Increased expression of *ompA*, *ompX*, *dedA*, and *gutS* genes in *Enterobacter* sp. YSU in the presence of selenite

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Abstract

Enterobacter sp. YSU is one of multiple metal-resistant bacteria isolated from East Fork Poplar Creek near a nuclear plant in Oak Ridge, Tennessee. This bacteria has a previously-detailed resistance to selenite toxicity, with the genes and proteins responsible yet to be fully elucidated. It can grow in the presence of 1 M sodium selenite in a rich LB broth and reduces the selenite to red selenium precipitates in culture. This study examines possible overexpression of *ompA*, *ompX*, *dedA*, and *gutS* genes in *Enterobacter* sp. YSU in the presence of selenite.

The four genes of interest were identified by previous studies. Overnight cell cultures of the *Enterobacter* sp. YSU strain were grown in a rich Luria-Bertani broth for 5 hours. One culture had 1 M sodium selenite added, the other water for control. Absorbance readings were taken every 30 minutes to monitor cell growth and RNA was extracted during log phase, 2.5 hours into the experiment, for an RT-PCR procedure. This RNA was subsequently converted to cDNA and amplified with primers for the four genes, then ran on 2% agarose gels. Both *ompX* and *dedA* displayed increased expression when grown with selenite but *ompA* and *gutS* had no difference in expression between the selenite culture and the control. It is possible that *ompA* had no overexpression because both of the omp proteins are hypothesized to form a single protein. Consistent low expression of *gutS* may be due to high amounts of this porin protein damaging the YSU cell membrane, due to selenite-induced apoptosis. Further work through qPCR could further elucidate the expression levels of these four genes in response to selenite.

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Chapter I: Introduction

1.1: The Y-12 Plant

During World War II, the American government constructed multiple facilities across the country for Uranium enrichment to make the first atomic bombs in the Manhattan Project. One such facility is the Y-12 Plant in Oak Ridge, Tennessee, where electromagnetic isotope separation is performed. In November 1943 the plant began separating uranium-235 from natural uranium, which is 99.3% uranium-238 (5). Radioactive uranium from the Y-12 Plant was used in the nuclear bomb "Little Boy" that America dropped on Hiroshima, Japan in 1945. Production was again heightened during the Cold War and the plant is still active today. During the Cold War, the plant imported massive amounts of mercury in order to process lithium for hydrogen bombs (5). Approximately 920,000 kg of mercury were spilled into the East Fork Poplar Creek due to poor handling of materials (5). Four S3 ponds were specifically constructed to dispose of acidic waste, but the design did not include a lining or any mechanism for decontamination of the uranium and other heavy metals within the acidic waste. Much of the toxic waste reached the nearby soil and creeks. These decades of processing heavy metals have significantly contaminated the nearby East Fork Poplar Creek, causing serious harm to plants, animals, and the ecosystem at large.

1.2: *Enterobacter* sp. YSU

Enterobacter sp. YSU was isolated from East Fork Poplar Creek and found to grow in the presence of heavy metals such as copper, mercury, silver, and selenium (6). Biochemical and 16s rDNA tests verified that it was related to the genus *Enterobacter* but

did not define it at the species level. Thus, it was named *Enterobacter* sp. YSU (7). Benchmark studies have already been conducted with regards to resistance to toxic quantities of heavy metals, with the *Enterobacter* sp. YSU displaying more mild resistance (6). The observed physical and chemical properties of the YSU species are standard for the genus *Enterobacter* as they are gram-negative bacteria and facultative anaerobes able to function in both aerobic and anaerobic environments (6). Its ability to withstand toxic concentrations of heavy metals like selenium may be related to reduction mechanisms, methylation mechanisms, detoxification, or ion efflux within the cell (9).

1.3: Selenium in Nature

The metalloid selenium was first scientifically discovered in 1817 by Swedish chemist Jons Jakob Berzelius who named it after the Ancient Greek "selene" meaning "Moon." (16). Its physical and chemical properties are most similar to adjacent elements on the periodic table, namely arsenic, tellurium, and sulfur (16). Elemental selenium rarely occurs in nature, located in metal sulfide ores. It is mainly found as an impurity, taking the place of sulfur. These stores of selenium replacing sulfur are common in Russia, China, and the USA (16). Nowadays selenium is mainly sourced from copper ores as a by-product of electrolytic refining. This commercial refining is done to produce pigments, glass, and photocell semiconductors (16). The largest producers globally are the USA, Canada, Japan, Peru, Sweden, and Belgium. Inorganic forms, including selenide, selenate, and selenite, are rare in nature and not used commercially.

While selenium is typically toxic in large amounts, it is an important trace element for many plants and animals, including humans. The human thyroid gland, and all other cells that require thyroid hormone, require selenium as a cofactor for 3 out of 4

thyroid hormone deiodinases (17). These 3 deiodinases need selenium in order to activate and subsequently deactivate thyroid hormone itself, as well as its metabolites. A daily intake of 0.2 mg of selenium has been shown to halt the effects of Hashimoto's disease, wherein the body attacks its own thyroid cells as foreign bodies (17). Other studies have also shown improved dietary selenium in meats, nuts, cereals, and mushrooms combats low-level cases of mercury toxicity (18). Selenoneine, a selenium derivative found in the blood of bluefin tuna, has displayed this capability for methylmercury detoxification, as well as rehabilitation for oxidative and inflammatory damages (18). Many other animals require selenium as an essential micronutrient (17). Plants will usually contain trace selenium as merely a bystander mineral but locoweed's growth can infer significant amounts of selenium in soil as the weed requires it. New evidence shows some plants will intake toxic doses of selenium to ward off prey (18).

These toxic doses of selenium are rare throughout nature but possible in human ailments. However, with the increasing anthropogenic climate change sweeping the globe, some animals are facing issues with selenium contamination. Aquatic birds and freshwater fish have seen spikes in birth defects from commercial refinement of selenium, as well (17). Within America, this selenium contamination is specifically stemming from coal ash at power plants seeping into nearby aquatic systems (18). Irrigation of highly seleniferous soils is another mechanism for aquatic environments to experience selenium contamination. Improper storage and disposal of radioactive wastes, as observed at the Y-12 Plant, can be deadly for the neighboring habitats (6). A 2007 study showed that approximately 40% of selenium emissions are due to human activity (18).

1.4: Selenium and Bacteria

1.4.1: Microbial Interactions with Selenium

For many years after its initial discovery, selenium was simply thought of as a toxin to be avoided. The element was blamed for "alkali disease" in the mid-19th century, a condition we now know as selenosis. Benefits of selenium were not known until it was proven to prevent liver necrosis in rats in 1957. Nowadays, we are aware that selenium is an essential trace element for much of life on Earth. Bacteria especially require it in specific amounts, as they do with the essential elements carbon, oxygen, hydrogen, nitrogen, phosphorous, and sulfur (8). While those elements are necessary components of nucleic acids, proteins, lipids, and carbohydrates, trace elements like selenium are vital for the function of certain enzymes in the cellular environment of bacteria and other organisms (8). Some of these physiological functions include biosynthesis of selenocysteine, coenzyme Q, thioredoxin reductase, and glutathione peroxidase. In bacteria, selenium metabolism is involved in a host of metabolic functions like methylation, detoxification, assimilation, and anaerobic respiration (9).

The biochemical selenium cycle was first proposed in 1964 as focus increased on this vital trace element. Attention now is on the ecological damage selenium pollution can cause, along with the effects of selenium-respiring bacteria on carbon, nitrogen and phosphorous cycles in nature. Both oxidation and reduction of selenium occur naturally and are mediated by environmental factors, chemical restraints, and biological mechanisms. Selenium metabolism is important in all domains of life, as well as in viruses (13).

1.4.2: Selenium-Reducing Bacteria

Reduction of selenium oxyanions is key for ecosystems to maintain balance. This reduction can be accomplished by both Archaea and Bacteria, some of which utilize selenium oxyanions as terminal electron acceptors during anaerobic respiration. Microorganisms may also conserve metabolic energy by reducing soluble selenate and selenite to insoluble, elemental selenium through dissimilatory reduction under anaerobic conditions (19). When bacteria live under aerobic conditions, they can reduce selenium oxyanions to elemental selenium via detoxification or redox homeostasis. Once oxyanions are reduced to elemental selenium, microbes are then able to cause further reduction to soluble selenides. Spontaneous emission of hydrogen selenide gas is a possible byproduct, but it is highly reactive and will be quickly oxidized to elemental selenium so long as oxygen is readily available (19).

1.4.3: Selenate-Reducing Bacteria

Selenate is reducible and a possible terminal electron acceptor within cells under anaerobic or anoxic conditions. Oremland et. al first detailed the role of selenate in 1989 experiments (20). They observed dissimilatory reduction of selenate to elemental selenium. Sediment slurries exhibited significant amounts of selenate to selenium reduction, with decreased rates of this reduction in portions of the sediment where preferred electron acceptors were more abundant. Higher presences of oxygen gas, chromate, and nitrate would decrease the amount of elemental selenium (20). This proved unknown bacteria were using selenate as a terminal electron acceptor. Carbon dioxide was an additional end product of the respiration process when acetate oxidation was coupled with selenate reduction. Also in 1989, Macy and her colleagues isolated a

co-culture in agriculture drainage waters from San Joaquin Valley, California that was capable of reducing selenate to elemental selenium (21). The co-culture specifically contained a strain of *Pseudomonas* which can respire selenate to selenite. An anaerobic minimal medium culture was then setup where selenate in this controlled environment reduced selenate to selenite and then finally to elemental selenium (21).

Subsequent phylogenetic studies showed many different microorganisms can couple anaerobic growth with selenate reduction and do so with a variety of terminal electron acceptors. *Gammaproteobacteria* are one example, coupling selenate reduction with oxidation of aliphatic and aromatic compounds (19). Scientists even isolated selenate-respiring bacteria from both India and New Jersey and grew them up with only selenate as a terminal electron acceptor. A diverse taxa belonging to *Gammaproteobacteria, Deltaproteobacteria, Chrysiogenetes,* and *Defferibacteres* thrived in these distant environments. A strain of *Desulfurispirillum indicum* was able to couple the reduction of selenate to the oxidation of many organic acids, like lactate and acetate (19).

Transmission electron microscopy has been used to localize selenium nanospheres within these bacterial cells (1). Reduction of selenate within the cytoplasm formed selenium precipitates that the cells needed to export. Breakdown of these precipitates is part of a two-step process wherein selenium is produced and exported from the cell. Details on the cellular mechanisms involved are still unclear and being investigated, but the enzymes and electron transport pathways involved in the initial selenium production, via selenate reduction, have been elucidated. Nancharaiah et. al studied the enzymes of two gram-positive and one gram-negative bacteria (19).

The gram-negative bacterium, *T. selenatis* (19), is another bacterium sourced from the seleniferous waters of San Joaquin, California and uses selenate as an electron acceptor. Large accumulation of red elemental selenium nanospheres were observed in its cytoplasm and selenate reductase was located in the cell's periplasmic space. Biochemical analysis showed that this enzyme was composed of a catalytic unit (SerA), an iron-sulfur protein (SerB), a heme B protein (SerC), and a molybdenum cofactor (19). Selenate reductase inhibitors used to decode the electron transport pathway with myxothiazol, a QCR inhibitor, indicated that quinol cytochrome c oxidoreductase inhibition was the pathway for electron transfer to SerABC, and that reduction of selenate to selenide was occurring in the periplasmic space of this bacterium (20). A sulfate transporter is known to deliver selenite to the cytoplasm where the mechanism for reduction to the observed red elemental selenium is unknown (20). *E. cloacae,* another gram-negative bacterium, was isolated from seleniferous waters in the San Luis drain in California. Its selenate reductase activity was inhibited by tungstate, suggesting the electron transport pathway in this bacterium also required a molybdenum enzyme (19). The reduction of selenate to selenide again occurred in the periplasmic compartment. One gram-positive bacterium, *B. selenatarsenatis,* was collected from sediment deposits of a glass manufacturing plant. Selenate was still the terminal electron acceptor, but lactate was the electron donor. A membrane-bound molybdoenzyme catalyzes this reduction process both within the periplasmic space and just outside of the cell (20).

1.4.4: Selenite-Reducing Bacteria

Selenite reduction can also be categorized into detoxification and anaerobic respiration processes. Here, very little reduction has been observed with lactate as the

electron donor, and zero reduction with acetate as the electron donor (21). Microorganisms will detoxify selenite to selenium through Painter-type reactions, thioredoxin reductase, sulfide-mediated reduction, and siderophore-mediated reduction (21). These processes are mediated by thiols in the cytoplasm. Selenite will react with glutathione to form selenodiglutathione, which will be further reduced to unstable selenopersulfide of glutathione by an NADPH-glutathione reductase within the cell (21). This unstable intermediate is hydrolyzed to elemental selenium and reduced glutathione.

Painter-type reactions have been visualized in *Escherichia coli* cells exposed to selenite by using selenium nuclear magnetic resonance. This occurs in a series of reactions, with the initial reaction following this proposed equation:

$$4\ RSH + SeO_3^{2-} + 2H \rightarrow RS\text{–}Se\text{–}SR + RSSR + 3H_2O$$

Kessi and Hanselmaan (22) also studied *E. coli* cells grown in the presence of selenite and observed glutathione reduction to selenium as a final step, but noted superoxide anions within the cell were used to protect the cell from oxidative stress in a 3-part sequence:

$$6\ GSH + 3\ SeO_3^{2-} \rightarrow 3\ GS\text{–}Se\text{–}SG + 3\ O_2 + 3\ H_2O$$

$$GS\text{–}Se\text{–}SG + NADPH \rightarrow GSH + GS\text{–}Se + NADP^+$$

$$GS\text{–}SeH \rightarrow GSH + Se^0$$

Thioredoxin [Trx(SH)2] reduction via thioredoxin reductase is another system for bacteria to reduce selenite to elemental selenium. First, *E. coli* cells will form oxidized thioredoxin ($Trx\text{-}S_2$), a selenopersulfide anion, and the reduced glutathione:

$$TrxSH_2 + GS\text{–}Se\text{–}SG \rightarrow Trx\text{-}S_2 + GSH + GS\text{-}Se\ H$$

Further reactions will finish the process when biogenic sulfide is available, and produce elemental selenium (22):

$$SeO_3^{2-} + 2\ HS\ 4\ H \rightarrow Se^0 + 2\ S^0 + 3\ H_2O$$

Siderophore-mediated reductions in *Pseudomonas stutzeri* have been observed with the iron siderophore pyridine-2,6-bis(thiocarboxylic acid) (PDTC; $[C_7H_3O_2S_2]$ 2) (21):

$$[C_7H_3O_2S_2]_2 + 2\ H_2O \rightarrow [C_7H_3O_4]_2 + 2\ HS^- + 2\ H^+$$

The hydrogen sulfide formed is then hydrolyzed, and finally, undergoes the same reducing agent step as with thioredoxin reductase to form elemental selenium:

$$SeO_3^{2-} + 2\ HS\ 4\ H \rightarrow Se^0 + 2\ S^0 + 3\ H_2O$$

1.4.5: Selenium-Oxidizing Bacteria

The other half of the natural selenium cycle is fulfilled by oxidation of elemental selenium and selenide back to selenate or selenite by some selenium-oxidizing bacteria (19). Chemolithoautotrophic soil bacteria were observed oxidizing selenium as far back as 1923, when the process was implemented to increase soil acidity. Cultured samples of soil slurries could grow with only selenium as an energy source. Expanded research from Sarathchandra and Watkinson discovered *Bacillus megaterium* could produce selenite from a selenium energy source (23). Autoclaving or addition of metabolic inhibitors such as antibiotics stopped this oxidation process in culture; addition of sulfide, acetate, or glucose enhanced oxidation. Researchers inferred that either chemoheterotrophic or

chemoautotrophic bacteria were involved (23). Losi and Frankenberger reported similar results around the same time (24). They detailed microbial oxidation and solubilization of selenium precipitates in soil and proved that some bacteria favored selenium oxidation over glucose oxidation in soil samples. This again implied chemoautotrophic oxidation. One drawback they noted was the relatively low rates of selenium oxidation; reduction rates are 3 to 4 times faster within the selenium cycle (24).

1.5: Selenium-Reducing Bacteria Ecology

Seleniferous habitats are often found near toxic waste or sedimental slurries, but selenium-respiring bacteria can be located in the most pristine of environments as well. Yet, there is still a gap in knowledge of the true abundance and ecological role of naturally occurring bacteria that reduce selenium (14). Selenate reduction is widespread in environments where selenium oxyanions should hypothetically not be present in large quantities. Researchers attribute this to anthropogenic activities like glass manufacturing, uranium refinement, agricultural irrigation, and dumping of toxic waste (4). Rapid appearance of selenium-reducing bacteria in these environments has been marked as proof of high metabolic activity (4). Their ability to couple the oxidation of inorganic and organic (aliphatic and aromatic compounds) molecules to selenium oxyanion reduction means these bacteria have a role to play in the carbon cycle of their habitats as well.

1.6: Bioremediation of Selenium Oxyanion Pollution

Advancements in biotechnology are elucidating microorganisms that may sequester selenium oxyanions as a potential strategy for decontamination of selenium

pollution. Multiple bacterial strains have demonstrated ability to uptake selenate and selenite using processes such as a sulfate transporter in *E. coli* (3). Several other methods exist for bacteria to incorporate sequestered selenium oxyanions into selenium amino acids such as seleno-cysteine. Sulfate permease in *Salmonella typhirium*, sulfite uptake in *Clostridium pasteurianum*, and the polyol ABC transporter in *R. Sphaeriodes* all construct these selenium amino acids for production of selenoproteins (25). Bacteria, like *Enterobacter cloacae* SLS1a-1, have also demonstrated ability to biomethylate selenium oxyanions into selenium methyl derivatives like dimethyl sulfide.

Recent projects for "green" decontamination of selenium pollution have targeted surface waters and wastewaters. They exploit bacterial strains capable of reducing selenate and selenite in order to detoxify these environments (25). Both aerobic and anaerobic microorganisms are being tested, although the aerobic bacterial strains face the complication of dissolved oxygen competing to be the terminal electron acceptor in the cell (25). Studies on large-scale applications of this technology are earmarked for future research.

As previously discussed, bacteria belonging to *Pseduomonas, Bacillus, Enterobacter*, etc. have displayed bioconversion of selenate to selenite under anoxic and aerobic conditions. Several of these anaerobic microorganisms have shown use of selenate as a terminal electron acceptor as well. Therefore, researchers are investigating *Pseudomonas stutzeri* as a facultative anaerobe that can be introduced into selenium-polluted environments to intake selenate (25). Being more soluble and reactive, selenite can more easily be removed through detoxification or anaerobic respiration. The aforementioned Painter-type reactions, thioredoxin reductase systems, and iron

siderophores are being manipulated and optimized for detoxification strategies (25). Laboratory tests show *Shewanella oneidensis* MR-1 and *Veillonella atypica* utilize these methods to reduce selenite to the safer elemental selenium (25).

1.7: Identification of the OmpA, OmpX, DedA, and GutS proteins

Studies characterize *Enterobacter* sp. YSU as resistant to several heavy metal salts, selenite included. Minimal medium experiments with both *E. coli* and *Enterobacter* sp. YSU showed that selenite enters the bacterial cell through two possible mechanisms: a specific pathway and a non-specific pathway. (7). A sulfate permease channel being the mechanism for the non-specific pathway's function. The specific pathway works through an unknown mechanism. When the selenite concentration was 100,000 times higher than the sulfate concentration, Jasenec et. al determined that the addition of 40 μg/ml L-cysteine conferred selenite resistance upon *Enterobacter* sp. YSU through reduction of selenite transport levels via the cell's -specific pathway (7). The non-specific pathway through sulfate permease appeared to be repressed.

Under selenite sensitive conditions in this experiment, *Enterobacter* sp. YSU increased expression levels of a tellurium resistance protein (7). As tellurium is one row below selenite in the periodic table, this was likely a non-specific response to the selenite. Two other identified proteins with high expression in high selenite environments included variations of the OmpA and OmpX proteins. It is hypothesized that, due to their sharing of multiple peptides, these proteins may form a single OmpA protein. Transposon mutagenesis revealed a variety of other proteins that may be involved in selenite uptake. Two of interest were sequenced and studied here: GutS and DedA, proteins found to be involved in selenite resistance in *Escherichia coli*. Protein motif

research has shown the GutS proteins to be up-regulated in response to both selenite and tellurite, but their functions in the cell are not yet elucidated (33). The DedA protein family also has currently unknown functions, but previous research has suggested it is strongly involved in selenite uptake in bacteria (34).

The Jasenec et al study employed proteomics and RT-PCR to demonstrate that selenite resistance could be conferred to bacterial cells through feedback inhibition of the synthesis of N-acetylserine (7). This intermediate acts as an inducer for the genes *cysA*, *cysW*, and *cysT*. The latter 3 are sulfate permease genes and their decreased expression is thought to have limited selenite transport into the cells by the non-specific pathway (7).

1.8: Specific Aims/Experimental Design

Previously studied *Enterobacter* sp. YSU is a gram-negative bacteria known to display strong resistance to many heavy metals, including selenite. Oligonucleotide primers were designed for *ompA*, *ompX*, *dedA*, and *gutS* genes to examine which genes in this bacteria are responsible for resistance to selenite. Purified RNA from cells that were grown in the absence and presence of selenite and converted it to cDNA. This cDNA was tested by RT-PCR to determine if there was an increase in expression of the *ompA*, *ompX*, *dedA*, and *gutS* genes in the presence of selenite.

Chapter II: Hypothesis

Since previous research in *Enterobacter* sp. YSU strains showed that *ompA*, *ompX*, *dedA*, and *gutS* genes are expressed at higher levels in the presence of selenite (7,33,34), we expect RT-PCR to show that they are expressed at a higher level in the presence of selenite.

Chapter III: Materials and Methods

3.1: Bacterial Strains and Growth Media

Enterobacter sp. YSU was isolated from East Fork Poplar Creek in Oak Ridge, Tennessee (6). Competent *Escherichia coli* EC100D™ *pir*-116 cells were obtained from Illumina (San Diego, CA).

Luria-Bertani (LB) medium (Fisher Scientific, Waltham, MA) contained 10 g/l tryptone, 5 g/l sodium chloride, and 5 g/l yeast extract (26). For preparation of LB agar plates, 1.6% agar (Amresco, Solon, OH) was added to the LB broth. Kanamycin (Amresco, Solon, OH) was added to LB media at 50 μg/ml to screen for resistance to the antibiotic.

3.2: Genomic Preparations

A DNA purification kit was utilized for isolation of DNA from the Gram-negative bacteria cells of *Enterobacter* sp. YSU. First, the cells were lysed with 600 μl nuclei lysis solution (Promega, Madison, WI). This solution was mixed with gentle pipetting and incubated for 5 minutes at 80℃. After cooling, 3 μl of 4 mg/ml RNase solution (Promega, Madison, WI) was added and mixed in (30). The cells were then incubated for 15 minutes at 37℃ and allowed to cool to room temperature. For protein precipitation, 200 μl of protein precipitation solution (Promega, Madison, WI) was added and the cells were vortexed for 20 seconds. This solution was incubated on ice for 5 minutes and then centrifuged for 3 minutes at 14000 rpm (30). Resulting supernatant was transferred to a clean tube containing 600 μl of room temperature isopropanol and mixed. DNA was

pelleted via centrifugation for 2 minutes at 14000 rpm and the supernatant was discarded. Next, 600 µl of 70% ethanol was added. The solution was mixed through pipetting and centrifuged for 2 minutes at 14000 rpm. Ethanol was aspirated and the pellet air dried for 10 minutes. To rehydrate the pellet, 100 µl of rehydration solution (Promega, Madison, WI) was added and left to sit overnight at 4℃ (30).

3.3: Growth Curves

An overnight cell culture of *Enterobacter* sp. YSU was grown in LB broth and in conditions with and without selenite. For the overnight culture 4.7 ml of LB broth were combined with 0.3 ml of sterile water, then a single colony of the *Enterobacter* sp. YSU strain was added and grown overnight in a 37℃ roller drum. The next day, two test tubes were each filled with 6 ml of LB broth and 60 µl of the overnight culture for a 1:100 dilution. Cell culture readings were taken with a NanoDrop 8000 (ThermoFisher Scientific, Waltham, MA) using a cuvette and 100 µl of cells per reading (31). The instrument was blanked with 100 µl of LB broth and cell culture readings were taken every 30 minutes, with the culture tubes being placed in a roller drum at 37℃. Readings were taken one tube at a time so the cells were not allowed to cool to room temperature. After 1.5 hours of growth, the experimental tube had 216 µl of 1 M selenite added for a final concentration of 37 mM selenite. The tube for the negative control had 216 µl of sterile water added to keep consistent volumes. Samples (200 µl) for RNA preps were taken 2.5 hours in, and readings continued for 5 total hours. Data from these readings was used to compile growth curves for the selenite and control conditions (31).

3.4: Total RNA Preparation

The 100 µl and 200 µl growth curve samples were added to 200 µl and 400 µl, respectively, of RNA Protect (QIAGEN, Germantown, MD). Then, 1.7 ml microfuge tubes with these solutions were immediately vortexed for 5 seconds and then incubated at room temperature for 5 minutes. Tubes were centrifuged for 10 minutes at 10000 rpm, supernatant was poured off, and the pellet was kept at -80℃ overnight.

Pellets were thawed out the next day and each was resuspended in 110 µl of a TE buffer mixture. This buffer consisted of 10 µl of proteinase K (QIAGEN, Germantown, MD) mixed with 100 µl of TE buffer (30 mM tris, 1 mM EDTA, pH 8.0) containing 15 mg/ml of lysozyme. For each sample, the total buffer solution was 45 µl proteinase K + 450 µl TE + 6.75 mg lysozyme. The resuspended cells were incubated for 10 minutes at room temperature, with vortexing for 10 seconds every 2 minutes. Each sample was then mixed with 350 µl of RLT buffer (QIAGEN, Germantown, MD) containing 10 µl □-mercaptoethanol/ml RLT buffer for a total of 1.575 ml RLT + 1.58 µl □-mercaptoethanol. All samples were centrifuged for 2 minutes at 10,000 rpm and the supernatant for each placed in new 1.7 ml tubes containing 250 µl of 100% ethanol. These solutions were transferred to RNA spin columns and centrifuged for 30 seconds to bind the RNA to the column (32).

The flow-through was discarded and 700 µl of Buffer RW1 (QIAGEN, Germantown, MD) was added to each of the 4 spin columns. Then, they were placed in the centrifuge for 30 seconds at 10,000 rpm and the flow-through was discarded. Two wash steps with 500 µl of Buffer RPE (QIAGEN, Germantown, MD) were centrifuged through the spin column, the first for 30 seconds and the second for 2 minutes. Again, the flow-through was discarded after each wash step. The columns were given a final 1

minute, 10,000 rpm centrifugation without buffer to ensure all wash solution had been removed (32).

Spin columns were placed into new 1.5 ml collection tubes and RNA was eluted via 30 µl of RNase-free water (QIAGEN, Germantown, MD). They were centrifuged for 90 seconds at 1,000 rpm and then this elution step was repeated again (32). Eluted RNA was transferred into 4 separate 0.65 ml microfuge tubes. A DNase treatment was performed with 6 µl of 10x TURBO DNase buffer (Fisher Scientific, Waltham, MA) and 1 µl of TURBO DNase (Fisher Scientific, Waltham, MA). Solutions were mixed by gently flicking the tube and then incubated for 20 minutes at 37℃. Next, 7 µl of DNase inactivation reagent (Fisher Scientific, Waltham, MA) was added to each microfuge tube and mixed in through pipetting up and down. Tubes were incubated for 5 minutes at room temperature and microfuged for 90 seconds at 10,000 rpm. The resulting supernatant was transferred to new, clean 0.65 ml tubes and tested on the NanoDrop 8000 for RNA concentrations in ng/µl (32).

3.5: cDNA Synthesis

Prepared RNA was then reverse transcribed into complementary DNA (cDNA). Measured RNA concentrations were used to calculate 100 ng of RNA for each sample. Nuclease-free water was added to bring the total volume of each sample up to 6 µl. Next, 2 µl of Random Primer Mix (NewEngland BioLabs, Ipswich, MA) was added for a total volume of 8 µl. A second tube, for a negative control, was assembled for each RNA sample. These RNA/primer mixes were denatured at 65℃ for 5 minutes in the thermocycler (Eppendorf, Enfield, CT). Tubes were cooled on ice before adding 10 µl of ProtoScript II Reaction Mix (NewEngland BioLabs, Ipswich, MA). For the controls, 2 µl

of nuclease-free water was added, and 2 µl of ProtoScript II Enzyme Mix (NewEngland BioLabs) was added for the experimental tubes. Synthesis of cDNA was completed with one cycle of 25℃ for 5 minutes, 42℃ for 60 minutes and 80℃ for 5 minutes. samples were held at 4℃.

3.6: Primers

Custom DNA oligonucleotides were designed using GenomeCompiler and purchased from IDT DNA technologies (Coralville, Iowa).

Primer	Nucleotide Sequence
OmpA1_1	5'-GAC TGA AGC GGC TGA AGA A-3'
ompA1_2	5'-GTT GCT GAC CCT CGA AAA C-3'
ompX1_1	5'-CGC GAT AAG TAT GAC TGC C-3'
ompX1_2	5'-CCG CCA GTT TAC ATT ACC A-3'
ompA_2_FWD	5'-CCG TTG TCT GGA CGA ACG CCA A -3'
ompA_2_REV	5'-CTG GTG GCG TTG AGT GGG CAA T-3'
ompX_2_FWD	5'-GCA CGG CAT CTT CAA GGT GGC T-3'
ompX_2_REV	5'-GAA CTC CCC TCC GGC CAG CTT A -3'
gutS_1_FWD	5'-GCT GCC CGT TTC CAG CAT GAG T-3'
gutS_1_REV	5'-CCA TCA GCA CGA AGC CGA AAC G-3'
dedA_2_FWD	5'-ACC CGC CCG ATG GTG TAG TTG A-3'
dedA_2_REV	5'-CCG TTC CTG CCG GGA GAT TCA

	C-3’
YSU-GAPDH_2_FWD	5’-GGA AAC GAT GTC CTG GCC AGC G-3’
YSU-GAPDH_2_REV	5’-ACA CAT CAC TGC GGG TGC GAA G-3’

Table 1.

3.7: PCR Amplification

Initially, primers for targeted sequences were designed using the GenomeCompiler software (Twist Biosciences, San Francisco, CA) for the outer membrane proteins of selenite-resistant bacteria. Primer pairs were chosen for the outer membrane proteins A and X in the *Enterobacter* sp. YSU bacterial strain (Table 1). Primer pellets were spun 10 seconds before dissolving in 100 µM with TE buffer. TE buffer contained 10 mM Tris-HCl (pH 8.0) (Amresco, Solon, OH) and 1 mM EDTA (Amresco, Solon, OH). A 10 µM working stock was made with 10 µl of dissolved primer mixed with 90 µl of nuclease-free water (28).

The first Q5 PCR was done using these primers with YSU strain genomic DNA as the template DNA. The reaction mixture contained 12.5 µl 2x Q5 DNA polymerase (NewEngland Biolabs, Ipswich, MA), 1.25 µl primer 1, 1.25 µl primer 2, 8 µl nuclease-free water, and 2 µl of YSU genomic DNA as a template. The final concentration of each primer was 0.5 µM. A PCR thermal cycler (ThermoFisher Scientific, Waltham, MA) was set to standard protocol and amplified DNA was stored afterwards at -20℃ (28). This protocol consisted of: 98℃ for 30 seconds, 98℃ for 10 seconds, 60±10℃ for 15 seconds (temperature here determined by the used primers’

annealing temperature), 70℃ for 40 seconds (20 seconds for every kilobase expected) and then go to step 2, repeat 34 times, and finally 72℃ for 60 seconds.

GoTaq Green polymerase (Promega, Madison, WI) was also utilized for multiple PCR reactions. First, 100 µM stock primers were diluted to 4 µM by mixing 4 µl of primer with 96 µl of nuclease-free water. Then a reaction mix was set up. For one tube, components were: 10 µl of 2xGoTaq Green polymerase, 2.5 µl of forward primer, 2.5 µl of reverse primer, 4 µl of nuclease-free water, and 1 µl of template or water for control. For the selenite-sensitive experiment done here, 6 tubes were set up for each set of primers, Tube 1 contained *Enterobacter* sp. YSU DNA template, tubes 2-3 contained cDNA mixed with ProtoScript II enzyme mix, tubes 4-5 contained cDNA without the enzyme, and tube 6 was completed with 1 µl of nuclease-free water. A PCR thermal cycler (ThermoFisher Scientific, Waltham, MA) was set for the following program: 95℃ for 120 seconds followed by 30 cycles of 95℃ for 60 seconds, and 62℃ for 60 seconds (temperature here determined by the used primers' annealing temperatures) 72℃ for 30 seconds. After a final incubation step of 72℃ for 10 minutes, the samples were held at 25℃

3.8: Agarose Gel Electrophoresis

Agarose gels (1%) were prepared by adding 1.3 g of BioExcell Agarose LE (WorldWide Medical Products, Bristol, PA) to 130 ml of 1x TBE buffer (Amresco, Solon, OH). For 2% agarose gels, 2.6 g of agarose were used with the same volume of TBE buffer. 1x TBE buffer contained 0.089 M Tris Base, 0.089 M Borate, and 0.002 M EDTA. Agarose/TBE mix was microwaved until the agarose had fully dissolved, then 13 µl (1:10,000 dilution of the stock solution) of GelGreen Nucleic Acid Stain (Embi Tec)

was added. After mixing it with a stir bar on a stir plate, the contents were poured into a gel tray with combs inserted to form wells. The gel was allowed to harden for approximately 30 minutes and the combs were removed. When needed, gels were placed in a RunOne Electrophoresis System (Embi Tec, San Diego, CA) and covered with 1x TBE buffer. DNA was separated at 100v current; gels were stopped once bromophenol blue was near the bottom (negative) end of the gel. Visualization was done using a PrepOne Sapphire illuminator with an Embi Tec camera.

Chapter IV: Results

4.1: Growth Curves

Experiments were conducted to determine which of the *ompA, ompX, gutS,* and *dedA* genes exhibited overexpression in response to selenite. This was accomplished by growing *Enterobacter* sp. YSU in cultures with and without selenite. RNA was extracted from these cell cultures, and converted to cDNA. This DNA was then used in a PCR reaction with primers for the target genes so their expression levels be could be observed qualitatively.

The initial experiment conducted is displayed in a growth curve in **Figure 1.** This figure is presented to show the effect of sodium selenite addition to the YSU bacterial strain grown in rich LB broth, and test the survivability of the cells. At first, the experiment was conducted with M-9 minimal medium but was then switched to rich LB broth after problems were encountered with obtaining a sufficiently high yield of RNA for analysis. An overnight culture of *Enterobacter* sp. YSU was diluted 1:100 in fresh medium and split into two different cultures. Optical density readings were taken every

30 minutes. After 1.5 hours of growth selenite was added to a final concentration of 37 mM to one culture and equal volume of water was added to the other culture. Cells were harvested for RNA purification after 2.5 hours of growth and 1 hour after the addition of selenite. This allowed ample time for the bacteria to respond to selenite, and samples to be taken during log phase. Reduction of selenite to elemental selenium in the experimental tubes can be seen via their red color in **Figure 2**. The experiment was conducted in triplicate, and once more, for a total of four data sets to ensure consistency. Data from **Table 2** was used to compile the growth curve. The data show that the selenite-treated culture had a slightly decreased *Enterobacter* sp. YSU cell density as compared to the untreated positive control. The decrease in density showing this occurs beginning at 2 hours in, meaning the addition of selenite caused the experimental culture to slow its growth. This may be seen in the growth curve graph of **Figure 1**, with the trend consistent throughout the final 3.5 hours of growth. Conclusively, addition of selenite hindered the growth of the YSU strain but did not kill it.

Table 2 : Optical density from growth curve of *Enterobacter* sp. YSU

TIME	NoSe	Se
0	0.03825	0.04325
0.5	0.07325	0.0745
1	0.15525	0.1555
1.5	0.29025	0.288
2	0.43325	0.4055
2.5	0.60575	0.51975
3	0.797	0.62375

3.5	0.9155	0.7795
4	1.00575	0.88675
4.5	1.1135	1.01625
5	1.1985	1.0605

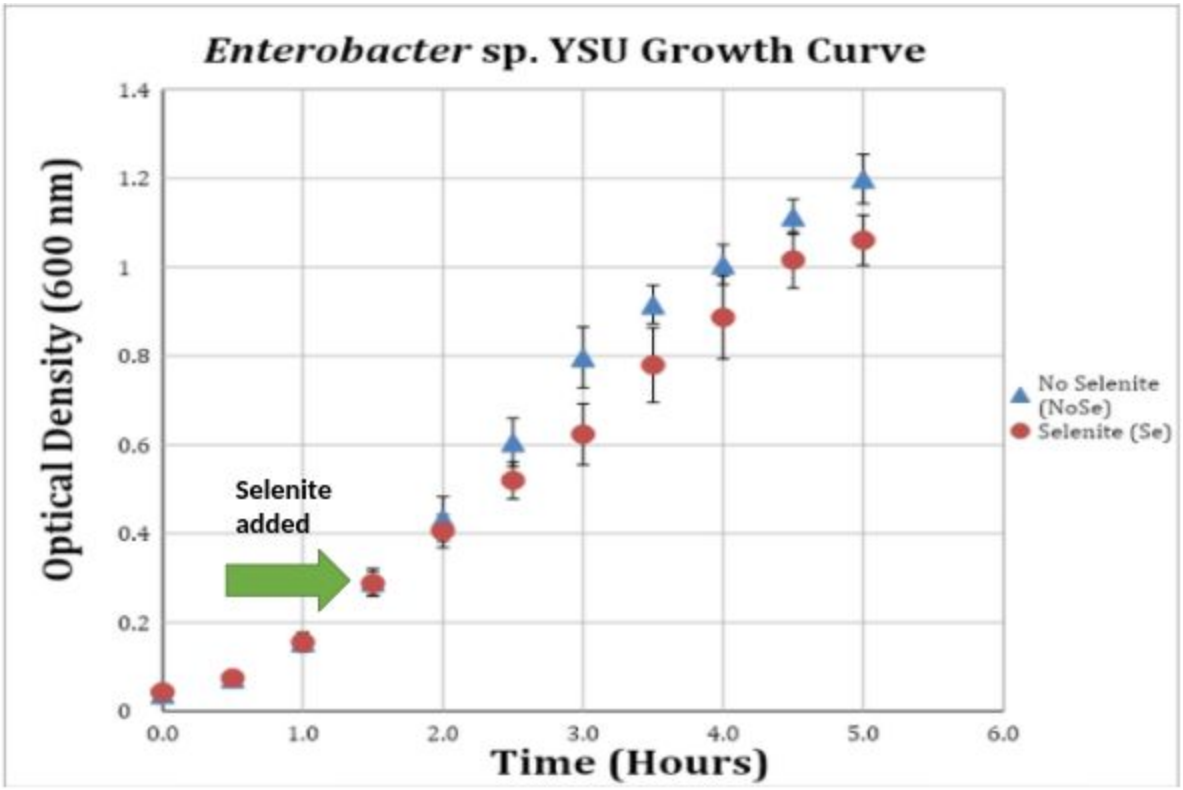

Figure 1: Optical density growth curves: Overnight culture was diluted 1:100 in LB broth and grown in a roller drum at 37℃ for five hours. After 1.5 hours of growth, water or sodium selenite was added to each culture. Optical density was measured every 30 minutes using a NanoDrop 8000. Values shown at each point in time are the average of 4 total experiments. Experiment was done in triplicate to ensure accuracy and consistency of the results, and was repeated again for 4 total trials that are seen in Figure 1.

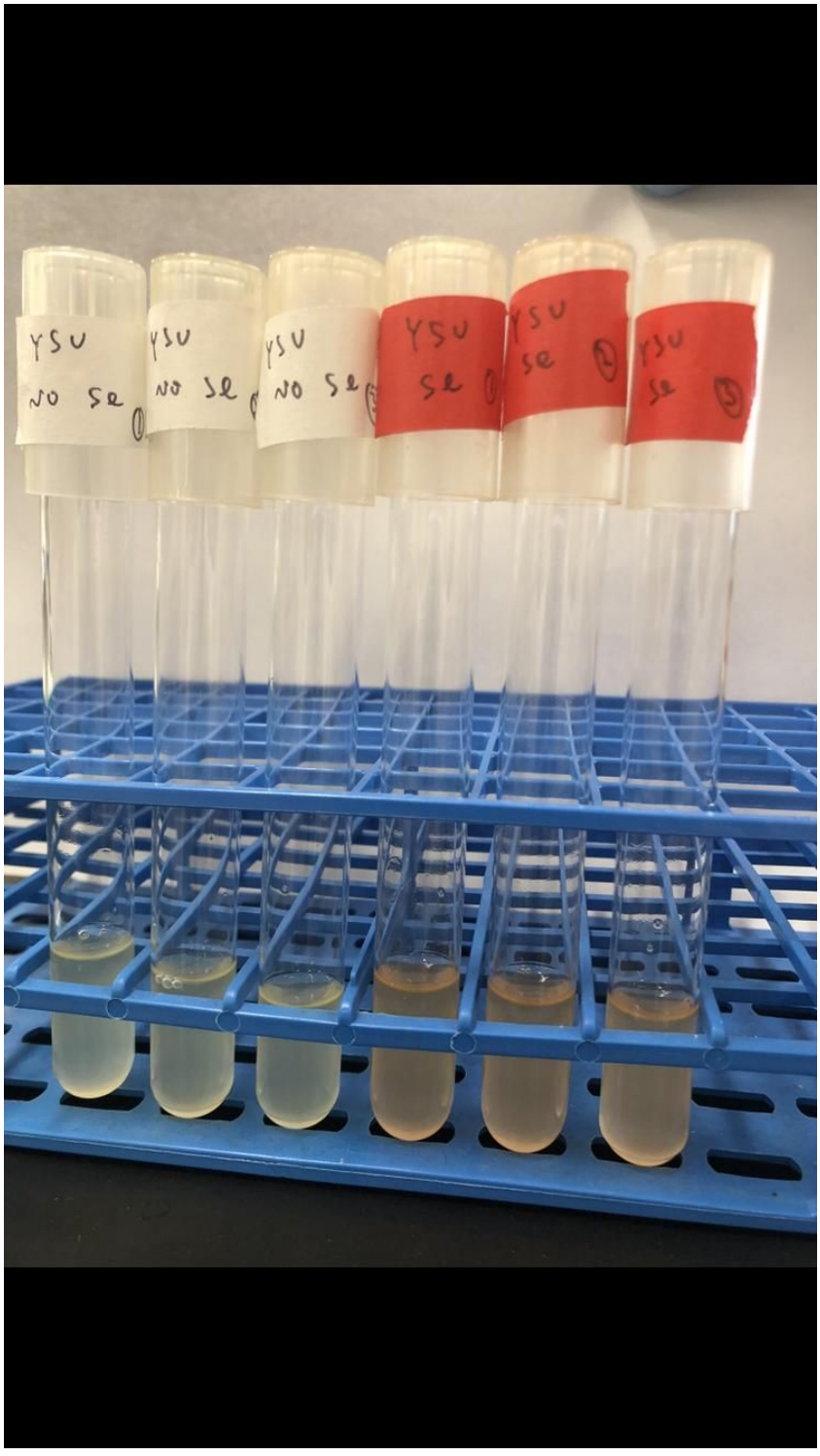
YSU
NO Se
YSU
NO Se
YSU
NO Se
YSU
Se
YSU
Se
YSU
Se

Figure 2: Selenite supplementation to LB cell cultures: An overnight culture of the *Enterobacter* sp. YSU strain was diluted (1:100) into LB growth media. Sodium selenite was added to one set of tubes and the cultures were grown in a roller drum at 37℃ for five hours. Experiment was repeated in triplicate for accuracy, with the three tubes on the right the no selenite conditions and the three tubes on the right as the selenite conditions. Faded red color of the selenite (Se) tubes shows production of reduced red elemental selenium.

4.2: Agarose Gel Electrophoresis

Research has shown the OmpA, OmpX, DedA, and GutS proteins are involved in the selenite resistance mechanisms of bacteria (33,34,35). An RT-PCR analysis was utilized here to determine if the genes for these proteins would display increased expression in *Enterobacter* sp. YSU when grown in selenite sensitive conditions. Primers (**Table 1**) were designed for genes of interest and included in a GoTaq PCR of the growth curve-synthesized cDNA. The housekeeping gene GAPDH was used as a positive control to show that transcriptional levels were normalized between the two growth conditions. Bands near 100 bp for the enzyme with and without selenite in **lanes 3 and 4 of Figure 3** confirmed this, with these bands having virtually the same intensity as the band for the positive control, genomic DNA of the YSU strain. Lanes 5 and 6 in **Figure 3** depicting the PCR mix without the enzyme required to synthesize cDNA are blank, demonstrating that there is no DNA contamination in the RNA samples. Those lanes match the lack of DNA shown by the negative control of nuclease-free water set up within the PCR.

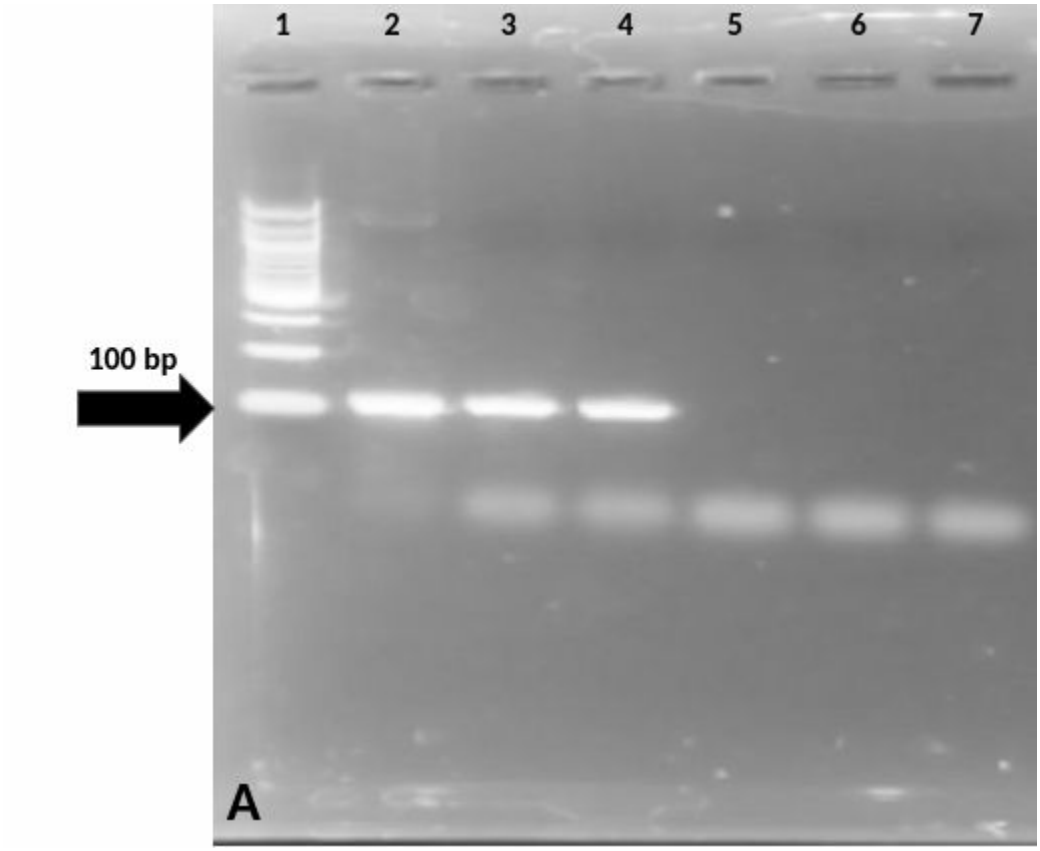

Figure 3: Separation of GAPDH PCR products on a 2% agarose gel. Lane 1, 100 base pair ladder. Lane 2, *Enterobacter* sp. YSU genomic DNA as positive control Lane 3, cDNA mix without selenite. Lane 4, cDNA mix with selenite. Lane 5, No selenite negative control – RNA in a cDNA synthesis reaction lacking reverse transcriptase. Lane 6, Selenite negative control - RNA in a cDNA synthesis reaction lacking reverse transcriptase. Lanes 5 and 6, without reverse transcriptase, were used to ensure there was no DNA contamination within the RNA prep. Lane 7, nuclease-free water as negative control 2.

Figure 4 shows the effects of selenite presence on expression of the *gutS* and *dedA* genes. The PCR products from the *Enterobacter* sp. YSU genomic DNA shows that the *gutS* primers are specific for their target (lane 2). The expression level of *gutS*

appears to be low under both conditions, but increases slightly in response to selenite (Lanes 3 and 4). A separate PCR was set up to test if running the DNA for 35 cycles rather than 30 would show a starker contrast between the two conditions, but the results were nearly identical. For *dedA*, the PCR products from the *Enterobacter* sp. YSU genomic DNA shows that the *dedA* primers are specific for their target (lane 8). The expression level of *dedA* appears to increase in response to selenite (lanes 9 and 10). Lack of 100 bp bands in lanes 11 and 12 shows that there was no DNA contamination of the RNA preps.

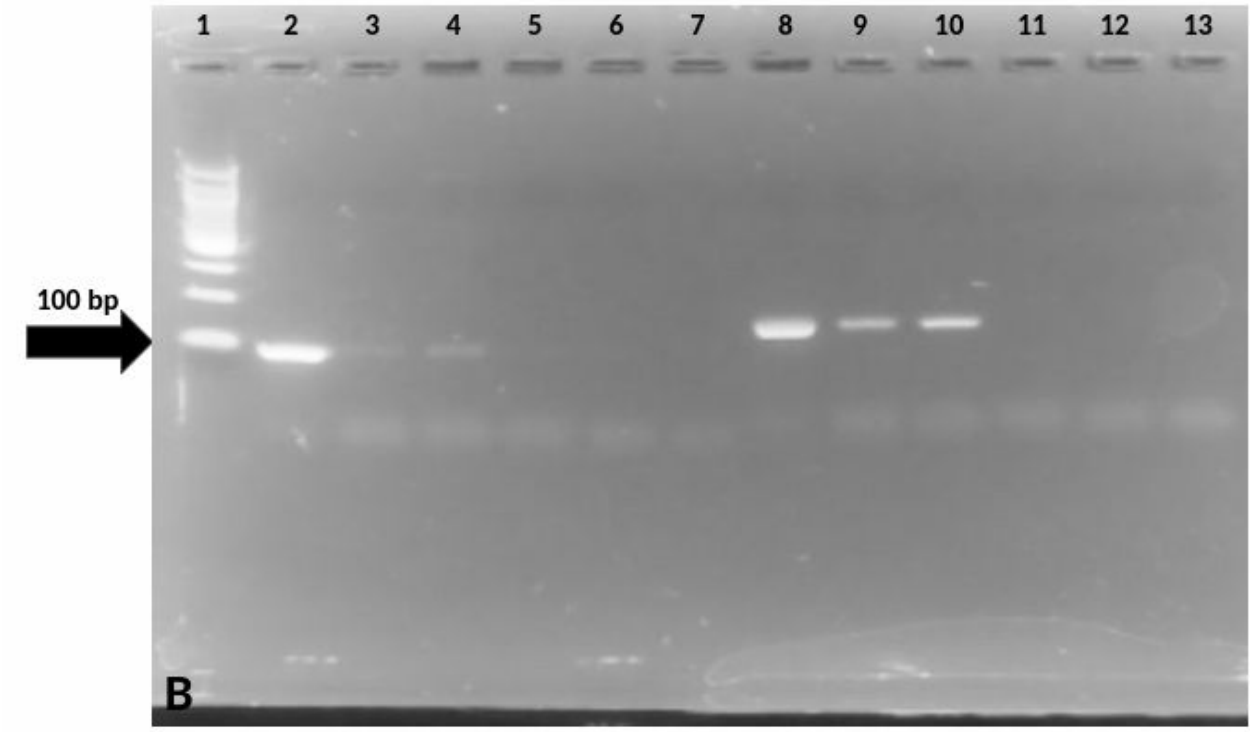

Figure 4: Separation of *gutS* and *dedA* PCR products on a 2% agarose gel. Lane 1, 100 base pair ladder. Lanes 2-7 utilized *gutS* primers. Lane 2, *Enterobacter* sp. YSU genomic DNA as positive control Lane 3, cDNA mix without selenite. Lane 4, cDNA mix with selenite. Lane 5, No selenite negative control – RNA in a cDNA synthesis

reaction lacking reverse transcriptase. Lane 6, Selenite negative control - RNA in a cDNA synthesis reaction lacking reverse transcriptase. Lanes 5 and 6, without reverse transcriptase, were used to ensure there was no DNA contamination within the RNA prep. Lane 7, nuclease-free water as negative control 2. Lanes 8-13 utilized *dedA* primers. Lane 8, *Enterobacter* sp. YSU genomic DNA as positive control Lane 9, cDNA mix without selenite. Lane 10, cDNA mix with selenite. Lane 11, No selenite negative control – RNA in a cDNA synthesis reaction lacking reverse transcriptase. Lane 12, Selenite negative control - RNA in a cDNA synthesis reaction lacking reverse transcriptase. Lanes 11 and 12, without reverse transcriptase, were used to ensure there was no DNA contamination within the RNA prep. Lane 13, nuclease-free water as negative control 2.

In **Figure 5**, the effects of selenite on the *ompA* and *ompX* genes can be seen. The *ompA* gene was heavily expressed with or without selenite presence during bacterial growth, with very bright bands mimicking the positive control. A separate PCR reaction run for 25 instead of 30 cycles showed no difference in *ompA* expression between the treated and untreated conditions. Therefore, we conclude that the presence of selenite did not affect expression of the *ompA* gene. A very strong band is noted in the lane with selenite and the cDNA enzyme, but not the lane with cDNA enzyme and a lack of selenite. Thus, the *ompX* gene has increased expression when the YSU strain is grown under selenite sensitive conditions.

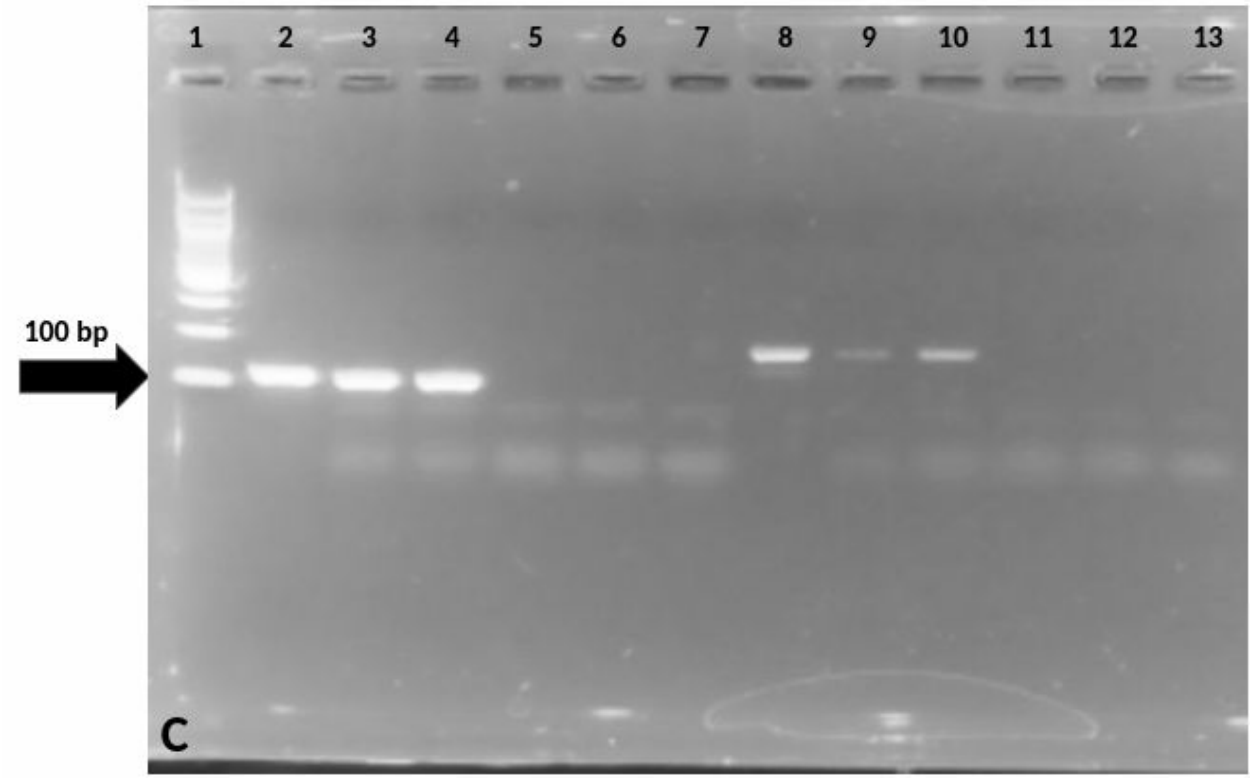

Figure 5: Separation of *ompA* and *ompX* PCR products on a 2% agarose gel. Lane 1, 100 base pair ladder. Lanes 2-7 utilized *ompA* primers. Lane 2, *Enterobacter* sp. YSU genomic DNA as positive control Lane 3, cDNA mix without selenite. Lane 4, cDNA mix with selenite. Lane 5, No selenite negative control – RNA in a cDNA synthesis reaction lacking reverse transcriptase. Lane 6, Selenite negative control - RNA in a cDNA synthesis reaction lacking reverse transcriptase. Lanes 5 and 6, without reverse transcriptase, were used to ensure there was no DNA contamination within the RNA prep. Lane 7, nuclease-free water as negative control 2. Lanes 8-13 utilized *ompX* primers. Lane 8, *Enterobacter* sp. YSU genomic DNA as positive control Lane 9, cDNA mix without selenite. Lane 10, cDNA mix with selenite. Lane 11, No selenite negative control – RNA in a cDNA synthesis reaction lacking reverse transcriptase. Lane 12, Selenite negative control - RNA in a cDNA synthesis reaction lacking reverse

transcriptase. Lanes 11 and 12, without reverse transcriptase, were used to ensure there was no DNA contamination within the RNA prep. Lane 13, nuclease-free water as negative control 2.

4.3: BLAST Analysis

The four proteins of interest studied here were obtained through BLAST and past 2-D gel electrophoresis. Both outer membrane proteins, OmpA and OmpX, were identified through a 2-D gel electrophoresis process (7). Protein spots with high intensities in selenite sensitive conditions were excised and analyzed by Mascot package software to obtain matches against known proteins. BLAST analysis of a known *E. coli* outer membrane protein with selenite resistance functions against the fully-sequenced genome of the YSU strain returned multiple results, seen in **Figure 6**. Both DedA and GutS from *Enterobacter* sp. YSU were identified using this BLAST search against the *Enterobacter* sp. YSU protein database at http://proteomics.ysu.edu/blast/blast.html.

YSU outer membrane proteins were aligned with counterpart *E. coli* proteins using the Clustal Omega online software. In **Figure 7** the conservation in the sequences for OmpA and OmpX within *Enterobacter* sp. YSU can be seen when compared to an OmpX protein within *E. coli*. The YSU OmpX protein had a conservation score of 84.12%, indicating a high likelihood of shared biochemical functions.

A tBLASTn search of the GutS protein sequence on the NCBI database revealed multiple selenite hydrogenase components within this protein. Results in **Figure 8** are consistent with literature outlining increased formate dehydrogenase activity when *E. coli* is exposed to selenite (36). Another database return (**Figure 9**) shows *gutS* has similarity

to an isocitrate dehydrogenase gene. Past work has shown that silencing these genes can enhance selenite-induced apoptosis, thus this gene's presence may play a key role in reduction and removal of the toxic selenite (42).

```
                                                                    Score     E
Sequences producing significant alignments:                         (bits) Value

contig_8_373_[68664_-_69179]_                                        259    5e-71


>contig_8_373_[68664_-_69179]_
          Length = 172

 Score =  259 bits (663), Expect = 5e-71
 Identities = 130/173 (75%), Positives = 136/173 (78%), Gaps = 3/173 (1%)

Query: 1   MKKIACLSALAAVLAFXXXXXXXXXXXXXXYAQSDAQGQMNKMGGFNLKYRYEEDNSPL 60
           MKKIACLSALAAVLA                YAQSD QG MNK  GFNLKYRYE+DN+PL
Sbjct: 1   MKKIACLSALAAVLAVSAGTAVAATSTVTGGYAQSDMQGVMNKTNGFNLKYRYEQDNNPL 60

Query: 61  GVIGSFTYTEKSRTASSGDYNKNQYYGITAGPAYRINDWASIYGVVGVGYGKFQTTEYPT 120
           GVIGSFTYTEK RT  +G YNK QYYGITAGPAYR+NDWASIYGVVGVGYGKFQ TE
Sbjct: 61  GVIGSFTYTEKDRT-ENGSYNKGQYYGITAGPAYRLNDWASIYGVVGVGYGKFQQTENQG 119

Query: 121 YKH--DTSDYGFSYGAGLQFNPMENVALDFSYEQSRIRRVDVGTWIAGVGYRF 171
               SDYGFSYGAG+QFNP+ENVALDFSYEQSRIR VDVGTWIAGVGYRF
Sbjct: 120 LNRTASNSDYGFSYGAGMQFNPIENVALDFSYEQSRIRNVDVGTWIAGVGYRF 172
```

Figure 6: BLAST of *E. coli* OmpX protein against the YSU database: An *E. coli* outer membrane protein (Query) with functions related to selenite uptake and resistance was searched against the genome of the *Enterobacter* sp. YSU strain (Subject).

Figure 7: Clustal Omega alignment of the three Omp proteins: The YSU OmpA and OmpX protein sequences were compared to the *E. coli* OmpX protein with known selenite resistance functions. A BLASTp at biocyc.org revealed the amino acid sequence

bracketed in yellow to be responsible for the outer membrane double barrel protein responsible for uptake of heavy metal ions.

```
gene            complement(383541..385220)
                /locus_tag="LI64_01835"
CDS             complement(383541..385220)
                /locus_tag="LI64_01835"
                /inference="EXISTENCE: similar to AA
                sequence:RefSeq:WP_020882829.1"
                /note="Derived by automated computational analysis using
                gene prediction method: Protein Homology."
                /codon_start=1
                /transl_table=11
                /product="formate dehydrogenase"
                /protein_id="AJB69371.1"
                /translation="MSNAINEIDNTDLVFIFGYNPADSHPIVANHVIRAKQNGAKIIV
                CDPRKIETARIADMHIALKNGSNIALLNAMGHVIIEENLYDQAFVASRTEGFEEYRKI
                VEGYTPESVEAITGVSAQEIRQAARMYAGAKTAAILWGMGVTQFYQGVETVRSLTSLA
                MLTGNLGKAHVGVNPVRGQNNVQGACDMGALPDTYPGYQYVKFPENREKFAKAWGVES
                LPEHTGYRISELPHRAAHGEVRAAYIMGEDPLQTDAELSAVRKGFEDLELVIVQDIFM
                TKTAAAADVILPSTSWGEHEGVYTAADRGFQRFFKAVEPKWDLKTDWQIISEISTRMG
                YPMHYNNTQEIWDELRHLCPDFYGATYEKMGELGYIQWPCRDESEADQGTSYLFKEKF
                DTPNGLAQFFTCDWVAPIDKLTDEYPMVLSTVREVGHYSCRSMTGNCAALAALADEPG
                YAQINTADAERLGIEDEALVWVNSRKGRIITRAQVSDRPNKGAVYMTYQWWIGACNEL
                VTENLSPITKTPEYKYCAVNVEPIADQHAAEQYVIDEYNKLKARLRESAMG"
```

Figure 8: tBLASTn result of GutS protein query: A formate dehydrogenase component was found in the sequence of the GutS protein.

```
CLUSTAL O(1.2.4) multiple sequence alignment


isocitrate      MSRGLELLIAQTI--LQGFDAQYGRFLEVTSGAQQRFEHADWHAVQLAMKQRIHLYDHHV    58
YSU_GutS        ------------------------------------------------------------    0
formate         MSNAINEIDNTDLVFIFGYN-----------------PADSHPIVA--NHVIRAKQNGA    40


isocitrate      GLVVEQLRCITEGKSPDAEFLLRVKKHYTHLLPDYPRFEIAESFFNSVYCRL----FDH-    113
YSU_GutS        ------------------------------------------------------------    0
formate         KIIVCDPRKIETARIADMHIALKNGS-NIALLNAMGHVIIEENLYDQAFVASRTEGFEEY    99


isocitrate      ----RSLSPERLFIFSSQPERRFRTIPRPLAKDFFPDRGWEKLLHRVLTDLPLRLPWENK    169
YSU_GutS        ----------------------------MTN-----------------SNRIKLTWIS-    13
formate         RKIVEGYTPESVEAITGVSAQEIRQAARMYAG-----------------AKTAAILWGMG    142
                                            :                       : *


isocitrate      A--------------------RDIGYIHAHLNEAFGAEVLSQSHLQVANELFYRNKAAWL    209
YSU_GutS        ---FFSYA-----------LTG--------------ALVIV--------------------    26
formate         VTQFYQGVETVRSLTSLAMLTGNLGKAHVGVNPVRGQNNVQ--------------------    183
                                                    :


isocitrate      AGKLVTPSAIVPFLLPIHRTDDGELFVDTCLTTSAEASIVFGFARSYFMVYAPLPAALVE    269
YSU_GutS        -TGMVMGNIADYFQLPVSSMSNTFTFL---------------------NAGILISIFLNA    64
formate         -GACDMGALPDTY--------PGYQYV---------------------KFPENREKFAKA    213
                             :            ::


isocitrate      WLREILPGKTTAELYMAIGCQKHAKTESYREYLR------------YVTTADEQ------    311
YSU_GutS        WLMEIVPLKTQLRFGFVLMVAAVAGLM------LSH-------SIALFSAAM--FVLGLV    109
formate         WGVESLPEHTGYRISELPHRAAHGEVR--AAYIMGEDPLQTDAELSAVRKGFEDLELVIV    271
                *  * :* :*  .:         .                         .  .


isocitrate      ---FIEAPGIRGMVMLV------------FTLPGFDRVF-----KVIKDKFAPQ------    345
YSU_GutS        SGITMS----IGTFLIT--------HMYEGRQRGARLLFTDSFFSMAGMIFPMVAAYLLA    157
formate         QDIFMTKTAAAADVILPSTSWGEHEGVYTAADRGFQRFF-----KAVEPKWDL-------    319
                    :      . .::                  *   .*     .     :


isocitrate      KEMSAAHVRACYQLVKEHDRVGRMADTQEFENFVLDKQQIDPALMALLMQEASAKITDLG    405
YSU_GutS        RSIEWYWVYACIGLVYVAIF--VLTFGCEFPVLG-KKAQTTS-------EPVAKEKWGIG    207
formate         -KTDWQII-----------S--EISTRMGYPMHY-N---------------NTQEIWDEL    349
                 . .   :              ::    :     .                : :  .


isocitrate      DKI---VISHLYIERRMVPLNIWLEQSEGQALRDAIEEYGNAIRQLAAANIFPG---DML    459
YSU_GutS        VL-FL-SIAALCYILGQLGFISWVPEYAKG-LGMSLNDAGKLVSDFWMSYMFGMWAFSFI    264
formate         RHLCPDFYGATYEKMGELGYIQWPCRDESE-ADQ-----G-------TSYLFKE------    390
                        .       :    *  .             *        : :*


isocitrate      FKNFGV-----TRHGRVVFYDYDEI-------------------------------CYMT    483
YSU_GutS        LRFFDLQRILTVLAGLATVLMYLF---INGSPEHMPWSI---LTLGFFSSAIYTS-IITL    317
formate         -K-FD------TPNGLAQFFTCDWVAPIDKLTDEYPMVLSTVREVGHYSCRSMTGNCAAL    442
                 : *.      .  * . .


isocitrate      EVNFRDIPPPRYPEDELSSEPWYSVSPGDVFPEEFRHWLCADPRIGPLFE----------    533
YSU_GutS        GSLQTKVASPKLVNFVL----TCG------TIGTMLTFV----VTGPIVAHSGPLAALHT    363
formate         AALA---DEPGYAQINTADAERLG------IEDEALVWV--NSRKGRIITRAQ-VSDRPN    490
                         *   :         .            ::      * :.


isocitrate      --EMHADLFRASYWRGLQTRIKNGHVED------------------------VYAYRRKQ    567
YSU_GutS        ANGLYAVVFIMCFVLGFVTRHRQ--HNP----------------ATATH*----------    394
formate         KGAVY---MTYQWWIGACNELVTENLSPITKTPEYKYCAVNVEPIADQHAAEQYVIDEYN    547
                   ::   :   :  *  ..      .


isocitrate      RFCIRFSPSPCGRGPG        583
YSU_GutS        ----------------        394
formate         KLKARLRESAMG----        559
```

Figure 9: Multiple Sequence Alignment of GutS and two dehydrogenase proteins: An isocitrate dehydrogenase component was also found in the sequence of the GutS protein, along with the formate dehydrogenase component. These two proteins were aligned with GutS to examine similarities, with little similarity found.

4.4: TMHMM Analysis

The four proteins of interest are all known membrane proteins and were examined with the TMHMM2.0 software program. This program utilizes a membrane protein topology prediction model to predict transmembrane helices, with the FASTA sequences of proteins as input (43). OmpX is predicted to have one transmembrane helix with the majority of the protein outside of the cell. Conversely, OmpA was found to be completely outside of the cell membrane. This is peculiar, as outer membrane proteins typically have a membrane spanning region. It is possible this protein is composed of beta barrels. There are 12 transmembrane helices hypothesized in GutS, with 6 helices inside the membrane and 7 outside. DedA is predicted to have 3 segments inside the cell and 2 outside, with 4 transmembrane helices.

```
# YSU_OmpX Length: 172
# YSU_OmpX Number of predicted TMHs:  1
# YSU_OmpX Exp number of AAs in TMHs: 22.29598
# YSU_OmpX Exp number, first 60 AAs:  21.6712
# YSU_OmpX Total prob of N-in:        0.86267
# YSU_OmpX POSSIBLE N-term signal sequence
YSU_OmpX        TMHMM2.0        inside       1     6
YSU_OmpX        TMHMM2.0        TMhelix      7    29
YSU_OmpX        TMHMM2.0        outside     30   172
```

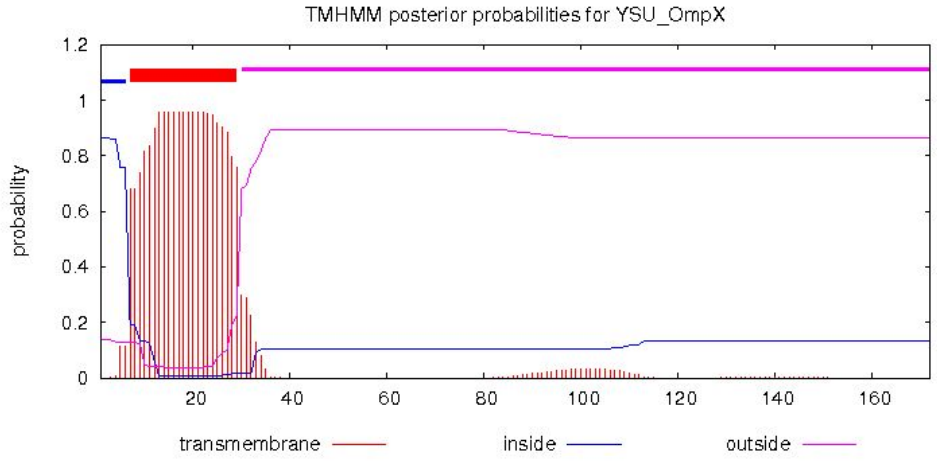

Figure 10: TMHMM Profile of OmpX Protein: Software at cbs.dtu.dk/services/TMHMM was used to analyze membrane protein properties of OmpX. One transmembrane helix was found, with the majority of the protein hypothesized to be outside of the cell membrane.

```
# YSU_OmpA Length: 350
# YSU_OmpA Number of predicted TMHs:  0
# YSU_OmpA Exp number of AAs in TMHs: 8.61161999999999998
# YSU_OmpA Exp number, first 60 AAs:  8.55845
# YSU_OmpA Total prob of N-in:        0.47400
YSU_OmpA        TMHMM2.0        outside      1   350
```

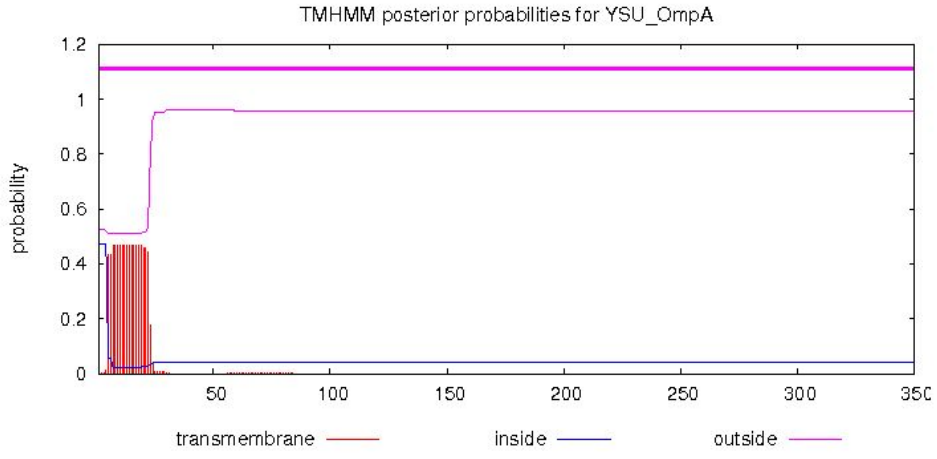

Figure 11: TMHMM Profile of OmpA Protein: Software at cbs.dtu.dk/services/TMHMM was used to analyze membrane protein properties of OmpA. The entire protein sequence is hypothesized to be outside of the cell membrane.

```
# YSU_GutS Number of predicted TMHs:  12
# YSU_GutS Exp number of AAs in TMHs: 260.34311
# YSU_GutS Exp number, first 60 AAs:  36.18899
# YSU_GutS Total prob of N-in:        0.99996
# YSU_GutS POSSIBLE N-term signal sequence
YSU_GutS        TMHMM2.0        inside      1   12
YSU_GutS        TMHMM2.0        TMhelix     13  35
YSU_GutS        TMHMM2.0        outside     36  44
YSU_GutS        TMHMM2.0        TMhelix     45  67
YSU_GutS        TMHMM2.0        inside      68  75
YSU_GutS        TMHMM2.0        TMhelix     76  98
YSU_GutS        TMHMM2.0        outside     99  101
YSU_GutS        TMHMM2.0        TMhelix     102 121
YSU_GutS        TMHMM2.0        inside      122 133
YSU_GutS        TMHMM2.0        TMhelix     134 156
YSU_GutS        TMHMM2.0        outside     157 160
YSU_GutS        TMHMM2.0        TMhelix     161 183
YSU_GutS        TMHMM2.0        inside      184 203
YSU_GutS        TMHMM2.0        TMhelix     204 226
YSU_GutS        TMHMM2.0        outside     227 245
YSU_GutS        TMHMM2.0        TMhelix     246 268
YSU_GutS        TMHMM2.0        inside      269 272
YSU_GutS        TMHMM2.0        TMhelix     273 290
YSU_GutS        TMHMM2.0        outside     291 299
YSU_GutS        TMHMM2.0        TMhelix     300 322
YSU_GutS        TMHMM2.0        inside      323 328
YSU_GutS        TMHMM2.0        TMhelix     329 348
YSU_GutS        TMHMM2.0        outside     349 357
YSU_GutS        TMHMM2.0        TMhelix     358 380
YSU_GutS        TMHMM2.0        inside      381 394
```

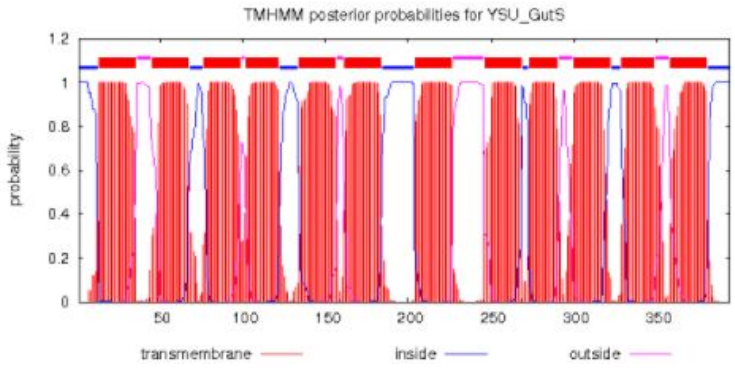

Figure 12: TMHMM Profile of GutS Protein: Software at cbs.dtu.dk/services/TMHMM was used to analyze membrane protein properties of GutS. There are 12 transmembrane helices theorized, along with 6 regions inside of and 7 regions outside of the cell membrane.

```
# YSU_DedA Length: 219
# YSU_DedA Number of predicted TMHs:  4
# YSU_DedA Exp number of AAs in TMHs: 91.18534
# YSU_DedA Exp number, first 60 AAs:  23.85016
# YSU_DedA Total prob of N-in:        0.94964
# YSU_DedA POSSIBLE N-term signal sequence
YSU_DedA        TMHMM2.0        inside       1    26
YSU_DedA        TMHMM2.0        TMhelix     27    49
YSU_DedA        TMHMM2.0        outside     50    70
YSU_DedA        TMHMM2.0        TMhelix     71    93
YSU_DedA        TMHMM2.0        inside      94   155
YSU_DedA        TMHMM2.0        TMhelix    156   178
YSU_DedA        TMHMM2.0        outside    179   187
YSU_DedA        TMHMM2.0        TMhelix    188   210
YSU_DedA        TMHMM2.0        inside     211   219
```

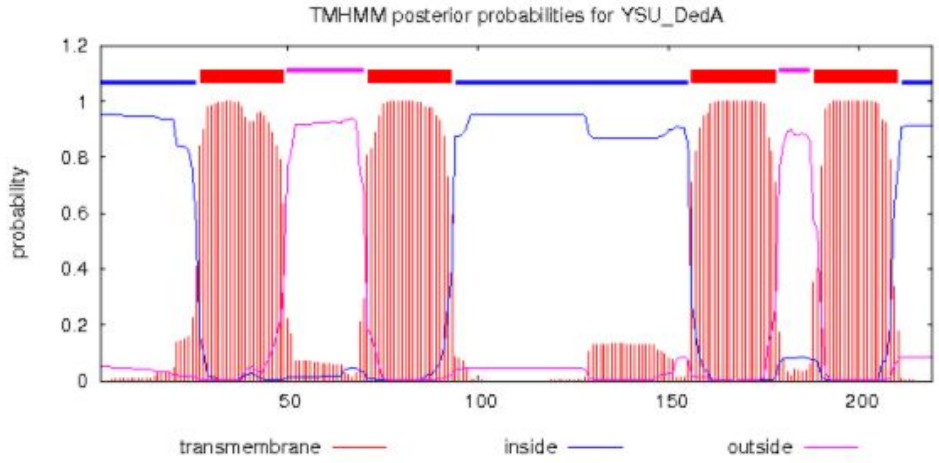

Figure 13: TMHMM Profile of DedA Protein: Software at cbs.dtu.dk/services/TMHMM was used to analyze membrane protein properties of DedA. There are 4 transmembrane helices theorized, along with 3 regions inside of and 2 regions outside of the cell membrane.

Chapter V: Discussion

5.1: Gene Analysis

Research was completed to gather further proof that the *ompA, ompX, dedA,* and *gutS* genes are overexpressed when *Enterobacter* sp. YSU is exposed to selenite sensitive conditions. The *ompA* gene was heavily expressed with or without selenite presence during bacterial growth, but the *ompX* gene only displayed high expression with selenite presence. Increased expression of *ompX* is consistent with research on the OmpX protein

increasing expression in the presence of selenite and tellurium. Previous studies have consistently shown the OmpA protein to be more expressed when bacteria are exposed to selenite though, so further research will need to be done and determine a possible link to these results and the hypothesis of OmpA and OmpX forming a single protein. Outer membrane proteins OmpA and OmpX are known to be some of the most abundant proteins in the membranes of gram-negative bacteria and are important in transport of molecules in and out of the cell (33). Thus, the increased expression of the *ompX* gene in *Enterobacter* sp. YSU is evidence of more selenite being pumped out of the cell via the specific pathway. Previous BLAST analysis of other Gram negative bacteria shows that these outer membrane polypeptides are capable of reducing selenite to elemental selenium by a dehydrogenase component during anaerobic respiration and this study could be further proof of that effect (36). A point mutation experiment could be utilized in the future to determine if the YSU proteins, GutS specifically, contain a dehydrogenase component.

Minimal difference is noted in expression of the *gutS* gene if the *Enterobacter* sp. YSU cells are exposed to selenite, but the *dedA* gene showed a stark increase in intensity of the 100 bp band that represented the selenite sensitive conditions. This was peculiar, as the literature has typically grouped these two genes together as selenite resistance genes (33,34). The DedA protein families have been linked to selenite resistance in bacteria and that is consistent with the data here.

Possible problems of this study include the possible lack of sensitivity, reproducibility, and specificity associated with RT-PCR. Also, there is the exponential growth of the cDNA that is possible with PCR methods. This can make accurate

quantification of RNA levels difficult (37). An alternate method for more accurate RNA content quantification would be the qPCR method. A separate set of primers would have to be designed as the original RT-PCR primers produced dimers which would cause background signal and false positive readings. The fluorescent dye utilized in qPCR would allow better monitoring of each cycle of PCR, for sharper quantification of RNA levels. Therefore, a qRT-PCR sequence would allow for increased sensitivity, and more accurate RNA measurement (38).

Based on similarities in the sequences of the four proteins studied in this experiment and known selenite resistance proteins in gram-negative bacteria such as *E. coli*, it may be deduced that the YSU proteins have similar functions within the cell. The OmpA and OmpX proteins likely are involved in a similar system as outer membrane porin proteins in *E. coli* which undergo a conformational shift and increase the uptake of selenite into the periplasm (39). Then, selenite couples with glutathione in the cytoplasm to form large selenium nanospheres to be exported from the bacterial cell. Through this mechanism, these outer membrane proteins are vital for gram-negative bacteria to reduce toxic selenite to manageable selenium and eventually rid the cell of it (39). Previous sequencing of the GutS protein revealed significant homology to multiple subcellular integral membrane transport proteins but no mechanism of action is currently known for this protein in any bacterial species (40). The DedA protein is a member of a protein family with mostly undiscovered functions. Recent literature has revealed a symporter/antiporter complex involving DedA but it is unclear how this would relate to uptake or removal of heavy metal ions like selenite (41).

5.2: Future Work

Future experiments could be done to examine why the *ompA* and *gutS* genes did not increase their expression in selenite sensitive conditions, as they were expected to. A gene knockout procedure could be performed on each of the four genes studied here to see if deleting any one of them, or any combination, can cause *Enterobacter* sp. YSU to lose its selenite resistance capabilities. This technique may also be able to elucidate if OmpA and OmpX are indeed a single protein family, by knocking out the *ompX* gene that was found to be overexpressed here. If that gene could be knocked out via deletion primers, with *ompA* kept intact, then a study could be completed to determine if the YSU strain still maintained its selenite resistance. If it did not, this could be evidence that the two proteins are indeed a single functioning protein within the bacteria's physiological processes. Further sequencing is necessary to determine if there is possibly a dehydrogenase component within the GutS protein. If a sequence of amino acids coding for this component is identified, a point mutation experiment may be done to verify the sequence. Work could also be done to understand all four studied genes grown in M-9 minimal media with and without L-cysteine addition, to examine the genes' expression in those conditions. Also, similar experiments could be performed with varying selenite concentrations and with other heavy metals, such as tellurite or mercury.

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