GENETIC STUDIES FOR SELENATE AND TELLURATE REDUCTION PROCESSES IN FACULTATIVE BACTERIA

By

JOANNE THEISEN

A thesis submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Master of Science

Graduate Program in Environmental Science

written under the direction of

Nathan Yee

and approved by

_____

_____

_____

New Brunswick, New Jersey

January 2013

ABSTRACT OF THE THESIS

Genetic studies for selenate and tellurate reduction processes in facultative bacteria

By JOANNE THEISEN

Thesis Director:

Nathan Yee

The bioavailability and toxicity of selenium in the environment is strongly affected by redox transformations. In this study, we conducted spectroscopic, genetic, and macroscopic chemical measurements to investigate the ability of *Citrobacter freundii* to catalyze the reduction of soluble selenate oxyanions [Se(VI)] to poorly soluble elemental selenium [Se(0)]. The results indicate that *C. freundii* forms a red precipitate on selenate containing agar after 48 hours of incubation. In liquid culture, bulk chemical measurements show the removal of selenate oxyanions from solution only after oxygen is completely removed from the bacterial media. Selenate reduction by *C. freundii* is a substrate specific process and does not simply bind or absorb to cells, as it does not reduce arsenic under the same conditions. X-ray absorption near edge spectra analysis of cell pellets collected from selenate incubations after 7 days show the formation of solid-phase elemental selenium. PCR amplification and DNA sequencing revealed that *C.*

freundii carries the ynfEGH operon, a gene cluster in the DMSO reductase family previously shown to be responsible for selenate reduction in other gamma proteobacteria. Sequence analysis of ynfEGH operon possess a FNR binding site (Fumarate nitrate reduction regulator), and twin-arginine translocation (Tat) signal sequence, The environmental implications of selenate reduction by *C. freundii* for bioremediation purposes are discussed.

The genetic identity and co-factor composition of the bacterial tellurate reductase are currently unknown. In this study, we examined the requirement of molybdopterin biosynthesis and molybdate transporter genes for tellurate reduction by *Escherichia coli*. The results demonstrate that mutants carrying deletions of the *moaA, moaB, moaE,* or *mog* gene in molybdopterin biosynthesis pathway lost the ability to reduce tellurate. Deletion of the *mod*B or *mod*C genes in molybdate transport pathway also resulted in complete loss of tellurate reduction activity. Genetic complementation by the wild-type sequences restored tellurate reduction activity in the mutant strains. These findings provide genetic evidence that the tellurate reductase in *E. coli* is a molybdopterin-containing enzyme.

Acknowledgements

I have been lucky enough to be in the Environmental Science department at Rutgers University not only for my graduate studies, but also my undergraduate career. I would like to extend my greatest thank you first and foremost to my advising professor, Dr. Nathan Yee, who always made time to give advice, discuss my work, and encourage me to be better. I am very thankful to my master's thesis and qualifying committee members: Dr. John Reinfelder, and Dr. Peter Strom. Not only were they gracious enough to be on my committee, they were great advisors and professors during my undergraduate career, and taught me a lot of what I know today. I am extremely grateful to Dr. Gerben Zylstra, and his post-doc Hung-Kuang Chang, for teaching me how to perform microbial genetic experiments, and making their laboratory resources available to me.

I can't express how appreciative I am to the present members of the Yee lab: Madhavi Parikh and Matt Colombo for all their help, advice, and ability to make long lab hours fun. Thank you to Dr. Lily Young's lab: Adam Mumford, Sarah Wolfson, and Alex Walczak for helping me with my genetic analysis and use of their various equipment. Also, thank you to Dr. Robert Tate's lab for teaching me the proper way to run a gel and how to analyze it.

I want to acknowledge the National Science Foundation for providing the funding resources necessary to make my research possible.

Last, but not least, I would like to extend me most sincere gratitude to my family and loved ones who have provided an endless supply of support throughout my time at Rutgers University. To my parents, Jerry and Karen, for always refusing to tell me how

to proceed in my academic career, forcing me to make my own decisions and follow my own path. To my brother Kevin, who believes in me enough to let me be Vice President of his company, and who is also now required to provide me with a steady paycheck for the rest of my life.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

Selenate Reduction by *Citrobacter freundii*

Selenium is a naturally occurring element in nature and elevated levels of Se-bearing contaminants have been released into the environment through a range of anthropogenic actions including irrigation, mining, fossil fuel combustion, and oil refining (Ohlendorf et al., 1986, 1988). As it is released into waters and soil selenium reacts with oxygen and becomes oxidized into soluble selenate Se(VI) and selenite Se(IV). While selenium is a micronutrient for organisms, the oxidized forms of selenium are teratogens at high concentrations that can cause death and abnormalities in fish and waterfowl (Ohlendorf et al., 1986, 1988). Figure 1.1 displays the toxic effects selenium has on fish found in Belews Lake, North Carolina, which was contaminated with selenium due to wastewater from a coal-fired power plant (Lemly, 2002). Redhead duck embryos from the Green River Basin in Utah are greatly affected by selenium contamination, as can be seen in Figure 1.2 (Seiler, Skorupa, & Peltz, 1999). Acute exposure in humans can cause respiratory and gastrointestinal problems, while chronic exposure leads to liver damage (Vinceti at al., 2001).

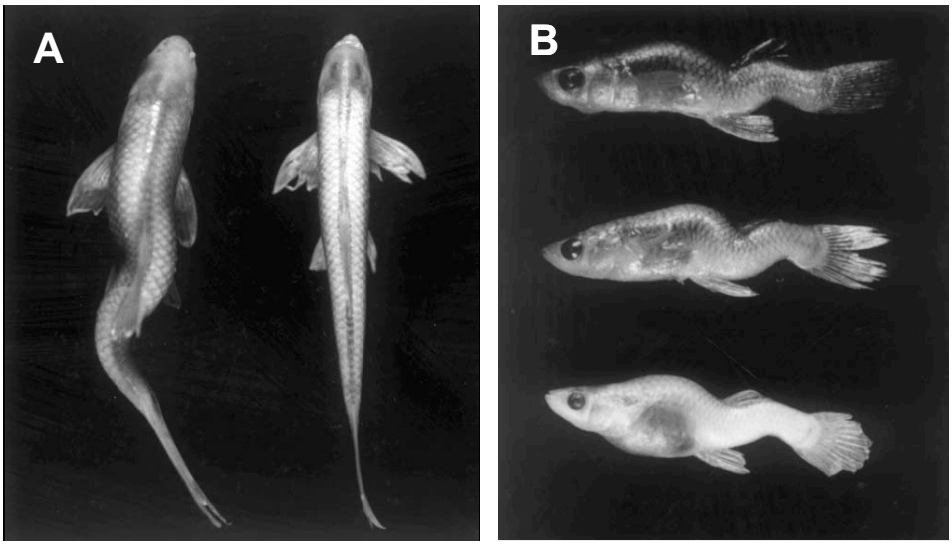

Figure 1.1: Fish from Belews Lake with (A) Lateral curvature of the spine and (B) dorso-ventral spinal deformities (Lemly, 2002).

Figure 1.2: Readhead duck embryo from the Green River Basin in Utah. Left is a selenium deformed bird embryo, right is a normal bird embryo.

The toxicity and bioavailability of selenium oxyanions are altered by redox transformations and their mobility in soil and groundwater is strongly affected by reductive precipitation, which can be facilitated by microorganisms. Oram et al. (2010) showed that in the hyporheic zones, the removal of selenate from interstitial waters is

concurrent with increasing solid-phase Se concentrations (Oram, Strawn, Morra, & Möller, 2010). Microbial mediated transfer of dissolved selenium oxyanions into solid selenium phases has been demonstrated in sediment incubations, which show selenate reduction coupled to organic carbon oxidation illustrating the biological nature of the reduction process (Oremland et al 1989).

Microorganisms that can reduce selenate are phylogenetically diverse, metabolically versatile, and have been isolated from microbial habitats around the world (Stolz, Basu, Santini, & Oremland, 2006; Stolz & Oremland, 1999). *Thauera selenatis* and *Bacillus selenatarsenatis* are examples of well studied selenate-respiring bacteria with genetically characterized selenate reductase operons. The selenate reductase in *T. selenatis* is part of the serABC operon (Krafft, Bowen, Theis, & Macy, 2000), while the selenate reductase respiratory activity in *B. selenatarsenatis* is conferred by the srdBCA operon (Kuroda et al., 2011). In contrast, *Salmonella enterica* and *Escherichia coli* have exhibited the ability to co-metabolize selenate through the activity of the selenate reductase *ynfE*.

Characterizing a new bacteria that has the ability to reduce selenate to elemental selenium and elucidating the mechanisms involved in the transformation process was the objective of the study described in Chapter 2.

Tellurate Reduction by *Escherichia coli*

Tellurium (Te) is a metalloid element used for a variety of industrial applications, including metallurgy, chemical manufacturing, and electronics (Chasteen & Bentley, 2003; Taylor, 1999). The disposal of tellurium-containing wastes has led to an increase

in environmental contamination. Te concentrations have reached 887 ng/m^3 in sewage gas and 75 ng/m^3 in landfill gases (Feldmann & Hirner, 1995). Gold mine tailings in south east Spain have tellurium concentrations between 4.6-14.8 mg/kg, while stream sediment Te in the area ranges from 0.9-13.8 mg/kg (Wray, 1998). Huang (2008) has shown that tellurate is the dominant species of tellurium in seawater with locations having 25.1 ng/L and 26.2 ng/L Te(VI) versus 6.9 ng/L and 3.9 ng/L Te(IV) respectively.

When released into the environment, tellurium undergoes oxidation and is mobilized as dissolved Te oxyanions tellurate [Te(VI), TeO_4^{2-}] and tellurite [Te(IV), TeO_3^{2-}]. Microorganisms have previously been shown to catalyze the reduction of toxic Te oxyanions into sparingly soluble and less toxic elemental tellurium. *Sulfurospirillum barnesii* and *Bacillus selenitireducens* can achieve growth by reduction of Te(VI) to elemental tellurium [Te(0)] (Baesman et al., 2007). Anaerobic respiration on tellurate has been seen by a *Shewanella* species isolated from deep ocean (1,543 to 1,791 m) hydrothermal vent worms (Csotonyi et al 2006) and gram positive isolate *Bacillus beveridgei* can grow by reducing Te(VI) to Te(0) (Baesman, Stolz, Kulp, & Oremland, 2009).

Genes have been identified for tellurite reduction including the tehAB system, klaABC genes, terZ-ABCDEF (*ter*) operon, and trgAB genes. Although tellurite resistant genes have been identified, the proteins required for tellurate reduction are still uncharacterized. The molybdenum cofactor forms the active site of several important bacterial redox enzymes, including the proteins in the DMSO reductase family. Sequence analysis indicates that pterin-containing molybdenum enzymes catalyze the reduction of selenium and arsenic (Bébien, Kirsch, Méjean, & Verméglio, 2002;

Malasarn, Keeffe, & Newman, 2008), two metalloid elements that share similar chemical characteristics to tellurium. This prompted the study presented in Chapter 3 to determine if the tellurate reductase in *E. coli* is a molybdopterin-containing enzyme.

Chapter 2: Reduction of Se(VI) to Se(0) by *Citrobacter freundii*

ABSTRACT

The bioavailability and toxicity of selenium in the environment is strongly affected by redox transformations. In this study, we conducted spectroscopic, genetic, and macroscopic chemical measurements to investigate the ability of *Citrobacter freundii* to catalyze the reduction of soluble selenate oxyanions [Se(VI)] to poorly soluble elemental selenium [Se(0)]. The results indicate that *C. freundii* forms a red precipitate on selenate containing agar after 48 hours of incubation. In liquid culture, bulk chemical measurements show the removal of selenate oxyanions from solution only after oxygen is completely removed from the bacterial media. Selenate reduction by *C. freundii* is a substrate specific process and does not simply bind or absorb to cells, as it does not reduce arsenic under the same conditions. X-ray absorption near edge spectra analysis of cell pellets collected from selenate incubations after 7 days show the formation of solid-phase elemental selenium. PCR amplification and DNA sequencing revealed that *C. freundii* carries the ynfEGH operon, a gene cluster in the DMSO reductase family previously shown to be responsible for selenate reduction in other gamma proteobacteria. Sequence analysis of the ynfEGH operon shows it to possess a FNR binding site (Fumarate nitrate reduction regulator), and a twin-arginine translocation (Tat) signal sequence. The environmental implications of selenate reduction by *C. freundii* for bioremediation purposes are discussed.

INTRODUCTION

Selenium is an environmental contaminant that is released into water and soil at elevated levels from various anthropogenic actions including mining, water irrigation, fossil fuel combustion, and oil refinement (Ohlendorf et al., 1988, 1986). The oxidized forms of selenium, selenate [Se(VI)] and selenite [Se(IV)], are teratogens at high concentrations that can cause death and abnormalities in fish and waterfowl (Ohlendorf et al., 1988, 1986). Acute exposure in humans can cause respiratory and gastrointestinal problems, while chronic exposure leads to liver damage (Vinceti et al., 2001). The toxicity and bioavailability of selenium oxyanions are altered by redox transformations (Brown, Foster, & Ostergren, 1999), and their mobility in soil and groundwater is strongly affected by reductive precipitation, which can be facilitated by microorganisms.

Prokaryotes that can enzymatically reduce selenate oxyanions are found throughout the bacteria domain. These microorganisms are phylogenetically diverse, metabolically versatile, and have been isolated from microbial habitats around the world (Stolz et al., 2006; Stolz & Oremland, 1999). *Thauera selenatis* and *Bacillus selenatarsenatis* are examples of well studied selenate-respiring bacteria with genetically characterized selenate reductase operons. The selenate reductase in *T. selenatis* is conferred by the serABC operon (Krafft et al., 2000), while the selenate reductase respiratory activity in *B. selenatarsenatis* is conferred by the srdBCA operon (Kuroda et al., 2011). In contrast, *Salmonella enterica* and *Escherichia coli* have exhibited the ability to co-metabolize selenate through the activity of the selenate reductase *ynfE* (Guymer et al., 2009). The selenate reductase gene in *E. coli* is controlled by the global anaerobic regulatory gene FNR (fumarate nitrate reduction regulator) (Yee et al., 2007) and requires export by the twin-argenine translocation (TAT) pathway, which moves folded proteins

from the cytoplasm through the cytoplasmic membrane (Ma et al., 2007). However, *Salmonella enterica* and *Escherichia coli* are bacteria typically found inside animals, and are not representative organisms in geologic environments. As such, the geochemical impact of the *ynfE* gene remains poorly understood.

Citrobacter freundii is a citrate metabolizing facultative anaerobe that is commonly found in soil, freshwater, and wastewater (Lipsky et al., 1980). This bacterium has been shown to strongly interact with a wide range of different environmental contaminants. Most notably, *C. freundii* can sequester uranium via phosphate precipitation (Macaskie et al., 1992) and reduce nitramine explosives in soil (Kitts et al., 1994). In metal-bearing streams and wastewater, *C. freundii* has been shown to absorb the heavy metals lead, cadmium, and zinc (Puranik & Paknikar, 1999). While *C. freundii* has been shown to sorb and precipitate various cationic contaminants its interactions with selenium oxyanions have not been studied.

In this study, spectroscopic, genetic, and macroscopic chemical measurements were conducted to investigate the interactions between *C. freundii* and dissolved selenate. The objective was to determine if *C. freundii* is able to reduce selenate to its elemental form, and to elucidate the mechanisms involved in the transformation process. The results of this work suggest that enzymes encoded by the *ynfE* gene, the tat pathway, and FNR regulator may be important to the reduction of selenium oxyanions in geochemical settings.

MATERIALS AND METHODS

Bacterial growth conditions. All experiments were performed using autoclaved and deionized Milli-Q water. *Citrobacter freundii* was grown and maintained on LB medium at 32°C unless otherwise noted. Initial selenate reduction activity was determined by plating cells on LB agar spiked with 1 mM and 10 mM selenate. Strains were obtained from Ward's Natural Science (Rochester, NY) and Dr. Max Häggblom (Rutgers University, New Brunswick, NJ). Single colonies were imaged with a Sigma macro lens every 24 hours for 3 days. In liquid medium, the growth of *C. freundii* was monitored using a Shimadzu (Kyoto, Japan) Bio-mini spectrophotometer at a wavelength of 595 nm.

Selenate Reduction Experiments. Selenate reduction by *C. freundii* was quantified in a defined liquid medium containing 0.34 mM KH_2PO_4, 0.85 mM NaCl, 1.87 mM NH_4Cl, 0.40 mM $MgCl_2$, 0.02 mM $CaCl_2$, 10 mM PIPES, 0.1 g/L casamino acids, 1 mL/L trace element solution, and 10 mM $Na_2HC_6H_5O_7$, adjusted to a pH of 7. Prior to inoculation 100 μM of SeO_4^{2-} was added to the medium. *C. freundii* cells were pre-grown aerobically at 32°C in LB broth to an optical density (OD_{595nm}) of 1.40 ± 0.05 and then transfered to the defined medium by 1:100 dilution. Test tubes were then sealed with a rubber stopper and samples were taken at periodic intervals up to 7 days. In a second experiment, *C. freundii* cells were pre-grown in the defined medium and allowed to exhaust the supply of oxygen in the sealed test tubes. When the cells reached stationary phase, the cultures were spiked with varying amounts of either selenate or arsenate, ranging from 10 μM to 10 mM, and incubated for 48 h.

A Dionex (Sunnyvale, CA) ICS-1000 ion chromatograph (IC) with an AS40 automated sampler, IonPac AS9-HC anion column, and ASRS-ULTRA II 4-mm

suppressor was used to measure anion concentrations. A mobile phase of 0.954 g/L sodium carbonate in Milli-Q water was filtered through a 0.2 μm filter and used. Each sample was syringe filtered through a 0.45 μm filter and the supernatant was run through the IC. Selenate in the final samples was subtracted from the original concentrations to determine the concentration of selenate removed. Standards used for calibration were made using the same minimal salt medium used in cultures.

Dissolved O_2 was measured with YSI (Yellow Springs, Ohio) 550A handheld dissolved oxygen probe that was calibrated assuming an atmospheric pressure of 1 atm. Samples for protein analysis were stored -20°C prior to being thawed and sonicated with a QSonica (Newtown, CT) Microson ultrasonic cell disruptor at an output power of 7-8 watts for 2 minutes and 35 seconds (alternating on 15 seconds, off 5 seconds). The samples were then centrifuged for 15 minutes at 9,300 g and the supernatant was removed. Bio-Rad (Hercules, CA) protein assay dye reagent was added and let sit for 10 minutes. Protein Assay Standard I from BioRad was used to create standards, and along with the samples, was quantified with a Shimadzu Bio-mini at a wavelength of 595nm. X-ray Absorption Near Edge Structure (XANES) was used to determine the speciation of the precipitate created by *C. freundii*. XANES experiments were carried out at the National Synchrotron Light Source at Brookhaven National Laboratory using Beamline X-11B. Samples for synchrotron analysis were collected by filtering liquid samples containing both cells and selenium particles through a 0.45 micron filter. Elemental selenium, sodium selenite, and sodium selenate powders were used as reference standards.

DNA isolation and PCR amplification. Experiments were conducted to amplify the functional selenate reductase gene in C. *freundii.* DNA from *C. freundii* was isolated using a bead beating PowerSoil DNA Isolation Kit with the protocol recommended by the manufacturer (Mo Bio, Carlsbad, CA). Primer sequences were designed using the genome sequence of the related strain *Citrobacter koseri*. Primers (Table 2.1) targeted 192 to 991 base pair sequences in the three genes of the ynf operon, ynfE, ynfG, and ynfH. PCR reactions were performed using a Taq PCR Kit (USB Corporation, Cleveland, Ohio). Briefly, a total volume of 25 µL consisted of 2.5 µL buffer, 0.5 µL dNTPs, 10 µM of each primer, 3 µL DNA extract, and 0.25 µL taq polymerase. PCR amplification was carried out using a BioRad IQ5 Multicolor Real-Time PCR Detection System. An initial denaturation step at 95°C for 5 minutes was followed by 30 cycles consisting of 30 s at 95°C, 60 s at 60°C and 90 s at 72°C. Gels were run on a 1% molecular-screening agarose gel in TAE buffer with a BioRad PowerPac 300 at 125V. Promega Blue/Orange 6X loading dye was mixed with PCR products, using a Promega 100bp DNA ladder. DNA was stained with an ethidium bromide solution and photographed with a BioRad Gel Doc XR+ System. DNA fragments were extracted from agarose gels using an UltraClean 15 DNA Purification Kit (Mo Bio).

Nucleotide sequencing was performed by GENEWIZ, Inc. (New Brunswick, NJ). The BLAST function from the National Center for Biotechnology Information (NCBI) was used to perform database searches and align nucleotide sequences.

RESULTS

Selenate Reduction by *Citrobacter freundii*. The growth characteristics of *C. freundii* was examined on selenate containing LB agar plates. *C. freundii* produced white colored colonies during the first 24 hours of growth (Figure 2.1a). By 48 hours (Figure 2.1b) the colonies develop a deep red-colored core interior to outer pink region, surrounded by a white rim. By 72 hours (Figure 2.1c) the red-colored center within the colony proceeded to develop outward as the bacterial cells continued to grow. The cultures in liquid medium formed pools of matching deep red precipitate after 2 days.

Experiments were conducted with *C. freundii* in liquid culture to determine the sequence of reduction reactions in the presence of oxygen and selenate. Figure 2.2 shows that, in sealed reaction vessels, *C. freundii* preferentially consumes oxygen concurrent with increasing cell biomass. Oxygen is depleted after 6 hours (Figure 2.2a), while protein biosynthesis continues until 36 hours (Figure 2.2b). Only during stationary growth phase and after oxygen is completely removed from the medium, does *C. freundii* begin to noticeably transform with the selenate. Dissolved selenate concentrations decrease at 36 hours and continue to decline for 5.5 days (Figure 2.2c). The formation of a red precipitate occurred concomitantly with selenate removal from solution. After 2 days, the culture turned bright red and dense accumulations of the red precipitate settled to the bottom of the test tubes. These results indicate that selenate reduction by *C. freundii* is an anaerobic process.

Figure 2.3 shows the varying rates of selenate reduction by *C. freundii* as the initial concentration of selenate increases. The rate of selenate reduction increases with increasing selenate concentrations up to 4 mM of selenate (Figure 2.3a). The rate of

selenate reduction was modeled using Monod kinetic equations and an analysis of the data resulted in a q_{max} of 89 μM/day and a K_s of 0.65 mM. The kinetic parameters generated by the Monod model yield an excellent fit to the experimental data.

Under the same growth conditions *C. freundii* does not reduce arsenate (Figure 2.4). At varying initial concentrations of arsenate, arsenate removal or transformation were not detected.

After reaction with selenate, the cell pellets containing the red precipitate were collected and analyzed using X-ray absorption spectroscopy to determine the oxidation state of the solid-phase selenium in the biological samples. Figure 2.5 shows the X-ray absorption near edge spectra (XANES) of cell pellets containing the red particles formed by *C. freundii*. The spectra show that the energy position Se K-edge position shifts to lower energies with decreasing oxidation states. The position of Se K-edge measured for selenate, selenite, and elemental selenium were 12.666 keV, 12.662 keV, and 12.658 keV, respectively. These Se K-edge values are in good agreement with previous XANES measurements of selenium reference compounds (Ma et al., 2007). Comparison of the XANES spectra collected for the Se(VI) reaction products confirm the red precipitate is elemental selenium.

PCR was employed to determine if *C. freundii* carried the functional selenate reductase operon found in related gramma proteobacteria. PCR amplification resulted in 11 overlapping DNA fragments between 200 to 1,100 base pairs in length. The selenate reductase operon, ynfEGH, was completely sequenced from *C. freundii* (Figure 2.6). The BLAST analysis shows that the selenate reductase operon has high similarity to the ynfEGH genes in *E. coli* with protein identities of 79%, 86%, and 73%, respectively. A

FNR binding site was located 95 base pairs upstream of *ynfE* gene, and the Tat signal sequence was identified 30 base pairs into the *ynfE* protein.

DISCUSSION

Macroscopic observation of the *C. freundii* colonies on agar suggested that selenium reduction occurred within anaerobic zones of the biofilm. Indeed, microscopic studies showed that *C. freundii* reduces selenate to elemental selenium in the absence of oxygen, confirming that the reduction reaction is an anaerobic process. This is in contrast to the aerobic metal sorption and uranium complexation activity (Macaskie et al., 1992) of well characterized *Citrobacter* species. Furthermore, *C. freundii* does not reduce arsenate oxyanions, suggesting that the enzyme that catalyzes anaerobic selenate reduction is substrate specific. Although, arsenate may be too toxic for *C. freundii* to remain viable, or the selenate reductase may reduce oxyanions that were not tested in this study. PCR amplification and sequencing showed that *C. freundii* carries the functional selenate reductase gene *ynfE*, which encodes for a putative molybdenum containing oxidoreductase protein. Previous knockout mutation experiments demonstrated that the related bacterial species *E. coli* and *Salmonella* mutants require the *ynfE* gene to reduce selenate (Guymer et al., 2009). Phylogenetic analysis of the *ynfE* gene in *C. freundii* indicates that this selenate reductase belongs to the dimethylsulfoxide (DMSO) reductase family (Figure 2.7). This protein family also includes the dissimilatory nitrate reductases (n*arG* and *napA*), formate dehydrogenases, trimethylamine-N-oxide reductases, and biotin sulfoxide reductases. In well characterized facultative anaerobic bacteria, these

terminal reductases are known to function only when oxygen is depleted from the environment.

DNA sequence analysis indicates that selenate reductase in *C. freundii* contains a FNR binding site 95 base pairs upstream of *ynfE* gene. FNR is a global transcription regulator that mediates physiological changes in response to anoxic environments by monitoring the presence of oxygen (Guest, 1995). In *E. coli*, FNR has been shown to regulate the expression of at least 103 operons (Constantinidou et al., 2006). The FNR protein is located in the cytoplasm, and contains a $[4Fe\text{-}4S]^{2+}$ cluster ligated by four essential cysteine residues that are involved in site-specific DNA-binding activity (Kiley & Beinert, 1999). Under oxic conditions the $[4Fe\text{-}4S]^{2+}$ cluster becomes swiftly oxidized into $[2Fe\text{-}2S]^{2+}$, which lacks the ability to bind onto DNA causing the FNR protein to only be active in anoxic environments (Khoroshilova, Popescu, Munck, Beinert, & Kiley, 1997). Yee et al. (2007) showed that mutation of the FNR gene in the Se-reducing bacterium *E. cloacae* results in complete loss of selenate reduction activity. The occurrence of the FNR gene in all sequenced *Citrobacter* genomes suggests that the anaerobic selenate reduction activity in *C. freundii* is controlled by the FNR regulator.

Protein sequence analysis of the selenate reductase in *C. freundii* shows the presence of a twin-arginine translocation (Tat) signal sequence 30 base pairs into the *ynfE* protein. The Tat pathway is known to transport folded proteins from the cytoplasm to the periplasm through the inner cytoplasmic membrane (Lee et al., 2006). Redox enzymes that function in anaerobic respiration are known to be excreted via the Tat pathway, these include hydrogenases, formate dehydrogenases, nitrate reductases, trimethylamine *N*-oxide (TMAO) reductases, and dimethyl sulfoxide (DMSO) reductases. Ma et al. (2007)

showed that disruption of the Tat pathway in *E.* cloacae *SLD1a-1* results in a complete loss of selenate reduction activity. Because sequenced *Citrobacter* genomes show the Tat transport system, it is likely that the selenate reductase in *C. freundii* is also secreted by this mechanism.

In addition to *ynfE*, the selenate reductase operon in *C. freundii* also contains the downstream units *ynfG* and *ynfH*. The *ynfG* gene is homologous to *dmsB*, which encodes for an iron-sulfur protein that contains 4 [4Fe-4S] clusters. The *ynfH* gene is related to *dmsC*, which encodes for membrane anchoring protein. *Citrobacter youngae* shares the same ynfEGH operon structure as *C. freundii*. However, the structural organization of the ynfEGH operon in *C. freundii* differs slightly from the selenate reductase in *E.coli*. Sequence analysis in *E.coli* has shown the presence of an addition gene, *ynfF*, located between *ynfE* and *ynfG*. *YnfF* has been identified as an oxioreductase enzyme, has been described as a tandem duplication (Lubitz & Weiner, 2003), and *E.coli* shares 74% identities between *ynfE* and *ynfF*. Several other organisms carrying *ynfE* do not possess *ynfF* (Table 2.2).

The reduction and precipitation of selenium by *Citrobacter freundii* and other related organisms may have utility for in situ bioremediation purposes. A survey of microbial genomes of organisms cultured from soil, sediments, and waters show that natural ecological habitats are teeming with a vast variety of bacterial strains containing the *ynfE* gene (Table 2.2). Analysis of these sequences indicate that these *ynfE* homologues invariably contain the FNR binding site, suggesting that the activity of the reductase will be controlled, in part, by oxygen levels. The results of our study suggest that the presence of the functional gene, and the depletion of oxygen are two of the

factors that affect Se reducing activity in *C. freundii*. If the activity observed in *C. freundii* can be extrapolated to other organisms in natural environments, then this microbial pathway may play an important role in selenium oxyanion reduction at oxic-anoxic zones in soil and sediments.

Table 2.1 ynf Operon Primers used in *Citrobacter freundii*

Forward Primer (5' to 3')	Reverse Primer (5' to 3')	# Base Pairs Amplified
TCAAAAGCGACATCGAACAG	GTGCGTTTCATCGGGTAGTT	665
ACGAAGTCGTATGGGTGGAG	GTTGTCGAGGAACGGTTGAT	599
CAAACTGGTGGTGATGTTCG	CACGCAGTAGTTGTCGAGGA	192
GGACGTGAAGACGAATGGAT	CAAATACAGGCCGGTTTAGC	247
TGGTAATGGGACTGGCGTAT	CAGCGTAGGCATACGTTCAA	330
AACCGGCCTGTATTTGTCAG	ATTGCCCGCATAGTCGTTAG	422
CGGGAAAGATAAGCTCGATG	AAAATGCCCGTTAGGGTCTT	449
AATGACCGTCGAACAGGAAG	TATCGCCATTTGCAATACCA	588
GCAGTTGTTTGGCTTCCATT	TCTTTCACATGCAGCCGTAG	991
ACGGGTTTTCTGGCTAATCC	ATGCAGGCCGTAAAACAATC	485
GTGGCGTTTCTCGTCAAAAT	CGGAGAAGGTAGCGCATAAG	727

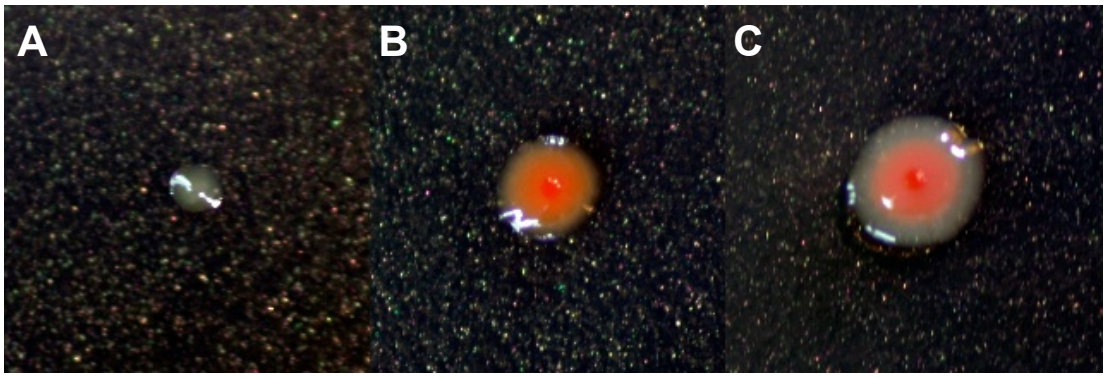

Figure 2.1. *Citrobacter freundii* plated on LB plates in the presence of 10 mM Selenate after (a) 1 day, (b) 2 days, and (c) 3 days. The centers of the colonies are comprised of elemental selenium that has been reduced from selenate by *C. freundii*.

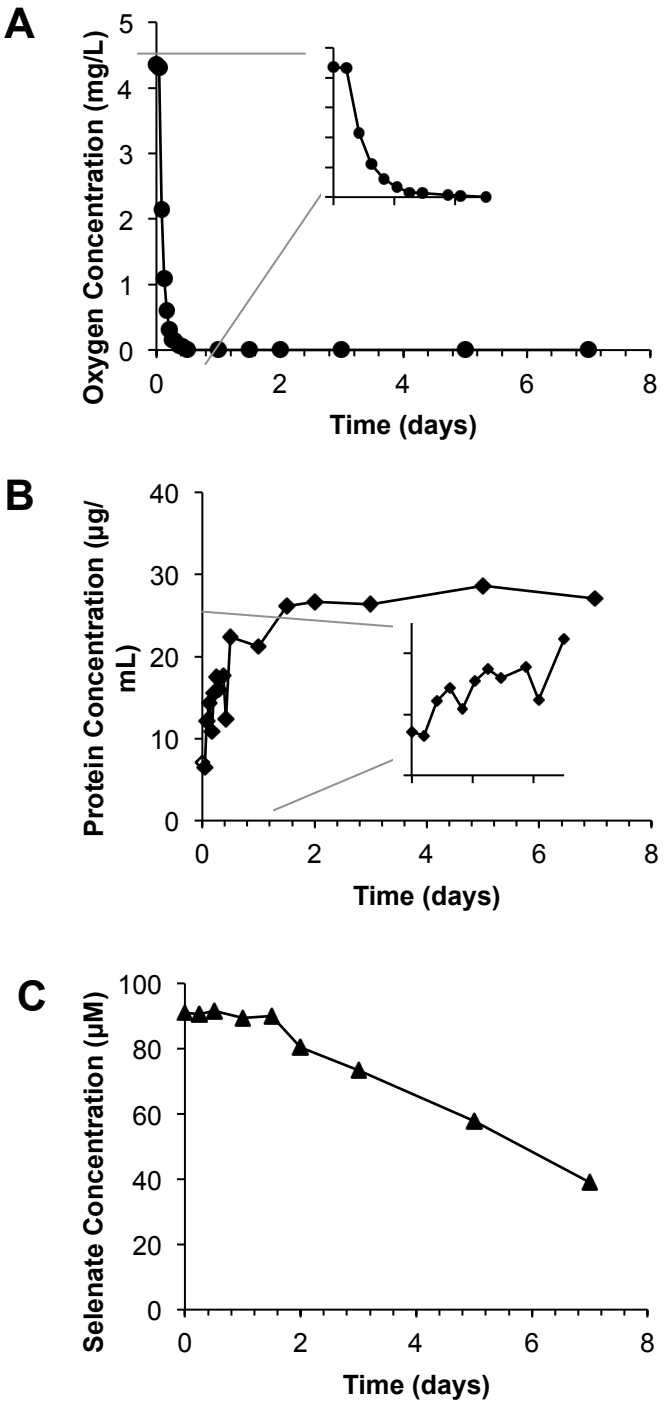

Figure 2.2. *C. freundii* in liquid culture: (A) oxygen; (B) protein; (C) selenate.

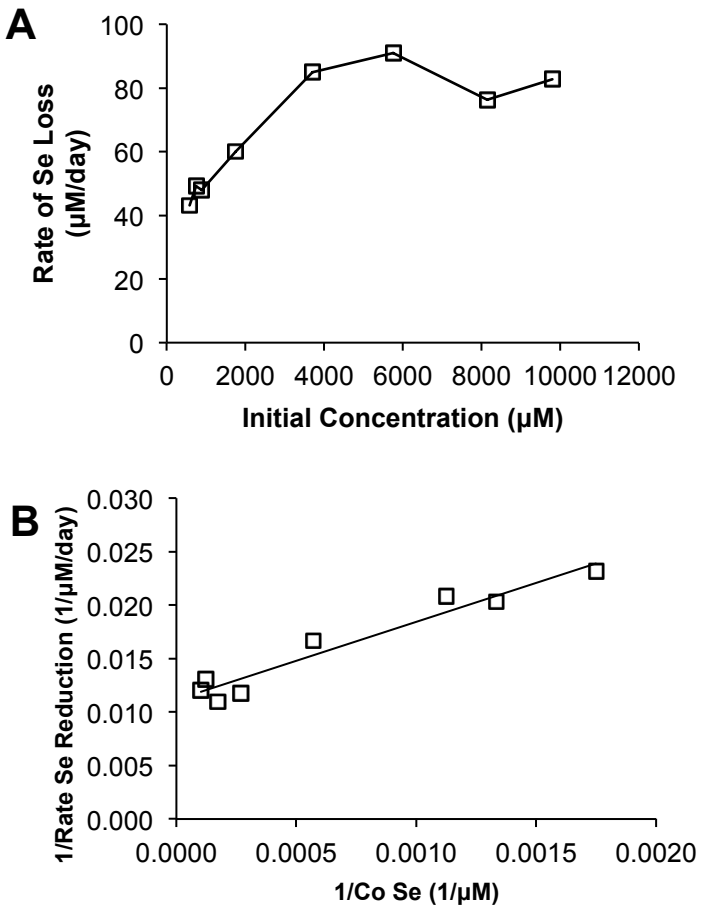

Figure 2.3. (A) Rates of selenate reduction by *C. freundii* at increasing initial concentrations and the corresponding (B) Monod kinetics of the rates where q_{max} is 89 µM/day, and K_s is 0.65 mM.

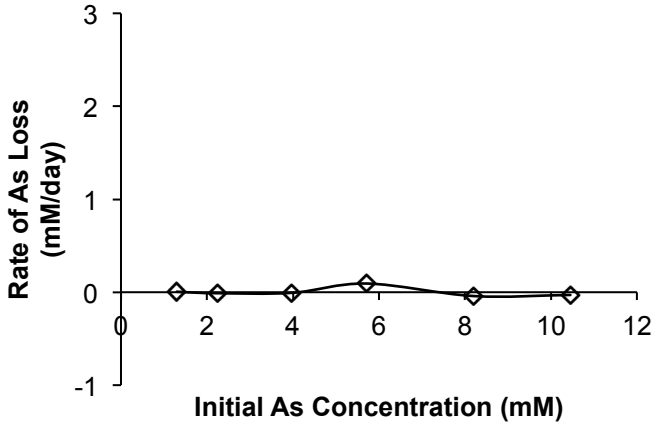

Figure 2.4. Rates of arsenate reduction by *C. freundii* at increasing initial concentrations.

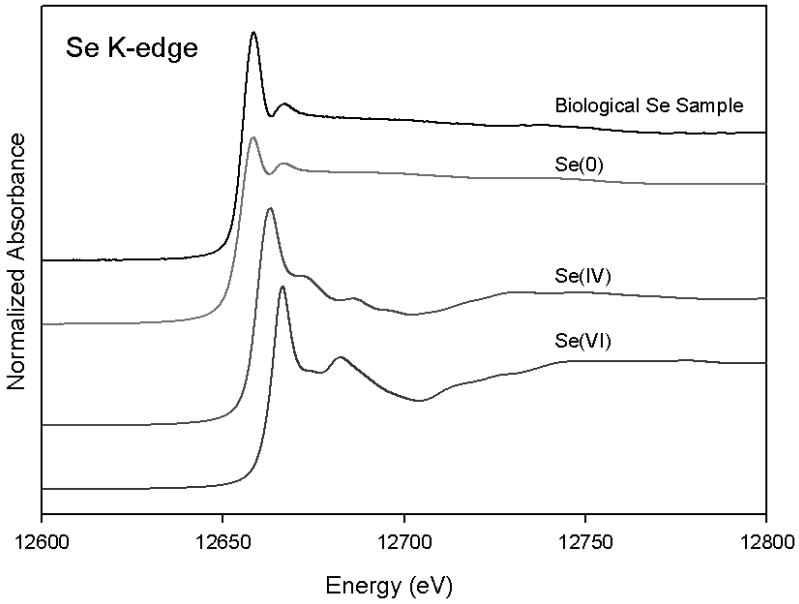

Figure 2.5. X-ray absorption spectra of selenate particles precipitated by *Citrobacter freundii*. Se(0) K-edge XANES most closely matches the biological selenate samples.

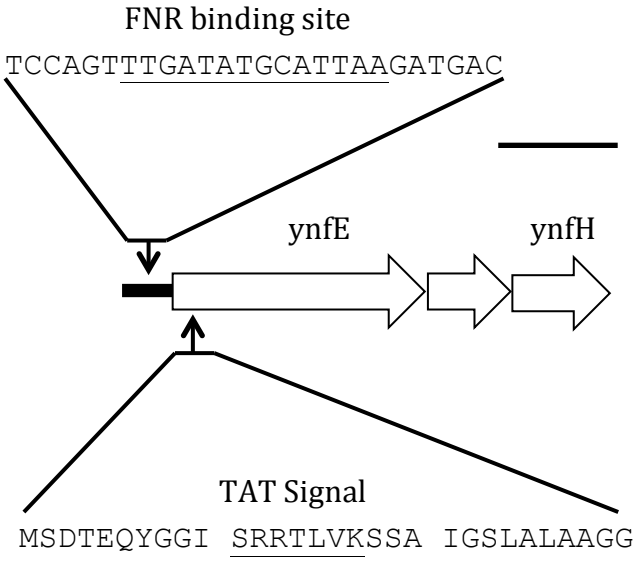

Figure 2.6. Molecular organization of the ynfEGH operon in *C. freundii*. The first arrow represents the FNR binding site, which is located 20 base pairs upstream of ynfE. Thirty base pairs into the ynfE protein is the Tat signal sequence.

Table 2.2. Bacterial Species Containing the *ynfE* Protein, FNR, TatABC, and their

Species	Identity to *E.coli* ynfE	Environmental Location	Operon Structure	FNR	TatA	TatB	TatC
Citrobacter koseri	78%	Water, sewage, soils, food		+	+	+	+
Citrobacter youngae	69%	Soil		+	+	+	+
Dickeya dadantii	72%	Soft rot on crops and ornamentals		+	+	+	+
Enterobacter cancerogenus	68%	Soil		+	+	+	+
Enterobacter cloacae	86%	Soil, water, wastewater		+	+	+	+
Klebsiella pneumoniae	82%	Water, soil, plant surfaces		+	+	+	+
Klebsiella variicola	88%	Fungus gardens		+	+	+	+
Rahnella	75%	Rhizosphere		+	-	-	+
Salmonella enterica	87%	Soil		+	+	+	+
Serratia proteamaculans	76%	Plant root endophytes		+	+	-	+
Shigella boydii	98%	Soil		+	+	+	+
Shigella dysenteriae	96%	Soil		+	+	+	+
Yersinia pestis	66%	Soil		+	+	+	+

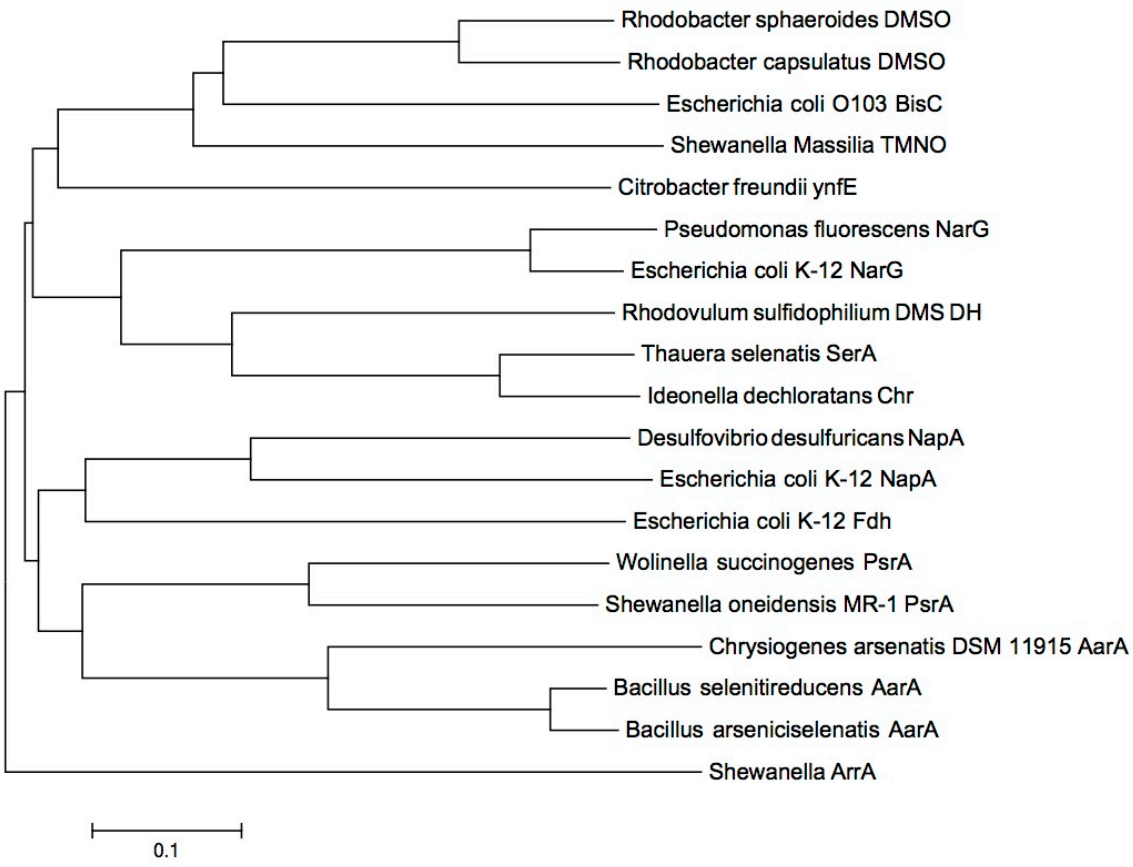

Figure 2.7. Phylogenetic analysis of the *ynfE* gene in *C. freundii* compared with known molybdoenzymes.

Chapter 3: Genetic Evidence for a Molybdopterin-Containing Tellurate Reductase

ABSTRACT

The genetic identity and co-factor composition of the bacterial tellurate reductase are currently unknown. In this study, we examined the requirement of molybdopterin biosynthesis and molybdate transporter genes for tellurate reduction by *Escherichia coli*. The results demonstrate that mutants carrying deletions of the *moaA, moaB, moaE,* or *mog* gene in the molybdopterin biosynthesis pathway lost the ability to reduce tellurate. Deletion of the *mod*B or *mod*C genes in the molybdate transport pathway also resulted in complete loss of tellurate reduction activity. Genetic complementation by the wild-type sequences restored tellurate reduction activity in the mutant strains. These findings provide genetic evidence that the tellurate reductase in *E. coli* is a molybdopterin-containing enzyme.

INTRODUCTION

Tellurium (Te) is a metalloid element used for a variety of industrial applications, including metallurgy, chemical manufacturing, and electronics (Chasteen & Bentley, 2003; Taylor, 1999). The disposal of mine tailing and tellurium-containing wastes has led to an increase in environmental contamination (Wray, 1998). When released into the environment, tellurium undergoes redox transformations and can be mobilized as the dissolved Te oxyanions tellurate [Te(VI), TeO_4^{2-}] and tellurite [Te(IV), TeO_3^{2-}]. These oxyanions are highly toxic to microbiota, and cause inhibitory effects to most microorganisms at concentrations as low as 1 μg/mL (Taylor, 1999). While naturally

occurring Te-resistant bacteria have been isolated that are able to grow in the presence of elevated Te concentrations (Baesman et al., 2007, 2009), and several genetic elements have been linked to Te-resistance (Chasteen at al., 2009), the molecular mechanisms of bacteria-tellurium interactions remain poorly understood.

Microorganisms are known to catalyze the reduction of toxic Te oxyanions into sparingly soluble and less toxic elemental tellurium [Te(0)]. Diverse species of bacteria have been shown to reduce tellurite to Te(0) (Castro et al., 2009; Chiong at al., 1988; Harrison at al., 2004; Moore & Kaplan, 1992; Summers & Jacoby, 1977). In comparison, little is known about the microbial reduction of tellurate, even though Te(VI) is the dominant form of Te in the hydrosphere (Huang & Hu, 2008; Lee & Edmond, 1985). Recently, anaerobic respiration on Te(VI) was shown by *Shewanella* species isolated from deep ocean hydrothermal vent worms (Csotonyi et al., 2006), and the anaerobic bacteria *Sulfurospirillum barnesii* and *Bacillus selenitireducens* were found to generate energy for growth on lactate by the reduction of Te(VI) to Te(0) (Baesman et al., 2007). Baesman et al. (2009) isolated a Gram positive bacterium *Bacillus beveridgei* that can also grow by reducing Te(VI) to Te(0). Currently, the genetic identity and cofactor composition of enzymes that catalyze tellurate reduction in these bacteria, as well as other Te(VI)-reducers, are unknown.

The molybdenum cofactor forms the active site of several important bacterial redox enzymes, including the proteins in the DMSO reductase family. Sequence analysis indicates that pterin-containing molybdenum enzymes catalyze the reduction of selenium and arsenic (Bébien et al., 2002; Malasarn et al., 2008), two metalloid elements that share similar chemical characteristics to tellurium. In selenate and arsenate reducing bacteria,

the assembly of the pterin-containing reductases is dependent on molybdendum import into the cell, and the biosynthesis of the molybdopterin cofactor. Previous studies have demonstrated that molybdate uptake in *E. coli* is facilitated by an ABC-type transporter, and the molybdopterin cofactor is constructed via a biosynthetic pathway encoded by the moa-mog gene system (Leimkühler at al., 2011; Self at al., 2001). To date, the role of molybdate transporter and molybdopterin biosynthesis genes in tellurate reduction remains poorly understood.

In this study, we tested *E. coli* mutants carrying single mutations in the molybdopterin biosynthesis (moa-mog) and molybdate transporter (modABC) gene systems for tellurate reduction activity. The objective was to determine if the tellurate reductase in *E. coli* is a molybdopterin-containing enzyme. The results indicate that the reduction of Te(VI), like Se(VI), is catalyzed by a molybdo-enzyme.

METHODS

Tellurate Reduction Experiments. All strains were grown and maintained on LB agar (Difco). Tellurate reduction activity was determined for the wild-type *E. coli* strain K-12, and *E. coli* mutant strains: *fnr, menA, menC, menD, menE, moaA, moaB, moaC, moaD, moaE, tatB, tatC, modA, modB, modC,* and *mog* (Table 3.1). Tellurate reduction activity was also determined for *moaA*, *modB*, and *mog* mutants carrying plasmids described in Table 3.1. Mutant strain tellurate reductase activity was determined by growing strains in LB broth overnight at 37°C reaching an OD_{600} of 0.85 ± 0.05 before spiking with 50 μM tellurate and taking a sample after 24 hours.

To determine tellurate reductase activity for wild-type *E. coli* strain K-12, cells were grown in LB broth overnight at 37°C reaching an OD_{600} of 1.6 ± 0.2. Cultures were centrifuged and a condensed culture was dispensed into flasks of LB containing 50 μM Na_2TeO_4. Samples were taken then at periodic intervals, and the black Te(0) precipitate was removed by filtration (0.45 μm). The total dissolved tellurium remaining in the media was analyzed using Inductively Coupled Plasma Optical Emission Spectroscopy (Vista-PRO Simultaneous, Varian Inc., Palo Alto, CA). Te standards for the calibration curve were prepared with the same LB broth used in the experiments. In a parallel wild-type *E.coli* K-12 experiment, 1 mM of Na_2Mo_4 was added to the LB medium prior to inoculation in order to test the effect of molybdenum amendment on tellurate reductase activity. Control experiments were conducted with cells that were heat-killed in a water bath at 80°C for 30 minutes. All experiments were carried out with triplicate cultures.

The mutant strains *ubiH, dmsA, arsC, ynfE, ynfF, bisC, torA, torZ, fdhF, fdoG, fdnG, napA, narG, narZ, ydeP, yheS,* and *yfgD* were also tested for tellurite, and selenate reduction activity. To test for tellurite reduction activity, overnight LB cultures were spiked with 50 μM Na_2TeO_3 and visually examined for the formation of black Te(0) over a period of seven days. To test for selenate reduction activity, the mutant strains were grown in LB broth amended with 1 mM sodium selenate and visually examined for the formation of red elemental selenium [Se(0)].

Genetic Complementation. In order to establish the role of the modA, moaA, and mog genes in tellurate reduction activity, genetic complementations were performed with the *E. coli* mutants. To clone the moaABCDE operon for complementation of the moaA mutant, a primer set was constructed that consisted of forward primer moa-F1 (5'-

GCGAAATAGCACGATCATGACGC-3') positioned 218 bp upstream of the start codon for the moaA gene and reverse primer moa-R1 (5'-GCGTAAACGTATGTACTGAGCGG-3') positioned 44 bp downstream of the stop codon for the moaE gene. The amplified product was purified using an UltraClean 15 DNA Purification Kit (Mo Bio, Carlsbad, CA), and then A tails were added to the blunt-ended DNA fragments through a second PCR cycle for 20 minutes at 72°C. The refined product was cloned into pCR2.1-TOPO vector using the One Shot TOP10 protocol from the Invitrogen TOPO TA Cloning protocol (Life Technologies, Grand Island, NY), resulting in pECA27. To clone the modABC operon for complementation of the modB mutant, a primer set was constructed that consisted of forward primer mod-F1 (5'-CAACTTCCTGCTTTTCCTGCCG-3') positioned 127 bp upstream of the start codon for the moaA gene and reverse primer mod-R1 (5'-GCCCAGTTCATTTATAGCCACC-3') positioned 8 bp downstream of the stop codon for the modC gene. The amplified product was cloned into pCR2.1-TOPO vector, resulting in pECD25. Finally to clone the *mog* gene for complementation of the mog mutant, a primer set was constructed that consisted of forward primer mog-F1 (5'- GGTCACGCTACCTCTTCTGAAGC-3') positioned 89 bp upstream of the start codon for the mog gene and reverse primer mog-R1 (5'-TTATTCGCTAACGTCGCGTCTTGC-3') consisting of the last 24 bp of the mog sequence. The amplified product was cloned into pCR2.1-TOPO vector, resulting in pECG07.

To confirm successful ligation of the PCR product and disruption of the lacZ reading frame, the plasmids were transformed into *E. coli Top10* cells, incubated on ice for 30 minutes, and then plated onto plates with 40 µg/mL of X-gal. White colonies were

picked for plasmid isolation and purification. After purification using a QIAprep Spin Miniprep Kit (QUAIGEN inc., Valencia, CA), the plasmids were visualized on 1% agarose gel and quantified on a nanodrop spectrometer.

The purified plasmids pECA27, pECD25, and pECG07 were subsequently inserted into MOAAM, MODBM, and MOGM respectively forming ECMOAC, ECMODC, and ECMOGC. MOAAM, MODBM, and MOGM were grown separately overnight in LB broth at 37°C before being placed into a sterile centrifuge tube and spun at 6,000 g for 10 minutes at 4°C. The resulting pellet was resuspended in 10 mL cold CG solution, which consists of 85% milliQ water, 10% glycerol, 5% 1M $CaCl_2$. The cells were allowed to sit on ice for 30 minutes before being centrifuged again at 6,000 g for 10 minutes at 4°C and resuspended into 1 mL cold CG solution. The purified plasmids pECA27, pECD25, and pECG07 were added to cultures of MOAAM, MODBM, and MOGM respectively, vortexed, incubated in an ice bath for 3 minutes, incubated at 45°C for 2 minutes, then incubated in an ice bath again for 5 minutes. The cells were subsequently added to 100 µL LB broth and incubated at 37°C for 2 hours. The complemented cells were then plated on plates with a concentration of 100 µM/mL ampicillin.

RESULTS

E. coli K-12 cells incubated with Te(VI) removed over 70% of the dissolved Te from the culture medium after 48 h (Figure 3.1). The loss of Te(VI) was concurrent with the formation of black Te(0) precipitate, which was visible after 3 h of incubation. No

loss of dissolved Te(VI) was observed in control experiments conducted with heat-killed cells.

To determine if molybdopterin biosynthesis genes were required for tellurate reduction, Te(VI) reduction experiments were conducted using *E. coli K-12* mutant strains containing single mutations in moaABCDE operon or *mog* gene. Deletion of the *moaA* gene resulted in the complete loss of tellurate reduction activity (Figure 3.2a). Te(VI)-containing medium inoculated with the Δ*moaA* strain showed no tellurate loss, and did not form black Te(0) precipitates. *E. coli* mutants containing a deletion of either the moaB or *moaE* gene were also unable to reduce Te(VI). Transformation of pECA27 into the mutant strain JW0747-1 carrying the *moaA* deletion restored the mutant's ability to reduce Te(VI), though the rates of tellurate reduction were slightly slower. The mutant strain JW0008-5 carrying a single deletion of the *mog* gene was also defective in Te(VI) reduction activity (Figure 3.2b). Complementation of the *E. coli mog* mutant with pECG07 fully restored the eliminated phenotype.

To determine if molybdate transporter genes are required for tellurate reduction, experiments were conducted using *E. coli* mutant strains containing single mutations in the modABC operon. Deletion of either the *modB* or *modC* gene resulted in the complete loss of tellurate reduction activity (Figure 3.2c). Complementation of the *E. coli modB* mutant with pECD25 fully restored the tellurate reduction activity.

Mutation of molybdopterin biosynthesis genes (moaABCD and mog) and molybdate transporter genes (modABC) had no affect on tellurite reduction activity (Table 3.2). All mutant strains tested in this study were able to reduce Te(IV) to Te(0), indicating that tellurite reduction occurs by a different pathway than tellurate reduction.

Interestingly, mutation of molybdate uptake and molybdopterin biosynthesis genes eleminated selenate [Se(VI)] reduction activity in *E. coli*. The mutation of fumarate nitrate reduction regulator gene (*fnr*) and genes in the twin arginine translocation pathway (*tatBC)* genes also resulted in the complete loss of Se(VI) reduction activity, but had no effect on the reduction of tellurate, nor the reduction of tellurite to elemental tellurium (Table 3.2).

Finally, we tested the tellurate reduction activity in a series of *E. coli* mutants carrying single mutations of genes encoding for molybdenum-containing oxidoreductases. Table 3.3 shows the activity of *E. coli K-12* mutants with single mutations of the genes *dmsA, ynfE, ynfF, bisC, torA, torZ, fdhF, fdoG, fdnG, napA, narG, narZ, ydeP, yheS,* and *yfgD*. All these mutant strains tested positive for tellurate reduction.

DISCUSSION

Mutants carrying gene deletions in the molybdopterin biosynthesis pathway lost the ability to reduce tellurate (Figure 3.2). Complementation by the wild type sequence restored tellurate reduction activity in mutants strains JW0747-1 and JW0008-5. In *E. coli*, the protein product of *moaA* is responsible for converting guanosine triphosphate to cyclic pyranopterin monophosphate (cPMP) (Leimkühler et al., 2011). Because cPMP is a critical intermediate in the biosynthesis of molybdopterin, mutation of the *moaA* gene prohibits the formation of the molybdenum cofactor. Furthermore, *moaE* is a critical component of the MPT synthase protein that inserts two sulfur atoms into cPMP. Mutation of the moaE gene prevents insertion of the dithiolene functional groups which

are required for Mo ligation by cPMP. In the final step of molybdenum cofactor synthesis, molybdenum is chelated and incorporated into molybdopterin by the action of the mog protein. Deletion of the mog gene precludes the incorporation of molybdenum into molybdopterin, and thus inhibits the formation of an active tellurate reductase.

Mutants carrying deletions of *modB* and *modC*, genes that are required for molybdate transport, also lost the ability to reduce tellurate (Figure 3.2c). The protein product of *modB* provides the transmembrane channel necessary to transfer molybdate across the cytoplasmic membrane (Self et al., 2001). Mutants carrying *modB* gene deletions are thus unable to transport molybdate ions from the periplasm into the cell. *ModC* is the ATPase subunit that serves as an energizing protein of the molybdate transport system. Mutation of the *modC* hampers ATP-binding and energization of molybdate transporter. The loss of the molybdate transporter system into the cell impedes the delivery of molybdenum to molybdopterin, which further supports the hypothesis that the active site of the tellurate reductase contains the molybdenum cofactor.

In addition to the loss of tellurate reduction activity, mutation of the molybdate uptake and molybdopterin biosynthesis genes in *E. coli* also eliminated the bacterium's ability to reduce selenate to elemental selenium. The selenate reductase in *E. coli* and *Salmonella enterica serovar Typhimurium* LT2 were recently shown to be encoded by the genes *ynfE* and *ynfF* (Guymer et al., 2009). The *ynfE* and *ynfF* proteins are predicted to bind a molybdopterin cofactor, and similar to other DMSO reductases, require molybdenum for their catalytic activity. The molybdopterin-containing selenate reductase in *E. coli* exhibits similar functions to the membrane-bound respiratory DMSO reductase. First, the selenate reductase carries an N-terminal twin-arginine translocation (TAT)

signal sequence. Here we show that mutation of the genes *tatB* and *tatC* in *E. coli*, which obstructs protein export via the TAT system, causes complete loss of Se(VI) reduction activity (Table 3.2). This is similar to the TAT-dependent selenate reductase in *Enterobacter cloacae* SLD1a-1 (Ma et al., 2007). Secondly, the *E. coli* selenate reductase is connected to the electron transport chain using electrons supplied by menaquinol to reduce selenate to selenite. Previously, we demonstrated that *E. coli mutants* carrying a deletion of either the *menD*, *menC* or *menE* gene in the menaquinone biosynthesis pathway was unable to reduce selenate (Ma et al., 2009). Finally, upstream of the *ynfE* gene is a conserved sequence for the FNR protein binding site, and we show that deletion of the *fnr* gene in *E. coli* eliminates selenate reduction activity (Table 3.2). This suggests that expression of the *ynfE* and *ynfF* genes are similar to the selenate reductase gene in *E. cloacae* SLD1a-1, which is regulated by the global anaerobic regulator FNR (fumarate nitrate reduction regulator) (Yee et al., 2007).

The tellurate reductase gene was not identified in this study. However, several *E. coli* mutants were examined for Te(VI) and Te(IV) reductase activity, and comparison with selenate reduction provide insights into the characteristics of the tellurate reductase. First, the deletion of TAT genes does not eliminate tellurate reductase activity (Table 3.2), suggesting that *E. coli* harbors a TAT-independent tellurate reductase. One possibility is that the tellurate reductase may be similar to the TAT-independent nitrate reductase, *narG*, which is a molybdopterin-contain enzyme that catalyzes oxyanion reduction inside the cytoplasm. Furthermore, deletion of the *fnr* gene also does not inhibit tellurate reduction (Table 3.2), indicating that unlike the selenate reductase, *E. coli* does not regulate the expression of the tellurate reductase under anaerobic conditions. We

were unable to grow *E. coli* using tellurate as the sole terminal electron acceptor, and it does not appear that tellurate reduction is an anaerobic respiratory process. Thus it is unlikely that the tellurate reductase in *E. coli* is associated with the electron transport chain. Finally, *E. coli* mutants lacking the *moaA*, *moaB, moaE, modB, modC*, or *mog* genes retain the ability to reduce tellurite, demonstrating that tellurate reductase is distinct from the tellurite reduction enzyme.

An interesting question is whether or not tellurate reducing bacteria, including Te(VI) respiring microorganisms, also employ a molybdopterin-contain enzyme for tellurate reduction. Intriguingly, genome sequences for tellurate respiring bacteria *Bacillus selenitireducens* and *Sulfurospirillum barnesii* contain the *modABC* transporter genes for molybdate uptake, and *moaE* gene for molybdopterin biosynthesis. Because *B. selenitireducens* was isolated from Mono Lake, CA, and *Sulfurospirillum barnesii* is a freshwater proteobacterium, we speculate that molybdopterin-containing tellurate reductases could be active in environmental settings. If this is the case, then pterin-containing molybdenum enzymes may play an important role in the reduction of tellurate oxyanions in the natural hydrosphere.

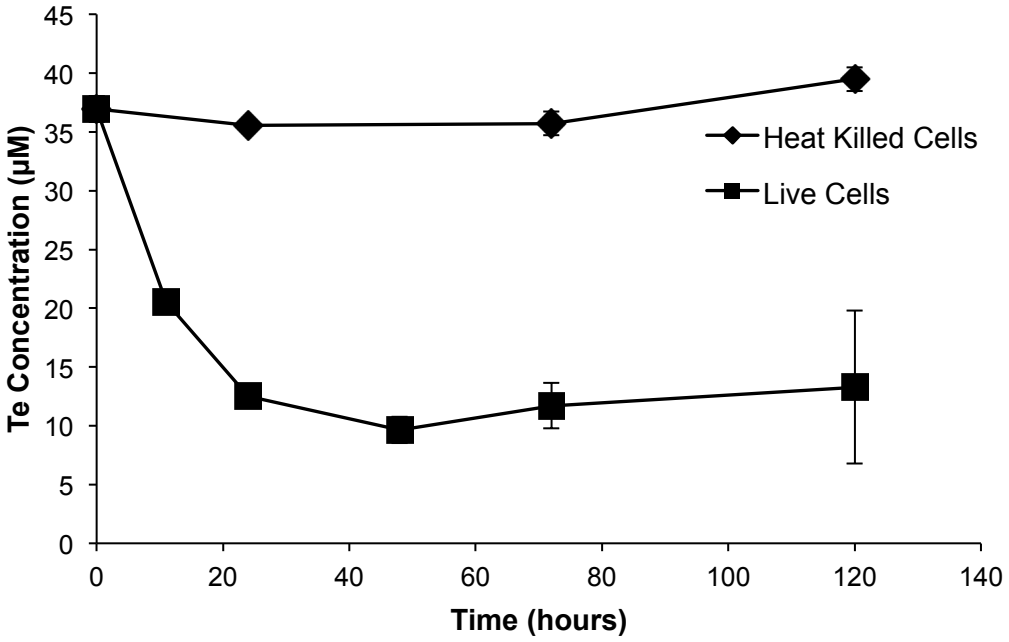

Figure 3.1: Tellurate Reduction by Escherichia coli K-12 wild-type at 37°C. Cells were heat-killed by being placed in a water bath at 80°C for 30 minutes. Symbols and error bars represent the average and standard deviation of triplicate experiments.

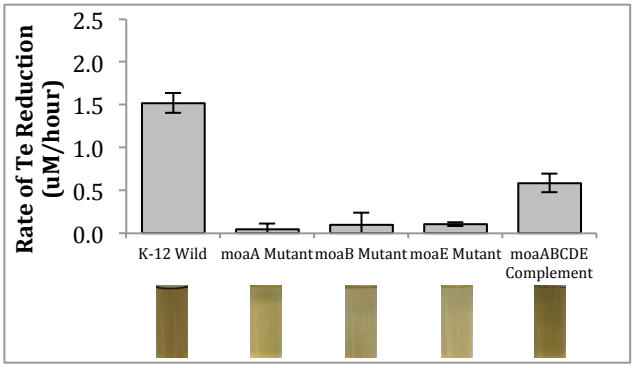

Figure 3.2a: Tellurate reduction rates for the molybdopterin biosynthesis pathway after 24 hours in LB broth at 37°C. *E.coli* K-12 wild-type, mutants JW0764-2, JW0765-1, JW0768-1, and JW0764-2 complemented with pECA27. Error bars represent standard deviation for parallel triplicate experiments. Pictures show corresponding strain after 24 hours.

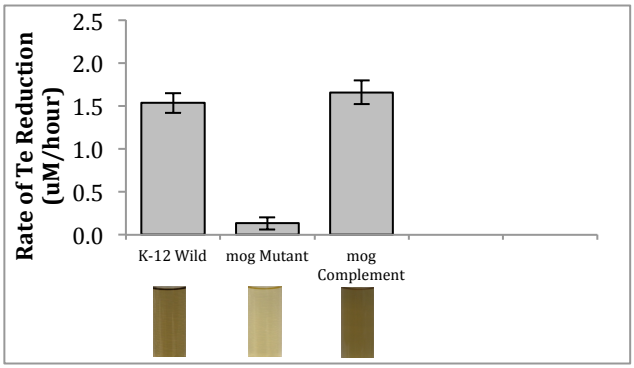

Figure 3.2b: Tellurate reduction rates for molybdenum cofactor synthesis after 24 hours in LB broth at 37°C. *E.coli* K-12 wild-type, mutant JW0008-5, and JW0008-5 complemented with pECG07. Error bars represent standard deviation for parallel triplicate experiments.

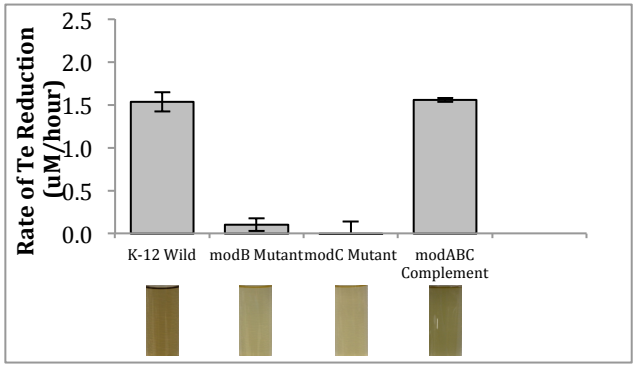

Figure 3.2c: Tellurate reduction rates for the molybdate transporter system after 24 hours in LB broth at 37°C. *E.coli* K-12 wild-type, mutants JW0747-1, JW0748-2, and JW0747-1 complemented with pECD25. Error bars represent standard deviation for parallel triplicate experiments. Pictures show corresponding strain after 24 hours.

Table 3.1: Bacterial strains and plasmids.

Strains or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli K-12		
JW0764-2 (MODAM)	*moaA*753(del)::kan	Baba *et al.* (2006)
JW0765-1	*moaB*754(del)::kan	Baba *et al.* (2006)
JW0766-1	*moaC*755(del)::kan	Baba *et al.* (2006)
JW0767-1	*moaD*756(del)::kan	Baba *et al.* (2006)
JW0768-1	*moaE*757(del)::kan	Baba *et al.* (2006)
JW0746-1	*modA*735(del)::kan	Baba *et al.* (2006)
JW0747-1 (MODBM)	*modB*736(del)::kan	Baba *et al.* (2006)
JW0748-2	*modC*737(del)::kan	Baba *et al.* (2006)
JW0008-5 (MOGM)	*mog*-729(del)::kan	Baba *et al.* (2006)
JW3901-1	*menA*789(del)::kan	Baba *et al.* (2006)
JW2256-1	*menC*743(del)::kan	Baba *et al.* (2006)
JW5374-5	*menD*746(del)::kan	Baba *et al.* (2006)
JW2255-1	*menE*742(del)::kan	Baba *et al.* (2006)
JW1328-1	*fnr*-771(del)::kan	Baba *et al.* (2006)
JW5580-1	*tatB*780(del)::kan	Baba *et al.* (2006)
JW3815-1	t*atC*781(del)::kan	Baba *et al.* (2006)
JW2875-1	*ubiH*758(del)::kan	Baba *et al.* (2006)
ECMOAC	JW0764-2 containing pECA27, Ap^r	This study
ECMODC	JW0746-1 containing pECD25, Ap^r	This study
ECMOGC	JW0008-5 containing pECG07, Ap^r	This study
Plasmids		
pCR2.1-TOPO	Cloning vector, Ap^r	Invitrogen
pECA27	2.7 kb moaABCDE PCR product cloned into pCR2.1-TOPO	This study
pECD25	2.5 kb modABC PCR product cloned into pCR2.1-TOPO	This study
pECG07	0.7 kb mog PCR product cloned into pCR2.1-TOPO	This study

Table 3.2: Anion Reduction by *E.coli* Mutants (+ represents positive activity)

	Te (VI)	Te (IV)	Se (IV)
K-12 wild-type	+	+	+
fnr	+	+	-
moaA	-	+	-
moaB	-	+	-
moaC	+	+	-
moaD	+	+	-
moaE	-	+	-
tatB	+	+	-
tatC	+	+	-
modA	+	+	-
modB	-	+	-
modC	-	+	-
mog	-	+	-
ubiH	+	+	-

Table 3.3: Molybdenum Enzyme Mutants Tested for Tellurate Reduction (+ represents positive activity).

Protein	Function	Tellurate Reduction
dmsA	Molybdopterin cofactor-binding dimethyl sulphoxide (DMSO) reductase subunit	+
ynfE	Tat-dependent selenate reductase enzyme	+
ynfF	Tat-dependent selenate reductase enzyme	+
bisC	Enzyme that reduces biotin-d-sulfoxide to biotin	+
torA	Trimethylamine N-oxide reductase enzyme	+
torZ	Homolog of torA	+
fdhF	Membrane-associated formate dehydrogenase isoenzyme	+
fdoG	Catalytic subunit of formate dehydrogenase-O	+
fdnG	Catalytic subunit of formate dehydrogenase-N	+
napA	Encodes the periplasmic nitrate reductase molybdoprotein with an Fe-S center	+
narG	Nitrate reductase	+
narZ	Nitrate reductase	+
ydeP	Putative oxidoreductase	+
yfgD	Putative oxidoreductase	+

Chapter 4: Summary and Conclusions

Selenate reduction by *Citrobacter freundii*

An abundance of bacteria have the ability to reduce selenate to elemental selenium. Selenate is a water soluble oxyanion and is highly toxic at low concentrations, while elemental selenium is insoluble and non-toxic. *Citrobacter freundii* was found to reduce selenate to elemental selenium in anoxic conditions, which was confirmed by XANES data. This bacterium has been shown to strongly interact with a wide range of different environmental contaminants. Most notably, *C. freundii* can sequester uranium via phosphate precipitation and reduce nitramine explosives in soil (Kitts et al., 1994; Macaskie et al., 1992). In metal-bearing streams and wastewater, *C. freundii* has been shown to absorb the heavy metals lead, cadmium, and zinc (Puranik 1999). This demonstrates the high probability that different enzyme processes are being employed in oxic and anoxic environments.

Previous knockout mutation experiments demonstrated that the related bacterial species *E. coli* and *Salmonella* mutants require the *ynfE* gene to reduce selenate (Guymer et al., 2009). PCR amplification and sequencing showed that *C. freundii* carries the functional selenate reductase gene *ynfE*, which encodes for a putative molybdenum containing oxidoreductase protein. Phylogenetic analysis of the *ynfE* gene in *C. freundii* indicates that this selenate reductase belongs to the dimethylsulfoxide (DMSO) reductase family.

C. freundii carries the operon ynfEGH and *Citrobacter youngae* shares this operon structure. The ynfEGH operon belongs to the DMSO reductase family. Included in the DMSO reductase family are dissimilatory nitrate reductases, formate

dehydrogenases, trimethylamine-N-oxide reductases, and biotin sulfoxide reductases, which serve as membrane bound terminal reductases to respective substrates, in anoxic conditions, in order to provide a more valuable energy metabolism compared to fermentation (Kisker 1997). DMSO contains three subunits, a molybdenum containing subunit (*DmsA*), an iron sulfur cluster subunit (*DmsB*), and a transmembrane subunit that binds and oxidizes menaquinol (*DmsC*). Electrons are passed from the oxidation of menaquinol, via the iron sulfur cluster to the molybdenum containing subunit, generating ATP (Kisker 1997). *YnfE* is a catalytic subunit molybdo-enzyme that is homologous to *dmsA*. It has also been shown through dms promoter control experiments that *ynfG* contains the 4 [4Fe-4S] clusters and is comparable to *DmsB*, and *ynfH* is a membrane protein related to *DmsC*.

Genetic studies have shown that selenate reductase activity requires the global anaerobic regulatory gene FNR (fumarate nitrate reduction regulator) (Yee et al., 2007). The FNR binding site was found 95 base pairs upstream of the *ynfE* gene. FNR is a global transcription regulator that mediates physiological changes, in response to anoxic environments, by monitoring the presence of oxygen. It has been shown to regulate at least 103 operons and is positioned in the cytoplasm (Constantinidou 2006). In oxic environments the $[4Fe\text{-}4S]^{2+}$ cluster becomes swiftly oxidized into $[2Fe\text{-}2S]^{2+}$, which lacks the ability to bind onto DNA causing the FNR protein to only be active in anoxic environments (Khoroshilova 1997).

The last piece to the puzzle is the Tat twin-arginine translocation (Tat) signal sequence, which was found in *C. freundii* 30 base pairs into the *ynfE* protein. The twin-arginine translocation (Tat) pathway transports folded proteins from the cytoplasm to the

periplasm through the inner cytoplasmic membrane. Redox enzymes that function in anaerobic respiration are known to be excreted via the Tat pathway, these include hydrogenases, formate dehydrogenases, nitrate reductases, trimethylamine *N*-oxide (TMAO) reductases, and dimethyl sulfoxide (DMSO) reductases (Lee 2006). Research has illustrated that when mutating the Tat pathway a complete loss of selenate reduction activity in *E.* cloacae *SLD1a-1* occurs.

C. freundii contains the three parts necessary for selenate reduction to elemental selenium. There is a great potential to harness microbial transformation of selenate to elemental selenium for bioremediation projects by reducing the bioavailability of selenate in the environment. Soil, sediments, and waters are teeming with a vast variety of bacterial strains containing the *ynfE* gene, as seen in Table 2.2. These strains also include the FNR binding site and the Tat signal, which are necessary for selenate reduction in bacteria. If the genes required for selenate reduction can be assessed fully, the bacteria can be employed to bioremediate soil and aquatic environments with toxic levels of selenate.

Tellurate Reduction by *Escherichia coli*

The deletion of genes responsible for parts of the molybdopterin biosynthesis pathway resulted in the loss of tellurate reduction activity. Complementation of these genes back into the mutants resulted in the restoration of tellurate reduction activity. In *E.coli* moaA converts guanosine triphosphate to cPMP, an essential intermediate in the biosynthesis of molybdopterin, and the mutation of *moaA* prohibits the formation of the molybdenum cofactor (Leimkühler et al., 2011). *MoaE* is part of the MPT synthase

protein that inserts two sulfur atoms into cPMP, and the mutation of the *moaE* gene prevents insertion of the dithiolene functional groups which are needed for Mo to bind to cPMP. Lastly, *mog* chelates and incorporates molybdenum into molybdopterin. Deletion of all these genes prevents the formation of molybdopterin, and in turn inhibits an active tellurate reductase.

The mutation of genes responsible for molybdate transport also caused a loss in tellurate reduction activity. *ModB* provides the transmembrane channel needed to transfer molybdate ions from the periplasm, across the cytoplasmic membrane, into the cell (Self et al., 2001). Mutation of *modC* terminates the ATP-binding and energization of the molybdate transporter, which halts the delivery of molybdenum to molybdopterin.

Even though the tellurate reductase gene was not identified in this study, the examination of tellurite and selenate reduction activity shed light on the characteristics of the tellurate reductase. Along with the loss of tellurate reduction activity, mutation of molybdate uptake and molybdopterin biosynthesis genes in *E.coli* prevented its ability to reduce selenate to elemental selenium. The mutation of TAT and *fnr* genes did not prohibit tellurate reduction, but it does inhibit selenate reduction. This suggests that unlike the selenate reductase, the tellurate reductase is TAT-independent and *fnr* does not regulate the expression of tellurate reduction under anaerobic conditions. The inability to grow *E.coli* with tellurate as the sole terminal electron acceptor suggests that tellurate reduction is unlikely an anaerobic respiratory process, and could possibly be a detoxification process. Lastly, *E. coli* mutants lacking the *moaA*, *moaB, moaE, modB, modC*, or *mog* genes retain the ability to reduce tellurite, demonstrating that tellurate reductase is distinct from tellurite reduction enzyme.

Genome sequences for tellurate respiring bacteria *Bacillus selenitireducens* and *Sulfurospirillum barnesii* contain the modABC transporter genes for molybdate uptake, and *moaE* gene for molybdopterin biosynthesis. Since *B. selenitireducens* was isolated from Mono Lake, CA, and *Sulfurospirillum barnesii* is a freshwater proteobacterium, we theorize that molybdopterin-containing tellurate reductases could be active in relevant environmental settings. Pterin-containing molybdenum enzymes may play an important role in the reduction of tellurate and selenate oxyanions in the natural environment.

References

Avazéri, C., Turner, R. J., Pommie, J., Weiner, J. H., Giordano, G., & Verméglio, A. (1997). Tellurite reductase activity of nitrate reductase is responsible for the basal resistance of Escherichia coli to tellurite. *Microbiology*, *143*, 1181–1189.

Baesman, S. M., Bullen, T. D., Dewald, J., Zhang, D., Curran, S., Islam, F. S., … Oremland, R. S. (2007). Formation of Tellurium Nanocrystals during Anaerobic Growth of Bacteria That Use Te Oxyanions as Respiratory Electron Acceptors. *Applied and Environmental Microbiology*, *73*(7), 2135–43. doi:10.1128/AEM.02558-06

Baesman, S. M., Stolz, J. F., Kulp, T. R., & Oremland, R. S. (2009). Enrichment and isolation of Bacillus beveridgei sp. nov., a facultative anaerobic haloalkaliphile from Mono Lake, California, that respires oxyanions of tellurium, selenium, and arsenic. *Extremophiles*, *13*, 695–705. doi:10.1007/s00792-009-0257-z

Brown, G. E., Foster, A. L., & Ostergren, J. D. (1999). Mineral surfaces and bioavailability of heavy metals: a molecular-scale perspective. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(7), 3388–95.

Bébien, M., Kirsch, J., Méjean, V., & Verméglio, A. (2002). Involvement of a putative molybdenum enzyme in the reduction of selenate by Escherichia coli. *Microbiology*, *148*(Pt 12), 3365–72.

Castro, M. E., Molina, R. C., Díaz, W. A., Pradenas, G. A., & Vásquez, C. C. (2009). Expression of Aeromonas caviae ST pyruvate dehydrogenase complex components mediate tellurite resistance in Escherichia coli. *Biochemical and Biophysical Research Communications*, *380*(1), 148–52. doi:10.1016/j.bbrc.2009.01.078

Chasteen, T. G., & Bentley, R. (2003). Biomethylation of Selenium and Tellurium: Microorganisms and Plants. *Chemical reviews*, *103*(1), 1–25. doi:10.1021/cr010210+

Chasteen, T. G., Fuentes, D. E., Tantaleán, J. C., & Vásquez, C. C. (2009). Tellurite: history, oxidative stress, and molecular mechanisms of resistance. *FEMS Microbiology Reviews*, *33*(4), 820–32. doi:10.1111/j.1574-6976.2009.00177.x

Chiong, M., Barra, R., González, E., & Vásquez, C. (1988). Resistance of Thermus spp. to Potassium Tellurite. *Applied and Environmental Microbiology*, *54*(2), 610–2.

Constantinidou, C., Hobman, J. L., Griffiths, L., Patel, M. D., Penn, C. W., Cole, J. a, & Overton, T. W. (2006). A Reassessment of the FNR Regulon and Transcriptomic Analysis of the Effects of Nitrate, Nitrite, NarXL, and NarQP as Escherichia coli

K12 Adapts from Aerobic to Anaerobic Growth. *The Journal of biological chemistry*, *281*(8), 4802–15. doi:10.1074/jbc.M512312200

Csotonyi, J. T., Stackebrandt, E., & Yurkov, V. (2006). Anaerobic Respiration on Tellurate and Other Metalloids in Bacteria from Hydrothermal Vent Fields in the Eastern Pacific Ocean. *Applied and Environmental Microbiology*, *72*(7), 4950–6. doi:10.1128/AEM.00223-06

Feldmann, J., & Hirner, A. (1995). Occurrence of Volatile Metal and Metalloid Species in Landfill and Sewage Gases. *International Journal of Environmental Analytical Chemistry*, *60*(2), 339–359.

Guest, J. R. (1995). The Leeuwenhoek Lecture, 1995. Adaptation to life without oxygen. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, *350*(1332), 189–202. doi:10.1098/rstb.1995.0152

Guymer, D., Maillard, J., & Sargent, F. (2009). A genetic analysis of in vivo selenate reduction by Salmonella enterica serovar Typhimurium LT2 and Escherichia coli K12. *Archives of microbiology*, *191*(6), 519–28. doi:10.1007/s00203-009-0478-7

Harrison, J. J., Ceri, H., Stremick, C., & Turner, R. J. (2004). Differences in biofilm and planktonic cell mediated reduction of metalloid oxyanions. *FEMS Microbiology Letters*, *235*(2), 357–62. doi:10.1016/j.femsle.2004.05.005

Huang, C., & Hu, B. (2008). Speciation of inorganic tellurium from seawater by ICP-MS following magnetic SPE separation and preconcentration. *Journal of Separation Science*, *31*(4), 760–7. doi:10.1002/jssc.200700405

Khoroshilova, N., Popescu, C., Munck, E., Beinert, H., & Kiley, P. J. (1997). Iron-sulfur Cluster Disassembly in the FNR Protein of Escherichia coli by 02: [4Fe-4S] to [2Fe-2S] Conversion with loss of Biological Activity. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(12), 6087–6092.

Kiley, P. J., & Beinert, H. (1999). Oxygen sensing by the global regulator, FNR: the role of the iron-sulfur cluster. *FEMS Microbiology Reviews*, *22*(5), 341–52.

Kitts, C. L., Cunningham, D. P., & Unkefer, P. J. (1994). Isolation of Three Hexahydro-1, 3, 5-Trinitro-1, 3, 5-Triazine-Degrading Species of the Family Enterobacteriaceae from Nitramine Explosive-Contaminated Soil. *Applied and Environmental Microbiology*, *60*(12), 4608–4711.

Krafft, T., Bowen, A., Theis, F., & Macy, J. M. (2000). Cloning and Sequencing of the Genes Encoding the Periplasmic-Cytochrome B-Containing Selenate Reductase of Thauera selenatis. *DNA Sequence*, *10*(6), 365–77.

Kuroda, M., Yamashita, M., Miwa, E., Imao, K., Fujimoto, N., Ono, H., … Ike, M. (2011). Molecular Cloning and Characterization of the srdBCA Operon, Encoding the Respiratory Selenate Reductase Complex, from the Selenate-Reducing Bacterium Bacillus selenatarsenatis SF-1. *Journal of Bacteriology*, *193*(9), 2141–8. doi:10.1128/JB.01197-10

Lee, D. S., & Edmond, J. M. (1985). Tellurium species in seawater. *Nature*, *313*(28), 782–785.

Lee, P. A., Tullman-Ercek, D., & Georgiou, G. (2006). The Bacterial Twin-Arginine Tanslocation Pathway. *Annual Review of Microbiology*, *60*, 373–95. doi:10.1146/annurev.micro.60.080805.142212

Leimkühler, S., Wuebbens, M. M., & Rajagopalan, K. V. (2011). The History of the Discovery of the Molybdenum Cofactor and Novel Aspects of its Biosynthesis in Bacteria. *Coordination Chemistry Reviews*, *255*(9-10), 1129–1144. doi:10.1016/j.ccr.2010.12.003

Lemly, A. D. (2002). Symptoms and implications of selenium toxicity in fish: the Belews Lake case example. *Aquatic Toxicology*, *57*(1-2), 39–49.

Lipsky, B. A., Hook, E. W., Smith, A. A., & Plorde, J. J. (1980). Citrobacter Infections in Humans: Experience at the Seattle Veterans Administration Medical Center and a Review of the Literature. *Reviews of Infectious Diseases*, *2*(5), 746–60.

Lubitz, S. P., & Weiner, J. H. (2003). The Escherichia coli ynfEFGHI operon encodes polypeptides which are paralogues of dimethyl sulfoxide reductase (DmsABC). *Archives of Biochemistry and Biophysics*, *418*(2), 205–216. doi:10.1016/j.abb.2003.08.008

Ma, J., Kobayashi, D. Y., & Yee, N. (2007). Chemical Kinetic and Molecular Genetic Study of Selenium Oxyanion Reduction by Enterobacter cloacae SLD1a-1. *Environmental Science & Technology*, *41*(22), 7795–801.

Macaskie, L. E., Empson, R. M., Cheetham, A. K., Grey, C. P., & Skarnulis, A. J. (1992). Uranium Bioaccumulation by a Citrobacter sp. as a Result of Enzymically Mediated Growth of Polycrystalline HUO2PO4. *Science*, *257*(5071), 782–4.

Malasarn, D., Keeffe, J. R., & Newman, D. K. (2008). Characterization of the Arsenate Respiratory Reductase from Shewanella sp. Strain ANA-3. *Journal of Bacteriology*, *190*(1), 135–42. doi:10.1128/JB.01110-07

Moore, M. D., & Kaplan, S. (1992). Identification of intrinsic high-level resistance to rare-earth oxides and oxyanions in members of the class Proteobacteria: characterization of tellurite, selenite, and rhodium sesquioxide reduction in Rhodobacter sphaeroides. *Journal of Bacteriology*, *174*(5), 1505–14.

Ohlendorf, H. M., Hoffman, D. J., Saiki, M. K., & Aldrich, T. W. (1986). Embryonic mortality and abnormalities of aquatic birds: apparent impacts of selenium from irrigation drainwater. *The Science of The Total Environment*, *52*(1-2), 49–63.

Ohlendorf, H. M., Kilness, A. W., Simmons, J. L., Stroud, R. K., Hoffman, D. J., & Moore, J. F. (1988). Selenium toxicosis in wild aquatic birds. *Journal of Toxicology and Environmental Health*, *24*(1), 67–92.

Oram, L. L., Strawn, D. G., Morra, M. J., & Möller, G. (2010). Selenium Biogeochemical Cycling and Fluxes in the Hyporheic Zone of a Mining-Impacted Stream. *Environmental Science & Technology*, *44*(11), 4176–83. doi:10.1021/es100149u

Puranik, P. R., & Paknikar, K. M. (1999). Biosorption of Lead, Cadmium, and Zinc by Citrobacter Strain MCM B-181: Characterization Studies. *Biotechnology Progress*, *15*(2), 228–37. doi:10.1021/bp990002r

Seiler, R. L., Skorupa, J. P., & Peltz, L. A. (1999). *Areas Susceptible to Irrigation-Induced Selenium Contamination of Water and Biota in the Western United States*. U.S. Dept. of the Interior, U.S. Geological Survey.

Self, W. T., Grunden, A. M., Hasona, A., & Shanmugam, K. T. (2001). Molybdate transport. *Research in Microbiology*, *152*(3-4), 311–21.

Stolz, J. F., Basu, P., Santini, J. M., & Oremland, R. S. (2006). Arsenic and selenium in Microbial Metabolism. *Annual Review of Microbiology*, *60*, 107–30. doi:10.1146/annurev.micro.60.080805.142053

Stolz, J. F., & Oremland, R. S. (1999). Bacterial respiration of arsenic and selenium. *FEMS Microbiology Reviews*, *23*(5), 615–27.

Summers, A. O., & Jacoby, G. A. (1977). Plasmid-determined resistance to tellurium compounds. *Journal of Bacteriology*, *129*(1), 276–81.

Taylor, D. E. (1999). Bacterial tellurite resistance. *Trends in Microbiology*, *7*(3), 111–5.

Vinceti, M., Wei, E., & Malagoli, C. (2001). Adverse Health Effects of Selenium in Humans. *Reviews on Environmental Health*, *16*(4), 233–251.

Wray, D. S. (1998). The impact of unconfined mine tailings and anthropogenic pollution on a semi-arid environment - an initial study of the Rodalquilar mining district , south east Spain. *Environmental Geochemistry and Health*, *20*, 29–38.

Yee, N., Ma, J., Dalia, A., Boonfueng, T., & Kobayashi, D. Y. (2007). Se(VI) Reduction and the Precipitation of Se(0) by the Facultative Bacterium Enterobacter cloacae SLD1a-1 Are Regulated by FNR. *Applied and Environmental Microbiology*, *73*(6), 1914–20. doi:10.1128/AEM.02542-06