was centrifuged at 14,000 x g for 10 minutes at room temperature. The clear lysate was transferred to the prepared Spin Column by decanting. A vacuum of at least 15 inches of mercury was applied to pull the liquid through the spin column. The vacuum was released when all liquid has been pulled through the spin column. 750 µl of the Column Wash Solution was added and a vacuum was applied to pull the Column Wash Solution through the Spin column. The wash procedure was repeated using 250 µl of Column Wash Solution. The Spin

Column was dried by applying vacuum for 10 minutes. The Spin Column was transferred to a 2 ml Collection Tube. It was centrifuged at a maximum speed for 2 minutes to remove any residual Column Wash Solution. The Spin Column was transferred to a new sterile 1.5 ml microcentrifuge tube. The plasmid DNA was eluted with 100 µl of Nuclease Free Water by centrifugation for 1 minute at room temperature. The plasmid DNA was stored at -20 ^{o}C until further use.

Ars- Kan PCR reactions:

The FRT-PGK-gb2-neo-FRT DNA template for the *ars*-Kan PCR product was obtained from GeneBridges, the *Ars* up primer had a sequence of 5'ACTTATCCGCTTCGAAGAGAGACACTACCTGCAACAATCAGGAGCGCAATAATTA ACCCTCACTAAAGGGCG 3' and the *ars* down primer had the nucleotide squence of 5' CCAGCGTCGCATCCGACATAAATAAAGCGCACTTTTCTAACAACCTGTGGTAATACG ACTCACTATAGGGCTC 3'. Phusion polymerase enzyme was used for the PCR reaction. After the PCR reaction, the Ars-Kan PCR product was cleaned using Qiagen® PCR Clean-Up kit.

Knock out of *arsR* (genes are lower case italics) gene in pOR1

The knock out was carried out using pRed / ET expression plasmid pRedET kit (GeneBridges GmgH, Heidelberg, Germany). HB101 was transformed by pSC101-BAD-gbaA. This plasmid contains the AraC regulated γ-phage *gam*, *beta* and *alpha* genes that promote homologous recombination in *E. col*i. It also contains a temperatue sensitive replication origin (repA) that is stable at 30 ^{o}C but unstable at 37 ^{o}C. A single colony of HB101 (pOR1) containing plasmid pSC101-BAD-gbaA (GeneBridges GmgH, Heidelberg, Germany) was inoculated into 5 ml of LB medium containing 100 µg/ml of Ampicillin and was grown over night at 30 ^{o}C in a TC8

Roller Drum (New Brunswick Scientific, Edison, NJ). The overnight cultures were then diluted 1:50 into 98 ml of fresh LB medium containing Ampicillin and grown at 30 ^{o}C with shaking in a C24 Incubator Shaker (New Brunswick Scientific, Edison, New Jersey) until the cells reached to an optical density between 0.3 at 600 nm (approximately 2 hours) as determined by an Eppendorf BioPhotometer spectrophotometer (Westbury, NY). Three ml of 10% Arabinose was added to the cells and the cells were incubated for an additional 45 min at 30 ^{o}C. The cells were prepared for electroporation as described above. About 40 µl of electrocompetent cells were mixed with 1 µl of the cleaned-up *ars*-Kan PCR product (1:10 dilution) and electroporated as described above. This DNA fragment was a PCR product of a kanamycin resistance cassette flanked by 50 pb regions that were homologous to the 3' and 5' ends of the *E. coli* chromosomal *ars* operon. Electroporation reactions were spread on LB plates containing kanamycin and incubated at 37 ^{o}C overnight to select against pSC101-BAD-gbaA. Four colonies were grown overnight in LB broth, frozen as glycerol stocks and named pRed ars 1, 2, 3 and 4.

Polymerase Chain Reactions (PCR) of *ars* genes:

To see whether the arsenic resistance genes were removed in the pRed ars1 and 2 strains, polymerase chain reaction was performed using Go Taq, 4 mM forward primer, 4 mM reverse primer, and nuclease free water. The ArsR F was 5'-ACT-3' and the ArsR R was 5'- ACC CAC TTACCTTGCTTCCGG -3', the Ars B F was 5' TTTTGAATGGGCGGCGCT- 3' and the Ars B R was 5' CCATCATTCGCAAACAGGGC – 3', the ArsC F was 5'- GCTGGGCCTTGCGGA AGAATAA- 3', the ArsC R was 5'- ACCACTTCTGAAGGGCGG CA- 3' and the Gor F was 5'- TTGTTGGCGCGGGTTTC- 3' and the Gor R was 5'- AATCAT GGGTCGAAGCTGCG- 3' were obtained from Integrated DNA Technologies, Inc. (Coralville, IA). For 4µL of each primer

96 µL of nuclease free water was added and the each template was diluted to 1:10 by adding 2 µL of genomic DCA to 18 µL of nuclease free water. A mix was made for 4.5 tubes by taking each primer pair in Go taq and 19 µL of mix was taken in PCR reaction tubes and 1 µL of template to 3 tubes and 1 µL of nuclease free water to the 4th tube. The PCR reaction was set up using the above ingredients and incubated overnight in a PCR Thermal Cycler set with the following program: 98 ^{o}C for 1 min, 55 ^{o}C for 20 sec, and 72 ^{o}C for 30 sec for 30 cycles followed by holding at 4 ^{o}C.

Minimum Inhibitory Concentrations (MIC) with pOR1, G50 and pRed ars strains:

MICs are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of the microorganism. MICs with sodium selenite and arsenite were carried out to confirm the sensitivity of pRed ars strains. MICs were carried out in M-9 minimal medium, pOR1 (without kanamycin), G50 and pRed ars strains were grown over night in 3 ml M-9 minimal medium with 3 µl of kanamycin at 37 ^{o}C in a TC8 roller drum incubator. Sometimes the overnight cultures did not grow in M-9 minimal medium, and the overnight cultures were grown in LB broth (Kanamycin was added to pRed and G50 all the times), 1 ml of cells were pelleted by centrifugation at 14,000 x g for 3 min and the supernatant was poured off and the cells were resuspended 1 ml of 1 X M-9 salts, the cells were again pelleted by centrifugation and resuspended in 1ml 1X M-9 salts. 1 ml of cell suspension was mixed in 49 ml of fresh M-9 minimal medium. 5 ml of the medium containing cells were taken in glass tubes. Arsenite concentrations ranging from 0 to 6000 µM were added to one set of tubes and selenite concentrations ranging from 0 to 40,000 µM were added to the other set of tubes. Initial readings at time 0 were taken using Klett colorimeter and the tubes were incubated at 37 ^{o}C in a TC8

roller drum incubator. Klett readings were taken again after 24 hrs and the readings were recorded and the data was plotted on Microsoft Excel spread sheet. The average difference between the 24 h Klett reading and the initial Klett reading was calculated for at least 3 different trials, and the standard error for each average difference was calculated using the student t distribution with a 95% confidence level. Then, the percent growth at each metal concentration was calculated by dividing the average difference of the treated cultures by the average difference of the untreated cultures. Percent error was calculated by dividing the error by the average difference of the untreated cultures.

Growth Curves for the RT –PCR Experiments:

RT –PCR Experiment was carried out to make RNA for RT-PCR reactions, overnight cultures of HB101 and pOR1 were grown in 5 ml LB medium without and with kanamycin at 37 ^{o}C in a TC8 roller drum incubator. For each strain 1 ml of overnight cells were added to 49 ml of fresh LB medium taken in a side arm flask. For each strain the medium containing the cells was split into half and taken in another side arm flask, Kanamycin was added to pOR1 cells, thus there were a total of four side arm flasks. The cells were grown at 37 ^{o}C with shaking in a C24 Incubator Shaker (New Brunswick Scientific, Edison, New Jersey). Klett reading were taken every 30 min and after 1.5 hrs of growth 1ml of sodium selenite was added to one flask and 1 ml of sterile water was added to the other flask, the same thing was done with the other two flasks. After 2.5 hrs, 2 ml of samples were taken from each culture, the cells were pelleted by centrifugation at 16000 rpm and the supernatant was poured off. The cells were resuspended in 1X M- 9 salts, the cells were again pelleted by centrifugation and the supernatant was poured off. The cells were resuspended again for RNA preps.

RNA Extraction:

RNA was extracted using Aurum Total RNA Mini Kit and iScript™ Select cDNA Synthesis Kit. For each tube 100µL of 500µg/ml lysozyme in TE was added and the cells were resuspened by pipetting up and down. The tubes were incubated at room temperature for 5 min. 350µL of lysis solution was added to each tube and the contents were mixed thoroughly by pipetting up and down. 250µL of 70 % isopropanol was added to each tube and the contents were mixed by pipetting up and down. The elution solution from the kit was warmed by placing in 70 ^{o}C water bath. An RNA binding column was inserted into a 2 ml cap less wash tube. The homogenized lysate was decanted into the RNA binding column. Centrifugation was carried out for 30 sec and the RNA binding column was removed from the wash tube and the column was replaced into the same wash tube. 700 µL of low stringency wash solution was added to the RNA binding column. Centrifugation was carried out for another 30 sec and the low stringency wash column was discarded from the wash tube and the column was replaced into the same wash tube. The DNase was provided as a lyophilized powder. The DNase was reconstituted by adding 250 µL of 10mM Tris to the vial and the contents were mixed by pipetting up and down briefly. For each processed column 315 µL of reconstituted DNase 1 with 75 µL of DNase dilution solution in a 1.5 ml centrifuge tube. 80µL of diluted DNase1 was added to the membrane stack at the bottom of each column. The digest was allowed for incubation at room temperature for 20 min. the column was centrifuged for 30 sec after the digest was complete. The digest buffer was discarded from the wash tube and the column was replaced into the same wash tube. 700 µL of high stringency wash solution was added to the RNA binding column. Centrifugation was carried out for 30 sec. The high stringency wash column was discarded from the wash tube and the column

was replaced into the same wash tube. 700 µL of low stringency wash solution was added to the RNA binding column. Centrifugation was carried out for 60 sec. The high stringency wash column was discarded from the wash tube and the column was replaced into the same wash tube. Centrifugation was carried out for an additional 2min to remove residual wash solution. The RNA binding column was transferred to a 1.5 ml capped micro centrifuge tube. 80 µL of warm elution solution was pipette onto the membrane stack at the bottom of the RNA binding column and it was allowed for 1 min for the solution to saturate the membrane. Centrifugation was carried out for 2 min to elude the total RNA. The eluted total RNA samples were used immediately in reverse transciptase reactions using equal concentration of RNA and iScript from BioRad. Then, 1.5 µl of cDNA was used in PCR reactions as described about

Chapter IV: Results & Discussion

Replica plating:

HB101 (pOR1) genes were subjected to knock out using a transposon carrying a kanamycin resistant gene. Transformations with Epicentre's EZ-Tn5 transposome, were spread on LB agar plates containing kanamycin. Transformants were then spotted on a grid and replica plated on M-9 minimal medium plates that lacked and contained 40 mM selenite. Colonies that failed to grow on the plates that contained selenite but grew on the plates that lacked it, were considered to be sensitive to selenite. One of the selected mutants was called G50 because it was located on plate G at spot 50.

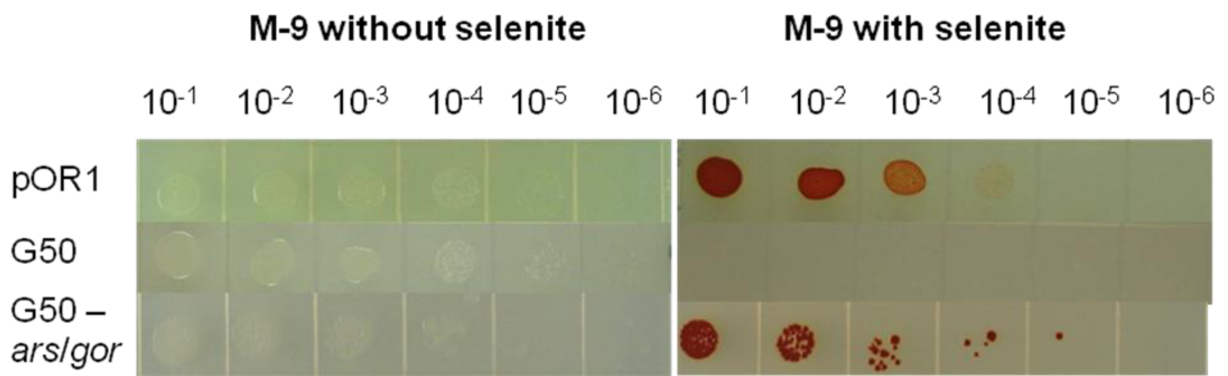
When DNA sequencing was performed, it was found that the transposon was inserted in the middle of a direct repeat that was located between the glutathione reductase gene and arsenic resistance gene on the chromosome of *E. coli* (Fig 1).

Spotting Experiments:

Dilutions of HB101 (pOR1) and G50 overnight cultures ranging from 10^{-1} to 10^{-6} were spotted on M-9 agar as well as LB agar plates with and without selenium. After incubation at 37 ^{o}C overnight, the growth of G50 was observed only in the plate without selenite. Whereas the growth of pOR1 was observed on both plates irrespective of the presence of selenium. Thus, the sensitivity was confirmed. When a plasmid containing the ars/gor genes was transformed into the G50 mutant, resistance to selenite was regained. This was also confirmed by growing pOR1, G50 and G50 *ars*/*gor* with different dilutions ranging from 10^{-1} to 10^{-6} on M-9 agar and LB agar

plates with and without selenium. After incubation overnight, HB101 (pOR1), G50 and G50 *ars*/*gor* grew on LB and M9 minimal medium agar plates without selenium. The HB101 (pOR1) and G50 *ars*/*gor* strains grew on the selenite plates but G50 failed to grow on selenite plates. You should delete figure 7 and just use figure 8 because figure 8 show the same thing as figure 7 plus the complementation information.

Figure 2: Complementation experiments were carried out in G50 strain. *Ars/gor* gene was introduced into G50 strain. It started growing in the medium without selenite and with selenite. The complemented G50 strain has shown growth up to 10^{-5} concentration.


M-9 without selenite
M-9 with selenite
10-1
10-2
10-3
10-4
10-5
10-6
pOR1
G50
G50 –
ars/gor

Liquid Culture Experiments and MICs:

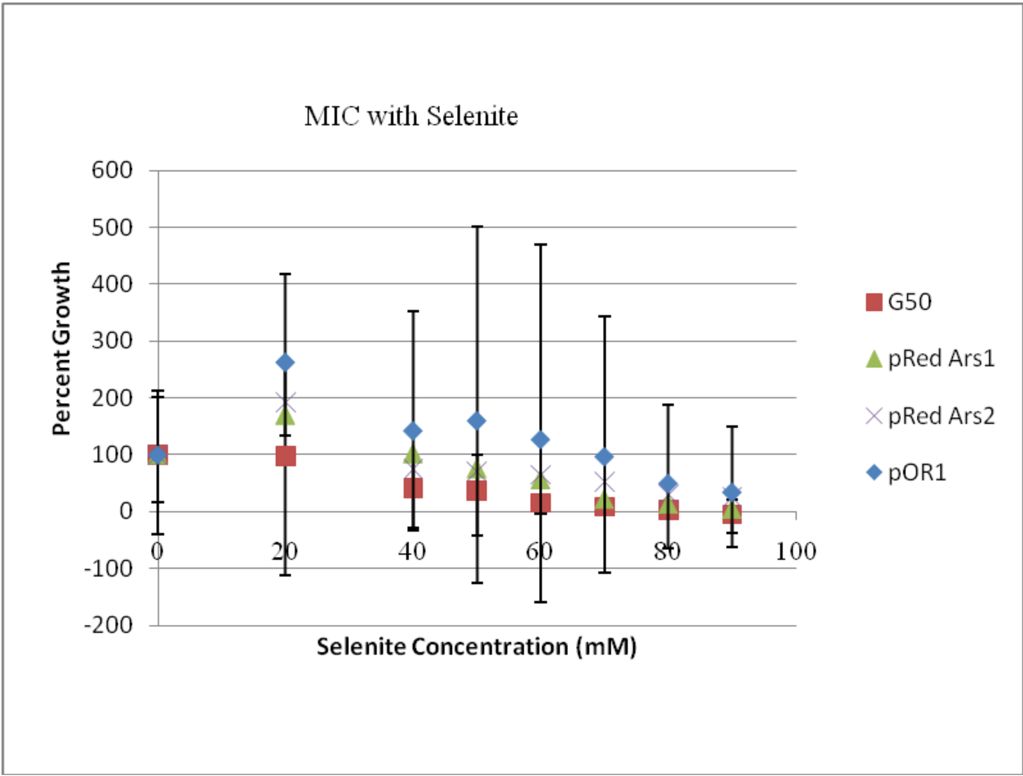
A single colony of pOR1 was picked using a sterile loop and transferred to 5 ml of LB medium without kanamycin and incubated overnight in a roller drum incubator at 37 ^{o}C. The liquid culture was taken in 1.5 ml tube and was centrifuged for 3 minutes and the supernatant was poured off, this was repeated until the whole 5 ml liquid was decanted from the culture tube and a pellet was obtained. The pellet was resuspended in 1X M9 salts and centrifuged and the supernatant was obtained this step was repeated for three times. The pellet was suspended in 1ml 1X M9 salts and was suspended into a 50 ml tube having 49 ml of M9 minimal medium. The cells were mixed well in the medium and it was transferred into 5 ml glass tubes to take the klett readings. The klett readings were noted in the book as time 0. The tubes were incubated in the roller drum incubator 37 ^{o}C for 24 hours. The tubes were taken out and vortexed and Klett readings were taken to measure the turbidity. The same experiment was performed for several times. Klett readings were tabulated in the M.S Excel spread sheet.

Table: 1 Table shows the Klett readings from Minimal Inhibitory Concentration experiment conducted in M9 minimal medium with pOR1 strain (conducted 3 different times) with a selenite concentration ranging from 0mM to 90mM. Klett reading were taken at time 0, the numbers in the first, fourth and sixth column of the table represent the time 0 Klett readings. The numbers in column 2, 5 and 7 represent the Klett readings that were taken at 24 hrs. The average difference between the 24 h Klett reading and the initial Klett reading was calculated for at least 3 different trials. Then, the percent growth at each metal concentration was calculated by dividing the average difference of the treated cultures by the average difference of the untreated cultures. Percent error was calculated by dividing the error by the average difference of the untreated cultures. And the standard error for each average difference was calculated using the student t distribution with a 95% confidence level.

MIC:

Klett Reading (Klett Units)												
pOR 1												
Trial 1			Trial 2			Trial 3						
T: 0	T:24	Difference	T: 0	T:24	Dif	T: 0	T:24	Diff	avg dif	%growth	error	%Error
16	92	76	5	161	156	8	135	127	120	100	101	84.08
10	280	270	5	292	287	10	395	385	314	262	154	128.9
10	270	260	5	98	93	12	170	158	170	142	209	174.7
15	360	345	5	84	79	13	163	150	191	160	342	285.9
15	315	300	5	32	27	7	136	129	152	127	343	286.4
15	244	229	5	51	46	7	80	73	116	97	245	205.1
15	135	120	5	17	12	6	53	47	60	50	137	114.4
12	105	93	5	7	2	8	37	29	41	35	116	97.02

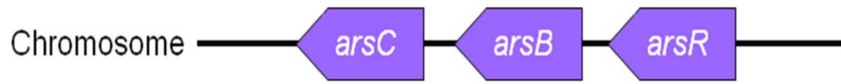
Figure 3: MIC conducted with pOR1, G50, pRedArs1and pRed ars 2. Symbols: red square, G50; green triangles, violet cross, pRed ars 1, and blue diamond- pOR1.


MIC with Selenite
Percent Growth
600
500
400
300
200
100
0
-100
-200
0
20
40
60
80
100
Selenite Concentration (mM)
G50
pRed Ars1
pRed Ars2
pOR1

Deletion of the HB101 (pOR1) chromosomal *ars* operon by homologous recombination:

Chromosomal *ars* operon was deleted using pRed ET recombination technique, the template DNA was obtained from Gene Bridges and it was amplified using phusion PCR reactions. The PCR product was cleaned and run on a gel to confirm that the PCR product was not loosed during clean up procedure, then the HB101 (pOR1) cells were electroporated with pRed ET expression plasmid. The cells were grown on LB kanamycin plate and they were again streaked on another fresh LB kanamycin plate. Six transformants were obtained on the plate and they were named as pRed ars 1, pRed ars 2 to 6.

Figures 4and 5: Knock-out of the *ars* resistance genes. The *arsR, arsB* and *arsC* genes were knocked out by the pRed ET kit. The plasmid contains the kanamycin resistant genes. The ars operon was replaced with Kanamycin resistant gene

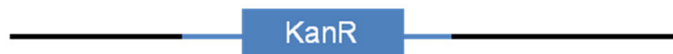


Plasmid

PCR

PCR Product

Chromosome



MICs of the pRed ars strains with selenite:

The graph shows the percent growth of pOR1, G50, pRed ars1 and pRed ars2 against time. MIC was carried out in M9 minimal medium and a different volume of Sodium selenite ranging from 0 mM to 90mM was added. Among all the strains pOR1 showed highest tolerance level towards the sodium selenite (Fig 3). pRed ars 1 also grew better than pRed ars 2 and pRed ars 4. G50 is showing least growth among all these strains. In the graph the tolerance with sodium selenite does not appear a lot among different strains, but there is lot of difference in th average klett readings. The average klett reading for pOR1 was 50, for G50 it was 13, for pRed ars1 the average klett reading was 17 and for the pRed ars 2 the average klett reading was 45.

MICs of the pRed ars strains with arsenite:

The MIC was performed in M9 minimal medium. Different volumes of sodium arsenite ranging from 0 mM to 9mM was added to the cells. Klett readings were taken at time 0 and time 24 hrs, the experiment was conducted 5different times and the average growth was calculated. From the average growth percent growth was obtained and plotted on M.S excel Spread sheet against time. pRed Ars 1 is showing maximum growth among all the other strains. There is a subtle dissimilarity among these strains when the percent growth was calculated, but the average Klett readings clearly distinguished with the percent growth of 4 different strains. The mean Klett readings for HB101 was 23 with 5mM sodium selenite, the mean Klett reading for pOR1 was 17, the mean Klett reading for G50 was 10 and for the pRed ars 1 it was 44. It is astonishing that pRed ars grew better than HB101.

Figure: 7 Illustrates the percent growth of HB101, pOR1, G50 and pRed ars1. The MIC was performed in M9 minimal medium. Different volumes of sodium arsenite ranging from 0mM to 9mM was added to the cells. Klett readings were taken at time 0 and time 24 hrs, the experiment was conducted 5different times and the average growth was calculated. From the average growth percent growth was obtained and plotted on M.S excel Spread sheet against time. pRed Ars 1 is showing maximum growth among all the other strains. There is a subtle dissimilarity among these strains when the percent growth was calculated, but the average klett readings clearly distinguished with the percent growh of 4 different strains. The mean klett readings for HB101 was 23 with 5mM sodium selenite, the mean kett reading for pOR1 was 17, the mean klett reading for G50 was 10 and for the pRed ars 1 it was 44. It is astonishing that pRed ars grew better than HB101.

Symbols: Blue diamond, HB101 (pOR1), red square-Ecoli HB101, green triangle- G50 and blue cross- pRed ars1

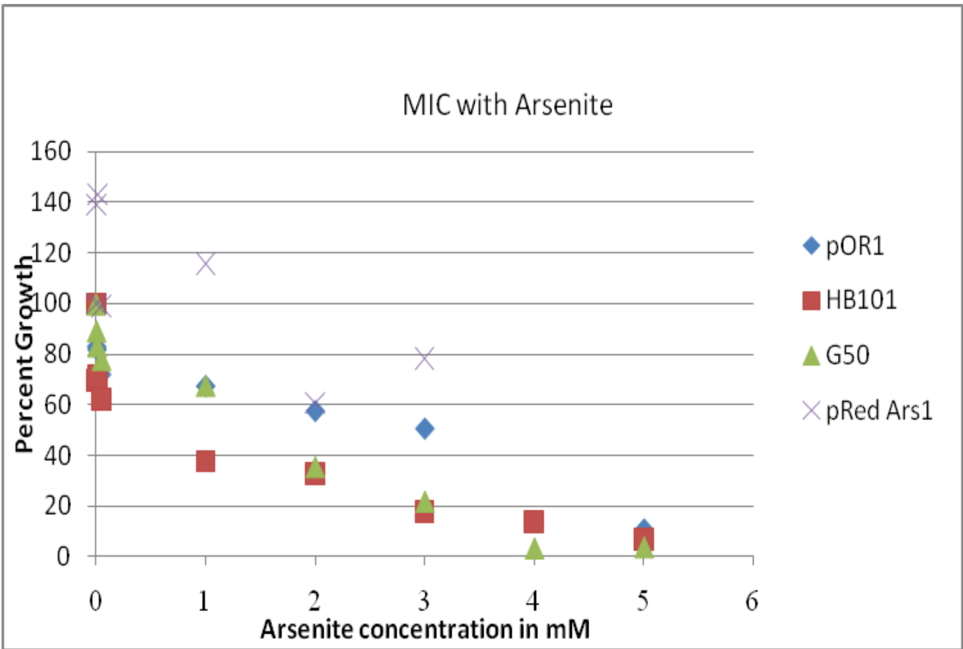


Figure 7: MIC experiment conducted with HB101, pOR1, G50 and pRed ars1 sodium arsenite

Note: No error bars included, as the error was too high and they were overlapping with each other.

Figure 8: Agarose gel image of cDNA. Four different primers, ArsR, ArsC, ArsB, Gor and Gap were used (to convert the RNA obtained from HB101 and pOR1 with and without selenite) to cDNA. The lanes are as follows

Lane1- ArsR HB101 ; Lane 2- ArsR HB101 Se; Lane3- ArsR pOR1 ; Lane 4-ArsR pOR1 Se; Lane5- Control; Lane6-100 bp Ladder; Lane 7-Ars C HB101; Lane8 -ArsC HB101 Se ; Lane9 - ArsC pOR1; Lane 10- ArsC pOR1 Se; Lane 11-Control; Lane12- 100 bp Ladder ; Lane 13- Ars B HB101; Lane 14-ArsB HB101 Se; Lane 15-ArsB pOR1; Lane16-ArsB pOR1 Se; Lane17- Control; Lane 18; 100 bp ladder; Lane 19-GOR HB101; Lane 20-GOR HB101 Se; Lane 21- GOR pOR1; Lane22- GOR pOR1 Se ; Lane23- Control; Lane24-100bp Ladder; Lane 25-GAP HB101; Lane 26-GAP HB101 Se; Lane27-GAP pOR1; Lane 28-GAP pOR1 Se; Lane 29- Control; Lane 30-100bp Ladder.

Figure:8 The strains pOR1 and HB101 were grown in four culture flasks and selenite was added two flasks and sterile water was added to other two flasks, when the cells reached the log phage. Samples were taken from the flasks 2 .5 hrs after the addition of selenite and RNA was immediately extracted. cDNA was made from the RNA using reverse transcript PCR and PCR reaction was performed with the cDNA using the primers of ars operon genes; *ars*R, *ars*B, *ars*C and gor forward and reverse primers. The bands in the lanes 4, 10 and 16 were expected to be brighter than the bands in the lane 3, 9 and 15.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

Discussion:

HB101 pOR1 is resistante to selenite with a minimum inhibitory concentration (MIC) of 40 mM selenite. From the transposon mutagenesis carried out in HB101 (pOR1), a new strain called G50 was obtained. This strain was visible sensitive to selenite in spotting experiments on LB and M-9 minimal medium (fig. 2). Complementation experiments carried out in G50 with *gor- ars* genes restored the selenite resistance. Because the transposon insert in G50 was found between the *ars* resistance genes and the *gor*, we hypothesized that it may have interfered with the expression of these genes. This work focused on the role of the chromosomal *E. coli* arsenic resistance proteins in selenite resistance.

Minimum inhibitory concentration experiments carried out in the liquid medium of M9 for number of times with G50 has given obscure results. Saw a general trend for better growth in HB101 (pOR1) strain, but the results were inconclusive because they were not statistically significant. Performing more trials of MIC with the strains, HB101 (pOR1), G50 and pRed ars may make them statistically significant. The error was calculated using student t- test, analyzing the data with other statistical techniques like ANOVA may give statistically significant data. There is a clear difference of growth between pOR1 and G50 on plates but there was not much difference in the liquid medium.

Minimum inhibitory concentration experiments that were conducted with HB101, pOR1, G50 and pRed a*rs* strains in M9 liquid medium in the presence of arsenite have given astonishing results. pRed *ars* strain grew better than any other strain that was tested, spotting experiments could be done to check the growth of this strain with the control strains.

The hypothesis was further tested by knocking out the *ars* operon. From the literature study it was interpreted that chromosomal encoded arsenic operons provide high level of resistance towards metals like arsenic and antimony, where as chromosomal encoded arsenic operons confer lower level of resistance. As it was discovered in *E. coli* strain JM109 (39), it was interpreted that other strains of *E. coli* chromosomal arsenic operon confer lower level of resistance towards arsenic. It was thought that it would be appropriate to postulate,that the transposon insert in G50 may control the arsenic operon or glutathione reductase gene (*gor*).

To see whether the γ-phage *redET* recombination was successful or not, PCR was carried out with different strains of pRed *ars*. The primers ArsR, ArsB, Ars C and Gor were used in the PCR reactions. Bands were found with ArsR,B and C primers in the strains of pRed *ars*1, pRed *ars*2, pRed *ars*3 and pRed a*rs*4 (data not shown) . The PCR results were not clear, maybe need to do a Southern blotting using KanR as a probe. Putative knock outs grew poorly in absence of arsenite. Surprisingly the arsenite MICs were not negatively affected. These knock outs grew better than the control.

To see if there is a difference in transcript levels or *ars* and *gor* genes in the presence and absence of selenite RT-PCR was performed. From the figure8, the cDNA bands of pOR1se (selenite) was not at all brighter than the bands of pOR1 without selenite. If the *ars* operon present in pOR1 and it is responsible for selenite tolerance the bands of pOR1 se must be brighter than the DNA obtained from the samples without selenite. RT- PCR could be done in the presence of sodium arsenite to check the transcript levels with the same genes.

Conclusion and future work:

From the research that was conducted with HB101 (pOR1) strain and its knock outs; G50 and pRed ars it appears that the *ars* operon might not be responsible for selenite tolerance in *E. coli*. Very interesting because the preliminary data suggest that this is not an arsenic resistance operon and may have some other function during normal growth. Future work involves Quantitative PCR analysis to measure expression levels of *ars* (*B* or *C*) and *gor* in response to selenite. Deletion analysis to identify the *ars*/*gor* region that complements (restores the activity of) the G50 mutations.

Chapter V References

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