FACTORS INFLUENCING THE FATE OF CHROMIUM IN SOILS: MICROBIAL ECOLOGY, PHYSIOLOGY AND METAL TRANSFORMATION STUDIES

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology

MONTANA STATE UNIVERSITY
Bozeman, Montana

April, 2011
ii

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April, 2011
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my advisor, Dr. Robin Gerlach for all the guidance, knowledge and support he has provided both professionally and personally throughout my graduate program here. I am deeply grateful for everything he has done for me. Thank you to the additional members of my committee Drs. Matthew Fields, Anne Camper, Brent Peyton, Gill Geesey and Isaac Klapper for everything they have done for me that far exceeded their obligations as my committee. Thank you to everyone at the CBE and especially my lab group for their help with my research and making the CBE such a great place to work. Additional thanks to John Blaskovich for all of his hard work and dedication in the laboratory.

Most importantly, I would like to thank my family, especially my father, for their never-ending moral support of which I could not have made it without. Similarly, thank you to Dr. Abigail Richards for everything, especially for being my biggest support system through these last five years. Thank you to my fellow students, both past and present, through which this experience would not have been the same without.

Thanks to the Inland Northwest Research Alliance, the Microbiology Department and U.S. Department of Energy Subsurface Biogeochemical Research Program for financial support through my graduate career.
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ABSTRACT

Chromium is a common contaminant in soil environments, especially at Department of Energy sites. The fate of chromium in the environment is influenced by a number of factors including the microbial processes and the interactions with soil constituents such as carbon sources, iron minerals and electron shuttling compounds. The overall goals of these studies were to isolate and characterize microorganisms that may play a role in the fate of chromium in soil systems, identify the microbial community associated with a simulated low-level waste site and determine the role carbon source, iron minerals and electron shuttles play on Cr(VI) toxicity and reduction by Arthrobacter spp. and Cellulomonas sp. strain ES6. Three strategies for isolating potential cellulose-degrading isolates (direct isolation on agar plates, liquid enrichments and soil laden continuous flow columns) were implemented through which thirteen isolates were obtained. Clone library, PhyloChip and quantitative PCR analyses of the bacterial community within a simulated low-level waste site at the Idaho National Laboratory suggested that the presence of cellulosic waste influenced the bacterial community structure with soil depth at the site. The influence of potential cellulose degradation products as carbon sources on Cr(VI) toxicity and reduction by Arthrobacter sp. isolate EF01 and A. aurescens TC1 was assessed through bench-scale studies. Results indicated that Cr(VI) toxicity to both Arthrobacter spp. was tied to carbon metabolism. Additional studies with Cellulomonas sp. strain ES6 demonstrated that Cr(VI) reduction by this organism was also influenced by carbon source as well as by the addition of electron shuttles and iron minerals. Overall, the results of this dissertation provide insight into the potential interactions between microorganisms, soil constituents and chromium in situ.
CHAPTER 1

INTRODUCTION

Chromium in Soils

Chromium is naturally occurring in the environment; however the majority of chromium present in the environment is due to anthropogenic sources (Barceloux, 1999). Chromium is used in many processes including metal-plating, wood treatment, leather tanning and mining and milling operations (Calder, 1988; Barceloux, 1999). Chromium can be in nine different valence states ranging from -2 to 6+ though only a few are stable in the environment (Barceloux, 1999). Hexavalent chromium (Cr(VI)) is highly water soluble, highly mobile and a known carcinogen (Yassi and Nieboer, 1988; Hayes, 1997; Barceloux, 1999; Nickens et al., 2010). The reduced trivalent form, Cr(III), is much less water soluble and less mobile and significantly less toxic (Yassi and Nieboer, 1988; Hayes, 1997; Barceloux, 1999; Nickens et al., 2010). Therefore, it is beneficial to reduce Cr(VI) to Cr(III) in the environment.

Many factors influence the state of chromium in the environment of which redox potential and pH are two of the most important (Calder, 1988; Barceloux, 1999). Chromium in soil is mostly found as either Cr(VI) or Cr(III) (Barceloux, 1999). Cr(VI) is dominant at high pH and high redox conditions while Cr(III) is dominant under low pH and low redox conditions (Calder, 1988). Cr(III) can complex with other compounds such as iron precipitating out as an insoluble oxide (Eary and Rai, 1988; James, 1994, 1996; Barceloux, 1999; Kumpiene et al., 2008). However, Cr(III) can also form
complexes with soluble organics and then become more water soluble (Calder, 1988). Additionally, if conditions the redox state of chromium could change and the precipitated chromium oxides. If chromium enters the soil environment as Cr(VI) then it will be mobile due to its increased solubility or can be reduced by natural reductants present including organic matter (Lovley et al., 1998). Cr(VI) enters most often as HCrO$_4^-$ and CrO$_4^{2-}$ and this anionic form does not significantly adsorb to soil particles as they are mostly negatively charged (Barceloux, 1999). It is clear that the state of chromium in soil environments can continually change and is influenced by a number of factors.

**Chromium at Department of Energy Sites**

Department of Energy (DOE) sites around the United States have been used for various purposes over the years. These include conducting research, the production and testing of nuclear materials, spent-fuel reprocessing and the construction and testing of nuclear reactors. Generated waste from these processes has been disposed of in ponds, cribs, basins, pits, storage tanks and landfills and has contaminated the subsurface through leaks in sewer lines, underground storage tanks and waste-containing drums (Riley and Zachara, 1992). The large-scale subsurface contamination across the DOE sites occurring in both soils and groundwater has led to the urgent need for remediation treatments.

Chromium is a wide-spread contaminant within the DOE complex in the United States and as of 1992, chromium was the second most common metal contaminant throughout the DOE complex in both groundwater and sediments (Riley and Zachara, 1992). Across the DOE complex, ground water concentrations of chromium ranged from
0.42 to 9,010 µg/L and sediment concentrations ranged from 5.1 to 3,950,000 µg/kg (Riley and Zachara, 1992). Many of these concentrations are well above the U.S. Environmental Protection Agency’s limit in drinking water which is 100 µg/L.

One of the overall goals of this dissertation was to determine factors that influence the fate of chromium in the subsurface at two DOE sites: the Idaho National Laboratory’s Remediation Waste Management Complex and the Hanford Site in Washington State.

Idaho National Laboratory Site

The Idaho National Laboratory (INL) site has generated wastes from the processing of nuclear materials as well as acquired waste transferred from other DOE sites such as Rocky Flats in Colorado. Since the 1950’s, this contaminated waste has been contained within the Radioactive Waste Management Complex (RWMC) and is mostly found in the 97-acre Subsurface Disposal Area (SDA) (Idaho Department of Environmental Quality, 2011a). The RWMC is located about 50 miles west of Idaho Falls, Idaho. There are many types of waste found within the RWMC; however, the majority of the waste at this location is classified as low-level waste. In fact, there is almost four times more low-level waste than all of the rest combined (Idaho Department of Environmental Quality, 2011b). Approximately 225,000 cubic meters of low-level waste has been disposed of at the SDA.

Low-level waste comes in a variety of forms including solids, liquids, sludge and debris (Figure 1.1). This is because low-level waste is broadly classified. Similar to an “other” classification, low-level waste contains all types of material that cannot be
classified as spent nuclear fuel, high-level waste or transuranic waste (Idaho Department of Environmental Quality, 2011b). While low-level waste is often contaminated with low concentrations of heavy metals, such as chromium and radioactive materials, that is not true for all of it. Soils in the Snake River Basin have also been classified as low-level waste due to the previous release of contaminants directly into the ground.

Low-level waste has been disposed of in pits, trenches and concrete vaults (Figure 1.1). Some has been incinerated and some repackaged and shipped to other DOE sites such as the Nevada Test Site. It has been determined that up to 90% of this waste contains cellulosic material (Vilks et al., 1998). The DOE intends to close the SDA as an
active waste site by capping buried waste and transferring waste that cannot be buried long-term to other sites including the Hanford Site and the Nevada Test Site (Idaho Department of Environmental Quality, 2011b). In the meantime, there is potential for contaminants associated with the waste to mobilize out of these pits.

One unique aspect of the SDA is that additional test pits for technology development purposes were established in which no contaminants were present. The Cold Test Pits, designated Cold Test Pit North and Cold Test Pit South, were constructed with the goal of having simulated buried waste pits in which technology demonstrations, new equipment testing and research could be conducted (Department of Energy, 2002). The Cold Test Pit South site was the study site for bacterial community characterization studies and is discussed in more detail in Chapter 3.

Hanford Site

In 1943, the Hanford Site, which is approximately 586 square miles, located near Richland, WA, was established as part of the Manhattan Project to manufacture plutonium for nuclear weapons. During the Cold War additional nuclear reactors were built. Most of these were constructed along the Columbia River so that water from the river could be used to cool the reactors and then be discharged back into the river. All reactors were shut down by 1990, but the radioactive and heavy metal waste from these processes remains. Approximately 450 billion gallons of liquid waste were released into the ground (Department of Energy, 2007). Additionally, 177 underground storage tanks currently contain liquid and semi-solid waste. Unfortunately, leakage from these
underground storage tanks has led to additional contamination of the subsurface by hazardous compounds including chromium (Department of Energy, 2005).

Significant progress has been made to decrease mobility and reduce further contamination through the plugging of wells, removal of pipelines and replacement of leaking pipelines (Department of Energy, 2005; Department of Energy, 2007). A pump and treat method has been employed to remove chromium before entering the Columbia River, but chromium contamination is still a problem at the Hanford Site (Department of Energy, 2007).

**Chromium Remediation in Soils**

Chromium can be treated in the environment through physical, chemical or biological methods. Currently, physical and chemical methods such as excavation and pumping of contaminated soil and water, soil washing and chemical reductants are used most often (Lloyd, 2003; Philip and Atlas, 2005). Unfortunately, at sites such as INL or Hanford, these methods are impractical or too expensive due to the large amount of contaminated soil and groundwater. In these cases, biological treatment offers a potentially more cost-effective and practical solution. Biological treatment can occur naturally by microorganisms present in the environment or can be stimulated through the addition of nutrients or microorganisms. Bioremediation has been shown to be successful in the treatment of a variety of contaminants including heavy metals, radionuclides and organic compounds in soil environments (Philip and Atlas, 2005).
Microorganisms and Chromium

Chromium Toxicity to Microorganisms

Small quantities of chromium are essential for living organisms; however, in larger doses it can be toxic. Cr(VI) can enter the cell due to its solubility and structural similarity to sulfate and phosphate in the form of chromate (CrO$_4^{2-}$) or dichromate (Cr$_2$O$_7^{2-}$). It can then react with a number of reducing compounds within the cell producing unstable intermediates such as Cr(V) and Cr(IV) as well as free radicals. These free radicals can cause oxidative damage to DNA and proteins (Cervantes and Campos-García, 2007; Cheung and Gu, 2007; Nickens et al., 2010). While Cr(III) is considered less toxic as it is less soluble and unlikely to enter the cell, if chromate entering the cell is reduced to Cr(III), it has been shown that uncomplexed Cr(III) can bind to phosphate in DNA or inhibit cell transcription and replication which can lead to cell damage and death (Plaper et al., 2002; Bencheikh-Latmani et al., 2007; Cervantes and Campos-García, 2007; Ramírez-Díaz et al., 2008).

Chromate Resistance by Microorganisms

Cr(VI) resistance in microorganisms can occur through the decreased uptake of Cr(VI), the increased use of efflux pumps, biosorption, and the upregulation of genes associated with oxidative stress response or Cr(VI) reduction (Cervantes et al., 2001; Cervantes and Campos-García, 2007; Cheung and Gu, 2007; Congeevaram et al., 2007; Ramírez-Díaz et al., 2008) (Figure 1.2). This has been observed for organisms such as Pseudomonas aeruginosa (Alvarez et al., 1999), Pseudomonas fluorescens (Bopp et al., 1983) and Streptococcus lactis (Efstathiou and McKay, 1977). Many of these resistance
mechanisms are plasmid-associated, especially in the case of efflux pumps (Cervantes and Silver, 1992; Cervantes et al., 2001; Cervantes and Campos-García, 2007). For example, *P. fluorescens* LB300 has been shown to possess plasmid-associated resistance genes and the loss of this plasmid can lead to the loss of Cr(VI) resistance (Bopp et al., 1983; Bopp and Ehrlich, 1988).

The chromate ion transporter (Chr) proteins have been demonstrated to be involved in chromate resistance as they act as efflux pumps (Nies et al., 1998; Cervantes and Campos-García, 2007; Díaz-Pérez et al., 2007; Ramírez-Díaz et al., 2008). These proteins have been hypothesized to act as chromate/sulfate antiporters (Nies et al., 1998; Díaz-Pérez et al., 2007). Functionally, ChrA is believed to act similar to a pump that removes Cr(VI) from the cytoplasm through transmembrane segments (TMS) and the use of the proton motive force (Alvarez et al., 1999; Pimentel et al., 2002; Ramírez-Díaz et al., 2008).

Chr proteins have been found primarily in bacteria, but more recently also in six fungal species and the archaeon *Methanococcus jannaschii* (Díaz-Pérez et al., 2007; Ramírez-Díaz et al., 2008). The ChrA proteins of *P. aeruginosa* and *Cupriavidus metallidurans* (formerly *Alcaligenes eutrophus*) are currently the best characterized of the identified Chr proteins (Cervantes et al., 1990; Nies et al., 1990; Díaz-Pérez et al., 2007). Bacterial Chr proteins fall into one of two categories based on their size: short-chain Chr proteins, with a sequence length of 123-234 amino acids, or long-chain Chr proteins, with a sequence length of 345-495 amino acids. Long-chain Chr proteins are thought to be larger as two homologous domains are present (Díaz-Pérez et al., 2007; Ramírez-Díaz et
al., 2008). The differences in sizes within each group are believed to be due to differences in the hydrophilic loops between the TMS of the protein (Díaz-Pérez et al., 2007). This may also play a role in the size of these proteins in fungi and archaea. The number of TMS varies between organisms, but up to 13 have been observed (Jiménez Mejía et al., 2006; Cervantes and Campos-García, 2007).

Overall protein sequences within the Chr family are not highly conserved and differences between these proteins have been observed in membrane topology orientation and genomic sequence (Díaz-Pérez et al., 2007). This is believed to be due to the diverse functions these proteins play within microorganisms. For example, ChrA protein has been detected in *Synechococcus elongatus* strain PCC7942 which is not Cr(VI)-resistant (Nicholson and Laudenbach, 1995; Díaz-Pérez et al., 2007). In fact, the deletion of the *chrA* gene within this organism led to increased Cr(VI) resistance. While these proteins may play a functional role in efflux of Cr(VI), it is likely that they have many purposes and these organisms may possess additional Cr(VI) resistance mechanisms.

Additional methods for chromate resistance include protection from oxidative stress through the upregulation of oxidative stress response genes (Juhnke et al., 2002; Ackerley et al., 2006; Chourey et al., 2006; Ramírez-Díaz et al., 2008). For example, under chromate stress *Escherichia coli* upregulates the genes encoding for superoxide dismutase and catalase while *Shewanella oneidensis* MR-1 increases thioredoxins and glutaredoxins in response to chromate addition (Brown et al., 2006; Chourey et al., 2006; Ramírez-Díaz et al., 2008). DNA repair mechanisms are also upregulated in the presence of chromate including DNA helicases and endonucleases (Llagostera et al., 1986;
Figure 1.2. Mechanisms of chromate transport, toxicity and resistance in bacterial cells. Mechanisms of damage and resistance are indicated by thin and heavy arrows, respectively. (A) Chromosome-encoded sulfate uptake pathway which is also used by chromate to enter the cell; when it is mutated (X) the transport of chromate diminishes. (B) Extracellular reduction of Cr(VI) to Cr(III) which does not cross the membrane. (C) Intracellular Cr(VI) to Cr(III) reduction may generate oxidative stress, as well as protein and DNA damage. (D) Detoxifying enzymes are involved in protection against oxidative stress, minimizing the toxic effects of chromate. (E) Plasmid-encoded transporters may efflux chromate from the cytoplasm. (F) DNA repair systems participate in the protection from the damage generated by Cr derivates. (Ramírez-Díaz et al., 2008).

Miranda et al., 2005; Chourey et al., 2006; Ramírez-Díaz et al., 2008). Interestingly, *Pseudomonas ambigua* G-1 has a thick cell wall envelope with lower membrane permeability which led to decreased Cr(VI) toxicity compared to its mutant which lacked this thicker cell wall envelope (Horitsu et al., 1983; Chen and Hao, 1998).
Chromate Reduction by Microorganisms

Numerous microorganisms are capable of reducing Cr(VI) to Cr(III) which decreases toxicity to the organism (Turick et al., 1996; Chen and Hao, 1998; Fredrickson et al., 2000). This can occur both aerobically and anaerobically. Aerobically, Cr(VI) can be reduced enzymatically by microorganisms through the use of soluble enzymes that utilize cofactors such as NADH or NADPH (Ohtake et al., 1990; Wang et al., 1990; Park et al., 2000; Cheung et al., 2006; Cervantes and Campos-Garcia, 2007; Cheung and Gu, 2007). The best-studied chromate reductase to date is the ChrR chromate reductase from *P. putida* and previous findings suggest this enzyme is NADH-dependent (Park et al., 2000). In *Enterobacter cloacae* HO1, electrons are transferred to Cr(VI) through the use of a NADH-dependent cytochrome (Ohtake et al., 1990; Wang et al., 1990).

Anaerobically, microorganisms such as *P. fluorescens* LB300 reduce Cr(VI) by utilizing it as an electron acceptor (Bopp and Ehrlich, 1988; Wang and Shen, 1995; Cervantes and Campos-Garcia, 2007; Cheung and Gu, 2007). With the exception of *Desulfotomaculum reducens* sp. strain MI-1, microorganisms have not been shown to be capable of utilizing Cr(VI) as the sole electron acceptor for energy generation (Tebo and Obraztsova, 1998; Cheung and Gu, 2007).

As previously mentioned, Cr(VI) can be reduced enzymatically by microorganisms through what are generally referred to as chromate reductases as they catalyze the reduction of Cr(VI) to Cr(III). These proteins can be membrane-bound or soluble (Chen and Hao, 1998; Cheung and Gu, 2007). *P. fluorescens* LB300 and *E. cloacae* HO1 both possess membrane-bound Cr(VI) reductases (Bopp and Ehrlich, 1988;
Wang et al., 1990; Chen and Hao, 1998). This is advantageous to the organism as it has the potential to reduce Cr(VI) outside the cell keeping it from entering. Some organisms such as *E. coli* ATCC 33456, *Desulfovibrio vulgaris*, *P. putida* PRS2000, and *P. ambigua* G-1 produce soluble Cr(VI) reductases (Ishibashi et al., 1990; Suzuki et al., 1992; Shen and Wang, 1993; Lovley and Phillips, 1994; Chen and Hao, 1998). These soluble enzymes can use a variety of electron donors and can be found both inside and outside the cell (Chen and Hao, 1998).

It has been proposed that many chromate reductases have a primary role other than the reduction of chromate (Ishibashi et al., 1990; Cervantes and Campos-García, 2007). Therefore, it may be hard to identify a specific chromate reductase within a microorganism as they may also function as nitroreductases (Kwak et al., 2003), iron reductases (Mazoch et al., 2004) or other metal reductases (Cervantes and Campos-García, 2007). ChrR from *P. putida* has been shown to act as a chromate reductase as well as be capable of reducing ferricyanide (Park et al., 2000; Ackerley et al., 2004). Additionally, YieF in *E. coli* has been shown to catalyze the reduction of Cr(VI), ferricyanide, vanadium, molybdenum and several quinones (Ackerley et al., 2004; Ramírez-Díaz et al., 2008).

There are numerous factors that influence the rate and extent of Cr(VI) reduction by microorganisms including oxygen concentration, biomass concentration, Cr(VI) concentration, carbon source, pH and temperature (Chen and Hao, 1998). A complete review of these topics can be found elsewhere (Chen and Hao, 1998), but it is clear that
Cr(VI) reduction is a complex process. This dissertation aims to identify some of the environmentally relevant factors that can influence chromium fate in two soil systems.

Cellulose in the Environment

Cellulose is one of the most abundant carbon sources in terrestrial environments as it is a key component of plant biomass. It is often a main constituent of hemicellulose and is also one of the main components of the low-level waste buried at the SDA site in Idaho (Vilks et al., 1998). Therefore, it is likely the most abundant carbon source available for microorganisms in low-level waste soil systems. This suggests that it may play an important role in the fate of chromium in these environments.

Cellulose is non-soluble, highly crystalline and is made up of repeating glucose residues (often 100 to over 10,000) that are bound by β-1,4 linkages. It is also considered to be a renewable energy source as it is continually cycled in the environment through degradation, often by microorganisms, and regeneration through photosynthesis (Coughlan and Mayer, 1992). Because of this, much research has been focused on the utilization of cellulose for the production of energy sources such as ethanol and hydrogen through both chemical and biological means (Lynd et al., 2002; Demain et al., 2005; Bayer et al., 2007).

Cellulose Degradation by Microorganisms

Even though cellulose is one of the most abundant forms of carbon in the terrestrial subsurface, few microorganisms are capable of carrying out its complete degradation (Coughlan and Mayer, 1992; Lynd et al., 2002; Kato et al., 2005). This is
mainly due to its complexity and the diversity of enzymes needed (Coughlan and Mayer, 1992). Cellulose is typically broken down aerobically by fungi in soil environments and the main end products are CO$_2$ and H$_2$O (Coughlan and Mayer, 1992; Lynd et al., 2002). When cellulose is degraded anaerobically, it is converted to various fermentation end products such as organic acids, ethanol, CO$_2$ and H$_2$ (Coughlan and Mayer, 1992; Lynd et al., 2002). Both aerobic and anaerobic degradation can lead to the formation of a variety of sugars, but they are not considered “end products” as they are easily metabolized by a variety of microorganisms. It is worth noting that cellulose degradation carried out aerobically by bacteria and fungi yields higher cell biomass because aerobic respiration provides more energy to the cells. When cellulose is degraded anaerobically through respiration or fermentation, biomass yields are much lower (Lynd et al., 2002).

Few fungi and even fewer bacteria are considered truly cellulolytic in which they can carry out complete degradation from crystalline cellulose to end products such as CO$_2$ and H$_2$O (Coughlan and Mayer, 1992). This is due to the need for many cellulase enzymes to carry out this process. Instead, it is common for a number of microorganisms to work together, each participating in part of the degradation. Once cellulose is converted to cellobiose or individual glucose monomers many microorganisms can utilize these carbon sources for growth.

Cellulolytic capabilities are not common among bacteria, but there are several phylogenetic groups with this ability. Cellulolytic bacteria include members of the genera Cytophaga, Sporocytophaga, Streptomyces, Bacillus, Thermobifida, Micromonospora, Fibrobacter, Erwinia, Cellulomonas, Cellvibrio, Arthrobacter,
Ruminococcus, and Clostridium (Stewart and Leatherwood, 1976; Coughlan and Mayer, 1992; Lynd et al., 2002; Desvaux, 2005; Bayer et al., 2006). These organisms vary in their oxygen, temperature and pH requirements. In general, it has been observed that cellulose degradation by aerobic bacteria occurs primarily through the use of extracellular cellulase enzymes (Lynd et al., 2002), while anaerobic organisms primarily use a complexed cellulase system such as a cellulosome (Leschine, 1995; Lynd et al., 2002).

To date, few facultative, cellulolytic bacteria have been identified that carry out cellulose degradation both aerobically and anaerobically. The most well-known and best characterized for this capability are Cellulomonas spp. (Stewart and Leatherwood, 1976; Bagnara et al., 1987; Lynd et al., 2002). It is believed that few organisms are capable of degradation under aerobic and anaerobic conditions because of the very distinct degradation strategies for each.

Cellulolytic Enzymes

Studying cellulolytic enzyme systems in microorganisms is extremely difficult. As cellulose is insoluble, cellulolytic enzymes must be extracellular or embedded in the cell membrane (Lynd et al., 2002). There are three general types of enzymes needed to degrade cellulose: exoglucanases, endoglucanases and β-Glucosidases. Evaluation of an organism’s cellulolytic abilities is often based on the detection of these enzyme types. Exoglucanases act on the ends of cellulose chains releasing cellobiose and glucose primarily. They often act on microcrystalline cellulose such as in the case of Avicel (Coughlan and Mayer, 1992; Lynd et al., 2002). Due to the tunnel-like structure of these enzymes they can only act on the chain ends. Endoglucanases act on the internal bonds
of cellulose creating new ends that can be hydrolyzed by exoglucanases. They are often involved in the degradation of soluble cellulose sources such as acid-swollen amorphous cellulose and carboxymethylcellulose (CMC) (Coughlan and Mayer, 1992). β-Glucosidases act on the β-linkages of cellobiose producing individual glucose molecules. There are additional enzymes involved in cellulose degradation including cellodextrinase, cellobiose phosphorylase, cellodextrin phosphorylase, and cellobiose epimerase however they have received much less attention and are often overlooked when evaluating the cellulolytic potential of a microorganism. It is important to remember that often the capabilities of these enzymes overlap and each enzyme may be capable of carrying out or be involved in other functions than those they are classified by (Coughlan and Mayer, 1992).

Cellulase enzymes are often associated with structures containing a catalytic site and a carbohydrate-binding module due to the insolubility of cellulose (Coughlan and Mayer, 1992; Lynd et al., 2002). The carbohydrate-binding module coordinates the identification and attachment to the cellulose source so that the cellulase enzymes can break it down into more easily utilizable components. Often these enzymes are soluble and not directly associated with the cell (Lynd et al., 2002).

Unlike the soluble cellulase enzymes produced aerobically, under anaerobic conditions bacteria use complexed cellulase systems called cellulosomes to degrade cellulose (Lynd et al., 2002; Demain et al., 2005). A cellulosome is a large complex of cellulases found on the outside of a bacterial cell. The development of this complex is believed to optimize efficiency of cellulose degradation and limit the potential release
and diffusion of products into the surrounding environment that could be utilized by other organisms.

**Research Goals and Objectives**

Chromium has been identified as a common contaminant in soil systems. However, its fate at many sites is largely unknown due to the complexity of the soil processes potentially involved. The interaction between soil microorganisms and chromium, whether through naturally occurring processes or stimulation for bioremediation purposes, is one important relationship affecting chromium mobility in the environment. The overall goals of the work discussed in this dissertation were:

1. *Isolate and characterize microorganisms that potentially play a role in chromium transformation in soil environments.* This was performed through the use of culture-based techniques and laboratory-based studies. The results will be discussed in Chapter 2 of this dissertation. A portion of the isolation methods have been published in Field et al. (2010) (Chapter 3 of this dissertation) and VanEngelen et al. (2010) (Appendix A).

2. *Identify the microbial community associated with a simulated low-level waste site and develop hypotheses regarding the interactions between cellulosic waste and the associated microbial community.* Molecular methods were used to evaluate soil core samples obtained from the Cold Test Pit South at the Subsurface Disposal Area at the Idaho National Laboratory. The results were published in Field et al. (2010) in *Applied and Environmental Microbiology* and are presented in Chapter 3 of this dissertation.
3. Determine what influence carbon source, electron shuttling compounds and iron minerals have on chromium toxicity and reduction by isolated microorganisms in soil environments. This was carried out through laboratory-based studies with environmentally derived bacteria. These results are presented in Chapters 4, 5, and 6 of this dissertation.

The results presented in Chapter 6 regarding factors influencing Cr(VI) reduction rates by Cellulomonas sp. strain ES6 have been submitted to Chemosphere for publication. Additionally, the work preceding the studies presented in Chapter 6, recently accepted to Biodegradation in which the factors influencing iron reduction by Cellulomonas sp. strain ES6 were assessed, is presented in Appendix B. The work presented in Chapter 4 is being prepared for submission to Environmental Toxicology and Chemistry.
References


Tebo, B.M., Obraztsova, A.Y., 1998. Sulfate-reducing bacterium grows with Cr(VI), U(VI), Mn(IV), and Fe(III) as electron acceptors. FEMS Microbiology Letters 162:193-198.


CHAPTER 2

ISOLATION AND CHARACTERIZATION OF POTENTIAL CELLULOSE-
DEGRADING MICROORGANISMS FROM A SIMULATED LOW-LEVEL WASTE SITE

Introduction

Laboratory-based experiments carried out with environmentally derived organisms provide insight about the fundamental interactions between cellulose, contaminants and microorganisms in situ. The goal of these studies was to isolate potential cellulose degrading microorganisms from a simulated low-level waste site and characterize their cellulose-degradation and hexavalent chromium (Cr(VI)) reduction capabilities. As cellulose is the dominant carbon source in the Idaho National Laboratory low-level waste sites and chromium is a contaminant present, isolates having cellulose degradation and Cr(VI) reduction capabilities would be useful in bench scale studies in which the fundamental interactions between the microorganism, cellulosic waste and chromium would be better assessed.

Select isolation methods were published in Field et al. (2010) (Chapter 3) and VanEngelen et al. (2010) (Appendix A). VanEngelen et al. (2010) further characterizes Pseudomonas sp. isolate (designated Isolate A) in regards to U(VI) toxicity. Further characterization studies with Arthrobacter sp. isolate (designated isolate EF01) can be found in Chapters 4 and 5 of this dissertation.
Materials and Methods

Isolation Methods

Sample Preparation: Soil cores spanning the depth of the pit were collected in sterile 3.2 cm diameter Lexan™ core tubes at the Cold Test Pit South site at the Idaho National Laboratory. Samples were placed in a cooler on ice for shipment to the INL laboratory where the samples were processed. Soil from the cores was then shipped on ice to Montana State University for further processing. The soil layers from which soil was used to isolate potential cellulose degraders were the fill-waste interface (FW), wood waste (WW), waste-clay interface (WC) and clay (C) layers. See Chapter 3 of this dissertation for more detailed information on the Cold Test Pit South site description. There was not enough material sent from Idaho National Laboratory at the time of inoculation to set up enrichments from the fill (overburden) soil layer. Soil from each layer was utilized separately for isolation. Soil from each layer was added to sterile 0.85% NaCl and vortexed. The soil slurry was then used to inoculate liquid enrichments and used for direct isolation on agar plates.

Direct Isolation Plates: A serial dilution was performed using the soil slurry from each of the soil samples diluted in 0.85% NaCl. One milliliter was added to 9 mL of 0.85% sterile NaCl, vortexed and one milliliter of this dilution was added to another 9 mL of 0.85% sterile NaCl. This was repeated twice after which the greatest dilution was $10^{-4}$. One hundred microliters of each dilution and the original soil supernatant were all spread plated onto agar plates containing 0.1% methyl cellulose and synthetic
groundwater (SGW) medium (as described by Borch et al. (2005)). Two plates from each of the dilutions for every soil layer were incubated both aerobically on the benchtop and anaerobically in the glovebag which contained a gas mixture of 90% N\textsubscript{2}, 5% H\textsubscript{2} and 5% CO\textsubscript{2}, (four plates in total per soil layer were inoculated). Because the original soil samples were obtained aerobically, the plates that were placed in the glovebag were used to potentially isolate facultative microorganisms as strict anaerobes were unlikely to be isolated. All plates were kept at room temperature (22 ± 2°C). Unique colony morphologies were re-streaked on new cellulose containing agar plates until pure cultures were obtained.

**Liquid Enrichments:** Liquid enrichments were set up with both a soluble cellulose (0.1% methyl cellulose, (Sigma-Aldrich, Inc., St. Louis, MO)) and a non-soluble cellulose (2.5 g/L Whatman No. 1 Filter Paper (Whatman, Piscataway, NJ)) in order to increase the likelihood of isolating microorganisms capable of degrading cellulose. Additionally, enrichments were set up both aerobically and anaerobically.

Aerobic liquid enrichments were set up in sterile screw-cap test tubes. They contained either the soluble methyl cellulose or non-soluble filter paper as the sole carbon source and SGW medium. The soil slurry from each soil sample (described in the above sample preparation procedure) was used to inoculate enrichments with each of the cellulose sources. There were a total of 12 enrichments including uninoculated controls. All enrichments were left at room temperature on the bench top. When enrichments turned visibly turbid or cellulose was visibly degraded they were vortexed well and transferred. After at least two transfers visually turbid enrichments were spread plated
onto cellulose containing agar plates. Unique colony morphologies were continually transferred on agar plates until pure cultures were obtained.

Anaerobic liquid enrichments were set up in the glovebag following the same procedure as the aerobic enrichments. Enrichments were set up in serum bottles in the glovebag under 90% N₂, 5% H₂, 5% CO₂ mixed gas and sealed with butyl rubber stoppers and sealed with aluminum crimps. The soil slurry was used to inoculate enrichments. Because the soil slurry was set up aerobically, some oxygen was added to enrichments, but it was expected that oxygen would be utilized by microorganisms at first and then when it was used up only those organisms capable of utilizing other electron acceptors would be able to grow. When enrichments appeared visibly turbid or cellulose was visibly degraded they were transferred. After at least two transfers visually turbid enrichments were spread plated onto cellulose containing agar plates. Unique colony morphologies were continually transferred on agar plates until pure cultures were obtained.

**Column Studies:** Four small scale columns were set up mimicking the low-level waste site conditions. Columns were made out of 10 mL syringes (BD, Franklin Lakes, NJ) of which the plunger was removed and replaced with rubber stoppers with glass tubing to close the top. Masterflex norprene tubing (Cole Parmer, Vernon Hills, IL) was used for all columns. Tubing ends were covered with aluminum foil and everything was autoclaved for 25 minutes. Glass beads (3 mm), nylon mesh (110 µM), sand (95% retained 40 mesh), and gravel were all autoclaved separately in beakers for 25 minutes. After cooling, the nylon mesh, glass beads, sand and gravel were aseptically added to the
syringes and then a mix of 10 g of the wood waste soil and 0.25 g sterile metal saturated Whatman No. 1 filter paper cut into 6 mm diameter circles (0.72 mg U in the form of UO$_2$Cl$_2$ and 0.41 mg Cr in the form of K$_2$CrO$_4$ per 0.25 g filter paper) were added and packed down to minimize uneven pore space, especially between columns. Another layer of sand, gravel, glass beads and nylon mesh were added to the top and the rubber stopper was put back in place. These metal concentrations were used based on reported contaminant concentrations (mg/kg soil) reported for the Idaho National Laboratory within the DOE Low-Level Waste Disposal Capacity Report (Anonymous, 2000). Exact contaminant concentrations saturated on the filter paper were determined by acid digestion followed by inductively coupled plasma mass spectrometry (ICP-MS) analysis of total U and Cr.

One column contained gamma irradiated wood waste soil as a sterile control. Two columns contained wood waste soil that was not irradiated and contained the indigenous microbial community of the CTPS site. The fourth column also contained non-sterilized wood waste soil from the site but was run in short-term batch mode to mimic conditions when high flow into the site occurs, possibly through snow-melt or rain, and the wood waste soil may become saturated with water. This column was filled with one pore volume (4 mL) of a defined, carbon-free medium, tubing was clamped off, and allowed to sit in batch mode for 46 hours. At this time media flow was turned on and flowed continuously through all four columns upwards at a rate of 0.074 mL/min. Carbon Source Screening (CSS) medium was used for this column study. CSS medium is a defined, carbon-free medium that simulates the site geochemistry and contains trace
quantities of potential terminal electron acceptors. This medium contained the following: 80 mg NaCl, 26 mg K₂HPO₄, 3.35 g PIPES Buffer, 0.6 mg KCl, 0.4 mg KOH, 12.7 mg Na₂SO₄, 3.5 mg CaO, and 7.5 mg MgCl₂·₆H₂O amended with 5 ml of Wolfe’s vitamin solution (Atlas, 2004) and 1 ml SL-4 trace elements solution (Atlas, 2004). Column effluents were collected aseptically daily and analyzed for Cr(VI), U(VI), pH, and total Cr, U, Fe, Cl, and K. Metal mobility results from these columns can be found in Appendix C.

Effluents from columns 2 and 3 (replicate columns with the wood waste soil) were visually turbid after 25 days. The collected effluent of column 4 was not visually turbid until day 33. The collected effluent of column 1 (the irradiated soil control column) was not visually turbid until day 53 when an unexpected increase in flow rate led to physical washout of soil plugged the effluent tubing after which the study was terminated. All isolation from column effluents was conducted before this event and there was no growth in this column effluent during isolation studies.

While samples were visually turbid for the other three columns, no significant increase in turbidity was measured when analyzed spectrophotometrically at 600 nm. On day 27, 20 µL of the collected effluents from all four columns was spread plated onto a Tryptic Soy Agar (TSA) (BD Difco, Franklin Lakes, NJ) plate to determine if this observed turbidity was due to microbial growth or chemical precipitation. These plates were incubated aerobically at room temperature. Unique colony morphologies were repeatedly re-streaked on TSA plates until pure cultures were obtained.
Sequencing and Identification

**Bacterial Isolates:** DNA was extracted from each of the bacterial isolates obtained through the use of the FastDNA Soil Extraction Kit (MP Biomedicals, Solon, OH) following the manufacturer’s protocol. The extracted DNA was then amplified with general bacterial primers specific for the 16S rRNA gene, 8F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5’-GGTTACCTTGGTACGACTT-3’). In the thermocycler reactions were heated to 94°C for 2 minutes, followed by 25 cycles of 94°C for 45 seconds, 55°C for 45 seconds and 72°C for 45 seconds, with a final extension at 72°C for 7 minutes. The amplified PCR product was purified using the Promega Wizard SV Gel and PCR Clean-Up kit (Promega Corporation, Madison, WI). The purified PCR products were sequenced at the Molecular Research Core Facility at Idaho State University. Each isolate was sequenced with four general bacterial primers specific for the 16S rRNA gene: 8F, 1492R, 533F (5’-GTGCCAGCMGCGGTAA-3’), and 907R (5’-CCGCTAACACCTTTRAGTTT-3’). The sequence results were aligned into one contiguous sequence using the Sequencher 4.5 software (Gene Codes Corporation, Ann Arbor, MI). The resulting aligned sequences, all of which were at least 1300 bp, were then identified using the BLAST function from the NCBI database (www.ncbi.nlm.nih.gov).

**Fungal Isolate:** The fungal isolate was grown on potato dextrose agar (BD Difco, Franklin Lakes, NJ) and a plate was sent to MIDI Labs (Newark, DE) for identification based on the partial LSU rRNA (D2)Gene alignment with sequences in the GenBank Database.
Characterization Methods

**Media and Culture Inoculum:** Isolates were grown aerobically from frozen glycerol stocks in Tryptic Soy Broth (BD Difco, Franklin Lakes, NJ) at room temperature (22 ± 2°C) and shaken at 150 rpm. As growth rates for all isolates varied, the amount of time these cultures were grown up varied, but late exponential phase was targeted which ranged from 20-40 hours. The culture was centrifuged, washed and resuspended with CSS medium three times. The last resuspended culture was diluted with sterile medium in order to obtain an optical density of approximately 0.2 for the culture inoculum. For anaerobic studies, the culture was washed and resuspended in the glovebag and sealed in serum bottles with butyl rubber stoppers.

**Carboxymethylcellulose Plates:** Carboxymethylcellulose (CMC)-containing agar plates were made as described by Kasana et al. (2008). In short, agar plates contained 2 g NaNO₃, 1 g K₂HPO₄, 0.5 g MgSO₄, 2 g CMC sodium salt, 200 mg peptone, 0.5 g KCl and 17 g noble agar per liter of nanopure water. Plates were inoculated with 5 µL of a liquid culture of each organism in CSS medium pipetted onto the center of the agar plate. One control plate contained 10 mg/mL *Aspergillus niger* cellulase enzyme (Sigma-Aldrich, St. Louis, MO) as a positive control. Plates were grown at 30°C for 72 hours and then flooded and stained with a Gram’s Iodine solution containing 2 g KI and 1 g iodine in 300 mL H₂O. After 20 minutes, the diameter of the zone of clearing, at its widest point if not completely circular, was measured.
**Pure Culture Studies**: Pure culture studies were set up with each isolate in order to determine their sole capabilities to degrade filter paper or methyl cellulose. These experiments were set up following the same procedure as described above in the liquid enrichments procedure. 1 mL of a pure culture that had been resuspended in CSS medium was used to inoculate liquid samples containing either filter paper or methyl cellulose and CSS medium. These were set up under aerobic and anaerobic conditions. In addition to samples set up for each individual isolate, one sample was inoculated with all the isolates that were found in the wood waste soil layer, as it is known that cellulose degradation typically occurs in a synergistic relationship between multiple organisms (Coughlan and Mayer, 1992; Lynd et al., 2002; Kato et al., 2005). Another sample was inoculated with all isolates regardless of the soil layer from which they were obtained. In both cases, 100 µL of each pure culture (resuspended in CSS medium and normalized to an OD of 0.2) was added in order to inoculate with approximately the same cell concentrations.

**Cellobiose Utilization**: Based on its filter paper degradation capabilities, additional studies were conducted with one of the isolates, *Cellulomonas parahominis*, to evaluate its ability to utilize cellobiose, an intermediate product of cellulose degradation. Cellobiose was dissolved in CSS medium at a concentration of 10 g/L which is comparable to the concentrations used for other growth medium (Atlas, 2004). Treatments were set up aerobically in screw-cap test tubes and anaerobically in anaerobic culture tubes, sealed with butyl rubber stoppers, and all were inoculated (10% inoculum) with a *C. parahominis* culture in CSS medium. Un-inoculated controls were also set up.
as well as carbon-free controls with *C. parahominis*. As cellobiose is soluble in water, absorbance measurements at 600 nm were taken over time.

**Carbon Source Utilization Studies:** All carbon source stocks were made in CSS medium and at a concentration of 150 mM carbon. The following carbon sources, all representing potential cellulose degradation products, were used to assess ability of select isolates to utilize them for growth: Glucose (dextrose), fructose, maltose, mannose, sucrose, xylose, sodium acetate, sodium butyrate, sodium formate, sodium fumarate, sodium propionate, sodium succinate, sodium lactate (60% w/w) and 100% ethanol (EtOH). In sterile, 24-well microtiter plates, duplicate wells were set up for each carbon source in which wells contained a final concentration of 15 mM carbon, CSS medium and *Arthrobacter* sp. isolate EF01. Duplicate cell-free control wells were also set up. Two wells contained CSS medium only. Plates were shaken at 150 rpm at room temperature (22 ± 2°C) and after 72 hours removed to take absorbance measurements at 600 nm.

This was repeated anaerobically following the same procedure, but was carried out exclusively in the glovebag. The microtiter plates were shaken at 150 rpm on a small shaker within the glovebag as well as absorbance readings taken in the glovebag at 600 nm using the PowerWave XS microplate spectrophotometer (BioTek Instruments, Winooski, VT).

**Cr(VI) Reduction Studies:** Select isolates were evaluated for their capabilities to grow in the presence of and reduce 50 µM Cr(VI). Aerobically, this was conducted using sterile, 24-well microtiter plates. Glucose was supplied as the carbon source unless
indicated otherwise. Triplicate wells were set up with CSS medium, 15 mM carbon, and
a pure culture of one of the isolates. Triplicate wells were set up containing CSS
medium, 15 mM carbon, pure culture of the isolate, and 50 µM Cr(VI). Appropriate cell-
free and carbon-free control wells were set up as well. Plates were shaken at 150 rpm at
room temperature (22 ± 2°C) and after 72 hours removed to take absorbance
measurements at 600 nm. Samples were taken at the end of the study for Cr(VI) analysis.

For each isolate these were repeated anaerobically. Due to the potential for
oxygen scavenging from the well-plates and potential evaporation over time, these
anaerobic studies were performed using anaerobic culture tubes sealed with butyl rubber
stoppers and aluminum crimp seals. The same treatments were set up in triplicate.
Absorbance measurements were taken over time and samples were periodically taken for
Cr(VI) analysis.

**Cr(VI) Quantification:** Cr(VI) concentrations were determined
spectrophotometrically using the diphenylcarbazide assay as described by Nyman et al.
(2002). 150 µL of the sample was added to 50 µL of 0.8N H₂SO₄ in a 96-well microtiter
plate followed by the addition of 20 µL of 0.25% 1,5-diphenylcarbazide (Sigma-Aldrich,
St. Louis, MO) in 100% acetone. Assay blanks were prepared by the addition of 20 µL
of 100% acetone without 1,5-diphenylcarbazide. Absorbance was measured at 540 nm,
adjusted to corresponding assay blanks and compared to standards made from a K₂CrO₄
stock solution (Ricca Chemical Company, Arlington, TX).
Results and Discussion

Isolation and Identification

Direct Isolation: Four bacterial isolates, a *Pseudomonas* sp., *Pedobacter* sp., *Streptomyces* sp., and *Flavobacterium* sp., were obtained from direct isolation on aerobic, methyl cellulose containing agar plates (Table 2.1, Table 2.2). One fungal isolate, *Lecythophora mutabilis*, was also obtained from direct isolation plates. All four of the bacterial isolates are likely to be strict aerobes as growth was not observed under anaerobic conditions. It is also possible that they are denitrifiers, though, this capability was not evaluated. The *Pseudomonas* sp., *Streptomyces* sp., and *Flavobacterium* sp. were all isolated from the FW, WW and WC layers. This suggests that oxygen may be present in the lower layers of this site as these organisms do not appear to be able to grow anaerobically. The *Pedobacter* sp. was only detected in the WW layer. No growth was observed on anaerobic plates before the plates dried out (~1 month) therefore no isolates were obtained from anaerobic direct isolation plates.

Liquid Enrichments: Three bacterial isolates were obtained through multiple transfers of anaerobic liquid enrichments and re-streaking onto methyl cellulose containing agar plates (Table 2.1, Table 2.2). Growth was very slow and was not visible until after a month of growth in liquid or on plates. All three isolates are facultative organisms as they were isolated through anaerobic methods, but can also grow in the presence of oxygen. The *Paenibacillus* sp. was isolated from both the methyl cellulose containing and filter paper containing clay layer enrichments. The *Cellulomonas*
*parahominis* isolate was obtained from all four soil layers and from enrichments of each of the two cellulose sources. *Serratia* sp. was obtained from the FW, WW and C layers all from filter paper enrichments. It was also isolated from the methyl cellulose-containing WW enrichment.

It is surprising that there was little growth in methyl cellulose-containing enrichments as this is a more easily utilizable carbon source compared to filter paper. It is possible that the amount of methyl cellulose in these enrichments was too low to support much growth especially under anaerobic conditions. Similarly, little growth was observed in all aerobic enrichments including methyl cellulose-containing enrichments. All aerobic enrichments were transferred at least once, but little to no growth was observed. Because aerobic isolates had been obtained through direct isolation methods, no additional isolation work was conducted with these enrichments.

**Column Studies:** Six days after the column effluents were spread plated, microbial growth was observed on three of the plates, including the plate inoculated with column 4 effluent that was not visibly turbid at the time of spread plating. There was no growth on the plate inoculated with effluent of the control soil column. This strongly indicates that the growth on the other plates was not due to contamination of the effluent from something in the laboratory or the agar plate itself. It is important to note that TSA, of which glucose (dextrose) is the main carbon source available, was used to isolate these organisms and therefore, these isolates are not necessarily cellulose-degraders. However, as cellulose is the dominant carbon source in the columns from which these organisms
Table 2.1. Isolation Method, Source, Oxygen Requirements and Colony Morphologies of Isolates Obtained from the Cold Test Pit South Site at the Idaho National Laboratory

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Type of Microorganism</th>
<th>Isolation Method</th>
<th>Soil Layer Source</th>
<th>O₂ Requirements</th>
<th>Colony Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas sp.</td>
<td>Bacterium</td>
<td>Direct Isolation Plates</td>
<td>FW, WW, WC</td>
<td>Aerobic</td>
<td>Yellowish, white smooth</td>
</tr>
<tr>
<td>Streptomyces sp.</td>
<td>Bacterium</td>
<td>Direct Isolation Plates</td>
<td>FW, WW, WC</td>
<td>Aerobic</td>
<td>Hard, white, grows into agar</td>
</tr>
<tr>
<td>Pedobacter sp.</td>
<td>Bacterium</td>
<td>Direct Isolation Plates</td>
<td>WW</td>
<td>Aerobic</td>
<td>Whitish yellow, round</td>
</tr>
<tr>
<td>Flavobacterium sp.</td>
<td>Bacterium</td>
<td>Direct Isolation Plates</td>
<td>FW, WW, WC</td>
<td>Aerobic</td>
<td>Yellow, smooth</td>
</tr>
<tr>
<td>Serratia sp.</td>
<td>Bacterium</td>
<td>Anaerobic Liquid Enrichments</td>
<td>FW, WW, C</td>
<td>Faculative</td>
<td>Light white, smooth</td>
</tr>
<tr>
<td>Cellulomonas parahominis</td>
<td>Bacterium</td>
<td>Anaerobic Liquid Enrichments</td>
<td>FW, WW, WC, C</td>
<td>Faculative</td>
<td>Small, transparent, smooth</td>
</tr>
<tr>
<td>Paenibacillus sp.</td>
<td>Bacterium</td>
<td>Anaerobic Liquid Enrichments</td>
<td>C</td>
<td>Faculative</td>
<td>Light greenish, rough edges, depressed center</td>
</tr>
<tr>
<td>Serratia sp.</td>
<td>Bacterium</td>
<td>Anaerobic Liquid Enrichments</td>
<td>FW, WW, C</td>
<td>Faculative</td>
<td>Light white, smooth</td>
</tr>
<tr>
<td>Arthrobacter sp.</td>
<td>Bacterium</td>
<td>Column Effluent</td>
<td>WW</td>
<td>Aerobic</td>
<td>White, small, raised, smooth</td>
</tr>
<tr>
<td>Paenibacillus sp. (LT)</td>
<td>Bacterium</td>
<td>Column Effluent</td>
<td>WW</td>
<td>Faculative</td>
<td>Larger, glossy, yellowish, slightly transparent</td>
</tr>
<tr>
<td>Paenibacillus sp. (2ST)</td>
<td>Bacterium</td>
<td>Column Effluent</td>
<td>WW</td>
<td>Faculative</td>
<td>Smaller, whitish, glossy, transparent</td>
</tr>
<tr>
<td>Paenibacillus sp. (2Y)</td>
<td>Bacterium</td>
<td>Column Effluent</td>
<td>WW</td>
<td>Faculative</td>
<td>Yellow, small, depressed center</td>
</tr>
<tr>
<td>Dietzia natronolimnaea</td>
<td>Bacterium</td>
<td>Column Effluent</td>
<td>WW</td>
<td>Aerobic</td>
<td>Orange, smooth</td>
</tr>
<tr>
<td>Lecythophora mutabilis</td>
<td>Fungus</td>
<td>Direct Isolation Plates</td>
<td>WW</td>
<td>Aerobic</td>
<td>White, fuzzy, hyphae</td>
</tr>
</tbody>
</table>

Table 2.2. 16S rRNA Gene Identification and Top BLAST Result of Bacterial Isolates from the Cold Test Pit South Site at Idaho National Laboratory

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Top BLAST Hit (% Similarity)*</th>
<th>Sequence Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas sp.</td>
<td>*Pseudomonas sp. JR1 (99%)</td>
<td>1427</td>
</tr>
<tr>
<td>Streptomyces sp.</td>
<td>*Streptomyces sp. HB200 (99%)</td>
<td>1301</td>
</tr>
<tr>
<td>Pedobacter sp.</td>
<td>*Pedobacter GR12-04 (98%)</td>
<td>1416</td>
</tr>
<tr>
<td>Flavobacterium sp.</td>
<td>*Flavobacterium sp. WB 3.1-83 (98%)</td>
<td>1376</td>
</tr>
<tr>
<td>Serratia sp.</td>
<td>*Serratia proteamaculans 568 (99%)</td>
<td>1413</td>
</tr>
<tr>
<td>Cellulomonas parahominis</td>
<td>*Cellulomonas parahominis strain W7385 (99%)</td>
<td>1374</td>
</tr>
<tr>
<td>Paenibacillus sp.</td>
<td>*Paenibacillus sp. GP26-03 (99%)</td>
<td>1429</td>
</tr>
<tr>
<td>Arthrobacter sp.</td>
<td>*Arthrobacter sp. OTSz_M_217 (97%)</td>
<td>1418</td>
</tr>
<tr>
<td>Paenibacillus sp. (LT)</td>
<td>*Paenibacillus sp. 4GH05-09 (99%)</td>
<td>1413</td>
</tr>
<tr>
<td>Paenibacillus sp. (2ST)</td>
<td>*Paenibacillus sp. AaDS (98%)</td>
<td>1410</td>
</tr>
<tr>
<td>Paenibacillus sp. (2Y)</td>
<td>*Paenibacillus sp. 4GH05-09 (98%)</td>
<td>1325</td>
</tr>
<tr>
<td>Dietzia natronolimnaea</td>
<td>*Dietzia natronolimnaea strain W5044 (98%)</td>
<td>1424</td>
</tr>
<tr>
<td>Lecythophora mutabilis</td>
<td>*Lecythophora mutabilis AF353604 (99%)</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Only top BLAST result for cultured organisms listed, uncultured clones were removed from search results
came, it is likely that they are breaking down cellulose or utilizing the products of degradation for growth.

*Arthrobacter* sp. was found on effluent plates from columns 2, 3 and 4 (Table 2.1, Table 2.2). It was the only strict aerobe isolated from the columns. Three *Paenibacillus* spp. isolates were obtained from the column effluents. Each of these isolates had a unique colony morphology; however, 16S rRNA gene sequencing indicated that they were all *Paenibacillus* spp. (Table 2.2). All three were isolated from the column 2 effluent. The isolates with large transparent (LT) and yellow (Y) colony morphologies were also found in the column 3 effluent and the isolate with small transparent (ST) colony morphology was also found in the column 4 effluent. One additional bacterial isolate was obtained from the column effluents. *Dietzia natronolimnaea* was observed on the transfer of one of the plates from the column 2 effluent. Only one colony with this morphology was found and as it did not appear until after one transfer, it is possible that this isolate may be a contaminant. However, its family, *Dietziaceae*, was detected through PhyloChip and clone library analyses using molecular techniques (see Chapter 3 for details regarding the molecular analyses) so it may have originated from the site.

**Phylogeny and Potential Cellulose Degradation Capabilities:** Of the twelve bacterial isolates obtained, there were members of four different phyla; *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Firmicutes* (Table 2.3). All of these phyla contain known cellulose degraders (Lynd et al., 2002). Three of these phyla also accounted for all of the changes in bacterial community structure with depth at the Cold Test Pit South
site which suggests they may be playing some role at this site (see Chapter 3 and Field et al. (2010) for more information). Additionally, if evaluated at the genus level, the *Pseudomonas, Streptomyces, Pedobacter, Serratia, Cellulomonas*, and *Paenibacillus* genera have all been shown to include members that can degrade cellulose to some extent (Coughlan and Mayer, 1992; Lynd et al., 2002; Bayer et al., 2006; Ogawa et al., 2007). While phylogeny cannot be directly linked to function, these findings do suggest that the isolation methods used were successful in obtaining potential cellulose-degraders.

**Characterization Studies**

**Cellulose Degradation Capabilities:** All of the isolates were screened for their ability to utilize carboxymethylcellulose (CMC) aerobically. As CMC is a soluble cellulose source and easily utilizable, it is commonly used for screening cellulose degradation capabilities (Coughlan and Mayer, 1992; Lynd et al., 2002). Ten of the thirteen bacterial isolates were capable of utilizing CMC and zones of clearing on the plates were observed (Table 2.4, Appendix D) *Pseudomonas* sp., *Pedobacter* sp., and *Dietzia natronolimnaea* were unable to utilize CMC within the agar plates after 72 hours as no zone of clearing was observed. Interestingly, *Flavobacterium* sp. had the greatest zone of clearing of all of the isolates. *Flavobacterium* spp. have been reported in the literature to not be capable of utilizing cellulose as a carbon source (Holmes, 2006). However, *Flavobacterium* spp. are closely related to *Cytophaga* spp, which are cellulolytic, it has been implied previously that some *Flavobacterium* spp. may be capable of cellulose degradation (Holmes, 2006). While CMC is commonly used to assess cellulolytic capabilities, it is important to point out that this is a soluble cellulose
Table 2.3 Taxonomical Levels of Bacterial Isolates Obtained from the Cold Test Pit South Site at the Idaho National Laboratory

<table>
<thead>
<tr>
<th>Genus</th>
<th>Family</th>
<th>Order (Suborder)</th>
<th>Class (Subclass)</th>
<th>Phylum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas</td>
<td>Pseudomonadaceae</td>
<td>Pseudomonadales</td>
<td>Gammaproteobacteria</td>
<td>Proteobacteria</td>
</tr>
<tr>
<td>Streptomyces</td>
<td>Streptomycetaceae</td>
<td>Actinomycetales</td>
<td>Actinobacteria</td>
<td>Bacteroidetes</td>
</tr>
<tr>
<td>Pedobacter</td>
<td>Sphingobacteriaceae</td>
<td>Sphingobacteria</td>
<td>Actinobacteria</td>
<td>Bacteroidetes</td>
</tr>
<tr>
<td>Flavobacterium</td>
<td>Flavobacteriaceae</td>
<td>Flavobacteriales</td>
<td>Flavobacteria</td>
<td>Bacteroidetes</td>
</tr>
<tr>
<td>Serratia</td>
<td>Enterobacteriaceae</td>
<td>Enterobacteriales</td>
<td>Gammaproteobacteria</td>
<td>Proteobacteria</td>
</tr>
<tr>
<td>Cellulomonas</td>
<td>Cellulomonadaceae</td>
<td>Actinomycetales</td>
<td>Actinobacteria</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>Paenibacillus</td>
<td>Paenibacillaceae</td>
<td>Bacillales</td>
<td>Bacilli</td>
<td>Firmicutes</td>
</tr>
<tr>
<td>Arthrobacter</td>
<td>Micrococcaceae</td>
<td>Actinomycetales</td>
<td>Actinobacteria</td>
<td>Actinobacteria</td>
</tr>
<tr>
<td>Dietzia</td>
<td>Dietziaceae</td>
<td>Actinomycetales</td>
<td>Actinobacteria</td>
<td>Actinobacteria</td>
</tr>
</tbody>
</table>

Table 2.4 Aerobic Carboxymethylcellulose Utilization by the Bacterial Isolates from the Cold Test Pit South Site on Agar Plates as Measured by Zone of Clearing

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Zone of Clearing Diameter (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas sp.</td>
<td>0.0</td>
</tr>
<tr>
<td>Streptomyces sp.</td>
<td>3.0</td>
</tr>
<tr>
<td>Pedobacter sp.</td>
<td>0.0</td>
</tr>
<tr>
<td>Flavobacterium sp.</td>
<td>4.5</td>
</tr>
<tr>
<td>Serratia sp.</td>
<td>1.0</td>
</tr>
<tr>
<td>Cellulomonas parahominis</td>
<td>1.0</td>
</tr>
<tr>
<td>Paenibacillus sp.</td>
<td>3.0</td>
</tr>
<tr>
<td>Arthrobacter sp.</td>
<td>1.5</td>
</tr>
<tr>
<td>Paenibacillus sp. (LT)</td>
<td>2.0</td>
</tr>
<tr>
<td>Paenibacillus sp. (2ST)</td>
<td>3.0</td>
</tr>
<tr>
<td>Paenibacillus sp. (2Y)</td>
<td>4.0</td>
</tr>
<tr>
<td>Dietzia natronolimnaea</td>
<td>0.0</td>
</tr>
<tr>
<td>Aspergillus niger cellulase 10 mg/mL</td>
<td>3.5</td>
</tr>
</tbody>
</table>
source, more easily utilizable, and truly cellulolytic microorganisms should also be capable of degrading non-soluble cellulose.

Also of interest, the four *Paenibacillus* spp. isolates with varying colony morphologies also had varying zones of clearing ranging from 2-4 cm. Cells were normalized to the same OD before inoculation, but it is still possible that there was enough variation in cell densities, since only 10 µL was used to inoculate the plate, for this to be a cell-dependent effect. However, as these isolates have morphological differences it is also possible that they may also have metabolic differences.

Isolates were also evaluated on their ability to utilize a variety of cellulose sources with varying crystallinities and solubilities. Unfortunately, the methods used to evaluate growth on these substrates were unsuccessful. The methods used included using DNA stains, taking absorbance measurements and protein assays. The problems with measuring biomass may have been caused by the interference of the cellulose with each of these methods. Cells were indistinguishable from the cellulose source as DNA-based stains used absorbed to the cellulose material. Non-soluble cellulose affected absorbance readings and protein could not be separated from the cellulose source through methods such as centrifugation after vortexing. Additionally, non-soluble sources changed color at higher temperatures used for protein assays also affecting the final absorbance readings. Cellulose degradation may also have not been extensive and therefore, biomass concentrations may have been below detection limits (approximately 5 mg/L for the Coomassie protein assay). It is worth noting that when all thirteen isolates, in equal concentrations, were used to inoculate an aerobic, liquid enrichment containing filter
paper as the sole carbon source the filter paper was degraded significantly within 10 days. As growth was significant, protein was able to be quantified. In the same enrichment set up anaerobically, there was little visual growth and filter paper degradation and protein was below detection limits.

When evaluated for their ability to degrade filter paper individually, *C. parahominis* was found to be the only microorganism capable of degrading filter paper anaerobically on its own. Aerobically, only the *Streptomyces* sp. isolate appeared to be capable of degrading cellulose to any extent based on visual observations. Growth and filter paper degradation by *C. parahominis* anaerobically appeared to be a slow process as it was only visually observed after a two month period. Protein was below the detection limit and breakdown products were evaluated through HPLC analysis, but degradation products were at the detection limit (< 2 ppm) and accurate quantification was not possible. Preliminary results indicated that acetate, fumarate and lactate were possible products. *C. parahominis* was not capable of degrading filter paper by itself aerobically. Additional studies with *C. parahominis* determined that cellobiose could be utilized for growth both aerobically and anaerobically (Figure 2.1). Maximum OD was reached at 72 hours under both aerobic and anaerobic conditions.

Further characterization studies focused on the three facultative isolates obtained from liquid enrichments: *Cellulomonas parahominis*, *Paenibacillus* sp., and *Serratia* sp. The isolates could either have been degrading cellulose and utilizing its products for growth or be a heterotrophic organism unable to degrade cellulose but utilizing the products provided by other cellulolytic microorganisms. The three facultative bacteria
Figure 2.1 Cellobiose utilization by *C. parahominis* under anaerobic and aerobic conditions. Error bars represent ± one standard deviation (n = 2) and are smaller than the symbols where not visible.

were isolated through multiple transfers of liquid enrichments and it was hypothesized that this method would best enrich for truly cellulolytic microorganisms as multiple transfers would dilute out a large portion of the heterotrophs utilizing cellulose degradation products. Their cellulose degradation potential and their facultative capabilities led to the interest in further evaluating their metabolic capabilities.

As discussed previously, cellulose degradation capabilities were difficult to assess especially in regards to non-soluble cellulose sources. Therefore, no conclusions were made regarding these capabilities and they will not be discussed further. Additional studies characterizing their carbon source utilization and Cr(VI) reduction capabilities were performed and are discussed below.
Carbon Source Utilization Capabilities: Of the thirteen potential cellulose degradation products tested, all three isolates were capable of utilizing glucose, sucrose, fructose and maltose for growth both aerobically and anaerobically (Table 2.5). *Serratia* sp. was capable of utilizing the organic acids and ethanol, the sole alcohol tested, for growth both aerobically and anaerobically. Neither *C. parahominis* or *Paenibacillus* sp. were capable of utilizing organic acids or ethanol for growth either aerobically or anaerobically. As studies were only conducted for 72 hours, it is possible that growth may occur on some of these carbon sources, but the time frame was too short to observe growth.

As CSS medium does not contain any additional external terminal electron acceptors, such as nitrate or sulfate, except in trace quantities, anaerobic growth of these

<table>
<thead>
<tr>
<th>Carbon Source (15mM)</th>
<th><em>Cellulomonas parahominis</em></th>
<th><em>Paenibacillus</em> sp.</th>
<th><em>Serratia</em> sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aerobic</td>
<td>Anaerobic</td>
<td>Aerobic</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fructose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mannose</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Xylose</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ethanol</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lactate</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Butyrate</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acetate</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Succinate</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Propionate</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fumarate</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Formate</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
isolates on these carbon sources is mainly fermentative. *Serratia* sp. being capable of utilizing a carbon source such as ethanol anaerobically is surprising as it is energetically unfavorable. The growth observed was over two standard deviations above the control treatments. It is possible that the organism was able to scavenge residual oxygen from the 24-well plate. This is why future anaerobic studies were conducted in anaerobic culture tubes that could be sparged with nitrogen to remove residual oxygen that may have been adhered to the glass. It is also possible that this increase in absorbance is due to cell clumping either due to starvation or cell death. In the future, these studies should be carried out in larger volumes in anaerobic culture tubes to minimize the potential influence of residual oxygen and protein measurements should be taken in addition to absorbance measurements to ensure the increase in absorbance is due to increased cell biomass. Also, *Serratia* sp. was observed to grow anaerobically on propionate but not aerobically. This again suggests these studies should be repeated on a larger-scale, for a longer period of time in order to provide more reliable and consistent results.

**Cr(VI) Reduction Capabilities:** All three isolates were capable growing in the presence of and reducing Cr(VI) when glucose was provided as the sole carbon source under both aerobic and anaerobic conditions (Figures 2.2-2.5, Appendix D). When utilizing glucose for growth aerobically, the maximum OD of *C. parahominis* in the presence of 50 µM Cr(VI) decreased by approximately 65% (Figure 2.2). After 78 hours, Cr(VI) concentration had decreased by 20% (Figure 2.3). Similar results were observed for *Paenibacillus* sp. demonstrating its ability to grow both aerobically and anaerobically
Figure 2.2. Aerobic growth of *C. parahominis* when glucose was the sole carbon source in the presence of 50 µM Cr(VI). Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.

Figure 2.3 Cr(VI) concentrations after 84 hours under aerobic conditions in the presence of *C. parahominis* when glucose was the sole carbon source. Initial concentration of Cr(VI) was 50 µM. Error bars represent ± one standard deviation (n = 3).
in the presence of 50 μM Cr(VI) as well as reduce Cr(VI) when utilizing glucose as the sole carbon source (Appendix D).

Under anaerobic conditions, \textit{C. parahominis} had an increased lag time when grown on glucose in the presence of 50 μM Cr(VI) (Figure 2.4). In the absence of 50 μM Cr(VI) \textit{C. parahominis} reached maximum OD after 83 hours while in the presence of 50 μM Cr(VI) maximum OD was reached after 395 hours. While there was no change in absorbance measurements for the first 395 hours in the presence of 50 μM Cr(VI), there was a continual decrease in Cr(VI) concentration in this treatment (Figure 2.5). When compared to the absorbance measurements, results indicate that growth in the presence of 50 μM Cr(VI) did not occur until Cr(VI) was almost completely reduced. Similar results were observed for \textit{Serratia} sp. in which growth on glucose anaerobically did not occur in the presence of 50 μM Cr(VI) until Cr(VI) concentrations approached zero (Appendix D). Under the same conditions, \textit{Paenibacillus} sp. was capable of growth in the presence of 50 μM Cr(VI) and a decrease in Cr(VI) concentration was observed (Appendix D).

In Chapters 4 and 5 of this dissertation, the influence of carbon source on Cr(VI) toxicity and reduction by \textit{Arthrobacter} sp. is discussed. To determine if the carbon source provided influences Cr(VI) toxicity and reduction by \textit{Serratia} sp. additional studies were conducted. These studies were not conducted with \textit{C. parahominis} or \textit{Paenibacillus} sp. as neither was observed to be capable of utilizing carbon sources other than sugars for growth. Results suggest that the carbon source available for growth aerobically clearly influenced both growth and Cr(VI) reduction by \textit{Serratia} sp. (Figures 2.6 and 2.7, respectively). If glucose or lactate were present, \textit{Serratia} sp. grew in the
Figure 2.4 Anaerobic growth of *C. parahominis* when glucose was the sole carbon source in the presence of 50 μM Cr(VI). Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.

Figure 2.5 Comparison of *C. parahominis* anaerobic growth (dark filled symbols) and Cr(VI) concentrations (unfilled symbols) over time when glucose was the sole carbon source in the presence of 50 μM Cr(VI). Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.
presence of 50 µM Cr(VI) and the Cr(VI) concentration decreased significantly after 96 hours (Figure 2.7). If ethanol or acetate were provided in the presence of 50 µM Cr(VI), no growth and no significant decrease in Cr(VI) concentration was observed after 96 hours. This should be repeated and carried out for longer times to determine if this lack of growth or reduction will occur if given more time. Additionally, this should be repeated anaerobically to determine if the presence of oxygen influences these results. These experiments were also carried out with xylose as the sole carbon source present.

Figure 2.6 Aerobic Growth of *Serratia* sp. when glucose (A), lactate (B), ethanol (C) and acetate (D) were the sole carbon sources in the presence (●) and absence (■) of 50 µM Cr(VI). Additional symbols: CSS media (▲), *Serratia* sp. only (♦), Carbon source only (x). Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.
and the results indicated that *Serratia* sp. was capable of growing in the presence of 50 µM Cr(VI). A significant decrease in Cr(VI) concentration was also observed (Appendix D). These results indicate that the carbon source present influenced Cr(VI) toxicity and reduction by *Serratia* sp.

![Figure 2.7](image)

Figure 2.7 Cr(VI) concentration after 96 hours when *Serratia* sp. was grown aerobically when ethanol (EtOH), acetate, glucose and lactate were the sole carbon sources provided in the presence of 50 µM Cr(VI). Initial concentration of Cr(VI) was 50 µM. Error bars represent ± one standard deviation (n = 3).

**Conclusions**

Three different enrichment and isolation strategies were successful in obtaining both bacteria and fungi from soil core samples obtained from the Cold Test Pit South site at the Idaho National Laboratory. Thirteen total isolates were identified with different phylogenies, colony morphologies, and oxygen requirements. By employing three
different techniques, a more diverse group of potential cellulose degraders was obtained from the CTPS site. The direct isolation method yielded both bacterial isolates and one fungal isolate all of which are aerobes. As these organisms were obtained through serial dilution before isolation on agar plates they may represent organisms that are more abundant at this site. The facultative isolates obtained anaerobically through liquid enrichment containing filter paper may be involved in degradation of the cellulosic waste regardless of oxygen availability. As the columns mimicked the low-level waste site, with media flow-through and contaminants present, it is likely these organisms may be playing a role in cellulose degradation at the site as well and possibly a role in metal mobility at low-level waste sites at the RWMC. These isolates will be used in future studies evaluating the interactions between cellulose, microorganisms and chromium in the environment.
References


CHAPTER 3

APPLICATION OF MOLECULAR TECHNIQUES TO ELUCIDATE THE INFLUENCE OF CELLULOSIC WASTE ON THE BACTERIAL COMMUNITY STRUCTURE AT A SIMULATED LOW-LEVEL-RADIOACTIVE-WASTE SITE

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Contributions: Experimental design, data analysis and editing

Co-author: Amber R. Miller
Contributions: Laboratory experiments, data analysis and editing

Co-author: Michael R. VanEngelen
Contributions: Laboratory experiments, data analysis and editing

Co-author: Robin Gerlach
Contributions: Experimental design, data analysis and editing

Co-author: Brady D. Lee
Contributions: Experimental design, data analysis and editing

Co-author: William A. Apel
Contributions: Experimental design, data analysis and editing

Co-author: Brent M. Peyton
Contributions: Experimental design, data analysis and editing
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Journal: Applied and Environmental Microbiology

Status of manuscript:
___Prepared for submission to a peer-reviewed journal
___Officially submitted to a peer-reviewed journal
___Accepted by a peer-reviewed journal
xPublished in a peer-reviewed journal

Published by the American Society for Microbiology

Appears in: Volume 76, Number 10, 2010, pgs 3106-3115

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Abstract

Low-level-radioactive-waste (low-level-waste) sites, including those at various U.S. Department of Energy sites, frequently contain cellulosic waste in the form of paper towels, cardboard boxes, or wood contaminated with heavy metals and radionuclides such as chromium and uranium. To understand how the soil microbial community is influenced by the presence of cellulosic waste products, multiple soil samples were obtained from a non-radioactive model low-level-waste test pit at the Idaho National Laboratory. Samples were analyzed using 16S rRNA gene clone libraries and 16S rRNA gene microarray (PhyloChip) analyses. Both methods revealed changes in the bacterial community structure with depth. In all samples, the PhyloChip detected significantly more operational taxonomic units, and therefore relative diversity, than the clone libraries. Diversity indices suggest that diversity is lowest in the fill and fill-waste interface (FW) layers and greater in the wood waste and waste-clay layers. Principal-coordinate analysis and lineage-specific analysis determined that Bacteroidetes and Actinobacteria phyla account for most of the significant differences observed between the layers. The decreased diversity in the FW layer and increased members of families containing known cellulose-degrading microorganisms suggests the FW layer is an enrichment environment for these organisms. These results suggest that the presence of the cellulosic material significantly influences the bacterial community structure in a stratified soil system.
Introduction

The processing of nuclear materials, operation of nuclear reactors, research and development activities at government sites, hospitals, universities, and radiochemical and radiopharmaceutical manufacturers have led to the generation of a substantial amount of low-level mixed radioactive and heavy metal wastes that have been disposed in pits, trenches, and other waste sites (Anonymous, 1994). Co-disposed with metals and radionuclides were large quantities of cellulose containing materials such as wood, paper towels, cardboard, cheesecloth, and other materials (Saunders and Toran, 1995). These wastes result from glove box operations, decontamination, housekeeping, maintenance, and construction activities, and can constitute up to 90% of the volume of typical low-level radioactive waste (low-level waste [LLW]) (Vilks et al., 1998). While there are over 20,000 commercial users of radioactive materials (Environmental Protection Agency, 1994), the Department of Energy (DOE) complex houses the majority of disposed LLW waste at sites including Savannah River, Hanford, Idaho National Laboratory (INL) and Nevada test sites (Anonymous, 2000). Prior to 2000, the DOE disposed of approximately 2 million cubic meters of LLW and has projected the disposal of an additional 10.1 million cubic meters by 2070 (Anonymous, 2000). Within the Subsurface Disposal Area at the INL alone, approximately 330 metric tons of U-238 have been buried with cellulose containing material (Hull et al., 2002; Keck and Seitz, 2002). While these LLW materials are generally classified as such due to their low radioactivity and metal concentrations, their large quantity suggests there is potential environmental concern if mobilization of these contaminants was to occur.
The mobility of heavy metals and radionuclides in the subsurface may be greatly affected by the decomposition of this cellulosic waste by cellulolytic or fermentative microorganisms. A number of soil microorganisms can degrade one or more lignocellulosic components (i.e. cellulose and hemicellulose) to their respective subunits, which include cellobiose, C₅ and C₆ sugars (i.e. xylose, mannose, and glucose) (Leschine, 1995; Chen et al., 2001; Lynd et al., 2002). The breakdown of cellulose itself may release the associated metals and radionuclides, potentially increasing their mobility. Additionally, fermentative bacteria can then use these cellulose breakdown products as carbon and energy sources producing a variety of fermentation products including short chain organic acids, alcohols and hydrogen (Francis et al., 1980). These fermentation products may significantly influence contaminant mobility, since organic acids can chelate metals and radionuclides potentially increasing their mobility (Means et al., 1978; Humphreys et al., 1997; Read et al., 1998; Francis et al., 1999; Choy et al., 2006). On the other hand, the work of numerous investigators has shown that these same compounds can serve as the carbon and energy source for metal and sulfate reducing bacteria that reduce and precipitate the metals and radionuclides found at these sites (Lloyd et al., 1997; Abdelouas et al., 2000; Kashefi and Lovley, 2000; Chew et al., 2001; Liu et al., 2002; Roh et al., 2002; Sani et al., 2002; Viamajala et al., 2002; Ortiz-Bernad et al., 2004; Shelobolina et al., 2004; Fox et al., 2006).

To better understand interactions between the bacterial community, cellulosic waste, and contaminants at LLW sites, the bacterial community must first be identified. Little is known about the bacterial community structure at LLW sites as previous studies
have focused on culture dependent techniques, the construction of small clone libraries, and Denaturing Gradient Gel Electrophoresis (Francis et al., 1980; Fox et al., 2006). Therefore, this study aims to perform a larger in-depth molecular analysis of the entire bacterial community at one of these sites. Soil cores from a surrogate waste pit at the INL were collected and samples from four depths within the pit were analyzed using 16S rRNA gene clone libraries and high-density 16S rRNA gene microarrays (PhyloChip). The overall goal of this study was to determine how the presence of buried cellulosic waste influences the bacterial community structure found at a LLW site.

**Materials and Methods**

**Site Description**

The Cold Test Pit South (CTPS) is located at the DOE INL Radioactive Waste Management Complex (RWMC) about 50 miles west of Idaho Falls, Idaho. The CTPS was constructed in 1988 and filled with simulated wastes that conform to the historical disposal practices at the RWMC between 1953 and 1970 (Thompson, 2002). The pit was constructed to provide a clean environment to test the implementation of innovative waste characterization, retrieval technology, performance and operational testing of remedial action scenarios. Cardboard was used as simulated waste containers to promote rapid deterioration and simulate up to 35 years of burial in shallow land filled pits. The bottom of the CTPS was lined with a crushed-sediment clay liner (Figure 3.1). The waste layer, designated as the wood waste layer, contains stacked cardboard boxes, drums of combustibles (scrap wood, cloth, paper, plastic and HEPA filters), metals (aluminum and steel), concrete, asphalt, glass, and simulated inorganic sludges (silica and carbonate...
based pastes). Evidence from previous activities in the CTPS suggests that most of the simulated waste forms were concentrated at the base of the pit between 2.4 and 4.9 m below grade. The simulated waste layer was then covered with an overlying fill soil layer using local unsaturated soil. Compaction over time reduced the size of the simulated waste layer to approximately 0.2 m.

CTPS Sampling

A truck mounted Powerprobe 9600TM (AMS, Inc., American Falls, ID) direct push sampling rig was used to obtain intact core samples from the CTPS. Soil cores spanning the depth of the pit were collected in sterile 3.2 cm diameter Lexan™ core tubes (Appendix E). Samples were placed in a cooler on ice for shipment to the INL laboratory where the samples were processed.

Lexan tubes were cut at four designated depths representing various layers of the pit (Figure 3.1). The four soil layers that were sampled were the overlying Fill soil layer (F), the Fill soil/Wood Waste interface (FW), the Wood Waste soil layer (WW), and Wood Waste/Clay interface (WC). Approximately 2.5 cm of soil was removed aseptically using a sterile spatula, then a sterile 50 ml conical centrifuge tube was used to subcore for samples from which DNA was extracted. For samples that were obtained at interfaces (FW and WC), the soil sample obtained spanned each of the upper and lower layers equally. Samples were stored at -20°C prior to DNA extraction. Triplicate soil samples were collected from each of the four soil layers for individual DNA extraction and molecular analysis.
Sampling Extraction and 16S rRNA Gene Amplification

DNA was extracted using the PowerMax® Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer’s protocol for the first set of soil samples from each layer (5 g soil per sample). DNA was extracted using the UltraClean Soil DNA Kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer’s protocol for the second and third soil sample (0.3 g per sample) from each soil layer. Since the WW layer soil was high in humic content, an additional clean-up step using a sephadex-based spin column was used according to instructions provided (illustra MicroSpin G-25 columns, GE Healthcare, UK) to remove compounds that would inhibit amplification.

![Diagram of soil layers and sampling points](image)

Figure 3.1 Schematic of the non-radioactive CTPS near the LLW site at the Idaho National Laboratory where soil samples were obtained. Brackets indicate sampling points. F, Fill; FW, Fill Waste interface; WW, Wood Waste; WC, Waste Clay interface.
PCR amplification of 16S rRNA genes was performed using 50 µL reactions containing a final concentration of 1x PCR buffer, 0.01 mg/mL bovine serum albumin, 0.5 Units JumpStart REDTaq DNA polymerase, (Sigma-Aldrich, St. Louis, MO), 0.4 µM 8F primer (5’-AGAGTTTGATCCTGGCTCAG-3’), and 0.4 µM 1492R primer (5’-GGTTACCTTGTTACGACTT-3’) (Integrated DNA Technologies, Coralville, IA). The reactions were heated to 94°C for 10 minutes, followed by 30 cycles of 94°C for 1 minute, 52°C for 1 minute, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes (Applied Biosystems, GeneAmp PCR System 9700). The amplicons were checked for the correct size on the Agilent 2100 Bioanalyzer with the Agilent DNA 7500 Kit (Agilent Technologies, Waldbronn, Germany).

Cloning and Sequencing

Triplicate clone libraries were created for each soil layer using the three individual soil samples and DNA extracts obtained. 16S rRNA gene amplicons were ligated into the pCR2.1 vector using the TOPO TA Cloning Kit and transformed into Top10 competent Escherichia coli cells, using the instructions provided (Invitrogen, Carlsbad, CA). Transformants were plated onto Sigma S-gal/LB agar and individual colonies containing vectors with inserts were chosen based on black/white selection and used to inoculate 1 mL 2xLB with kanamycin in deep well plates. The plates were incubated between 16 and 18 hours at 37°C. The plasmid DNA was purified as per manufacturer’s protocol (Montage Plasmid MiniprepHTS Kit, Millipore). The average concentration of the plasmid DNA was between 100 – 300 ng/µL as determined using a NanoDrop, ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).
The purified plasmid DNA from one clone library of each of the four soil layers was sent to Idaho State University Molecular Research Core Facility (ISU MRCF) for sequencing. The purified plasmid DNA from the other two clone libraries of each of the four soil layers was sequenced at INL. At both locations, Sanger cycle sequencing reactions with dye-terminators were prepared using between 100 and 200 ng template DNA, 1 µL BigDye v3.1 (Applied Biosystems, Carlsbad, CA), and one of three primers: M13F (5'-GTAAAACGACGGCCAG-3’), 515F (5'-GTGCCAGCMGCGCGGTAA-3’), or M13R (5'-CAGGAAACAGCTATGAC-3’) in a reaction volume of 10 µL (primer concentrations were 3.2 pmol/µL at ISU and 5 pmol/µL at INL). Reactions were denatured at 96°C for 1 minute, followed by 40 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. At the ISU MRCF, excess reagents and dye were removed using Millipore™-seq plates (Millipore, Billerica, MA) and DNA was analyzed on an Applied Biosystems 3130 Analyzer (Applied Biosystems, Carlsbad, CA). At INL, excess reagents and dye were removed using Performa DTRPlates (Edge Bio, Gaithersburg, MD) and DNA was analyzed on a 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA).

**Sequence Analysis**

Individual clones were sequenced using the forward, internal, and reverse primers, M13F, 515F, and M13R, respectively. Vector sequences were removed before assembly. Contiguous sequences were assembled using Phrap (Ewing and Green, 1998; Ewing et al., 1998) to make full-length 16S rRNA gene sequences. Clones were trimmed to remove poor quality regions using Phred (Gordon et al., 1998) (Q<20), NAST-aligned
(DeSantis et al., 2006a), and checked for chimeras with Bellerophon (Huber et al., 2004) all through the use of tools provided by Greengenes (DeSantis et al., 2006b) (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi). Non-chimeric sequences were compared to public databases in Greengenes and classified using the G2_Chip taxonomy classification system.

16S rRNA Gene Microarray Analysis

Amplification of the 16S rRNA gene from one of the DNA extractions obtained from each of the four soil layers was performed using 2 µg per reaction. Hybridization and subsequent analysis on a 16S rRNA gene-based microarray (PhyloChip) was carried out as previously described (DeSantis et al., 2007). Duplicate microarrays were analyzed for each soil layer sampled. A probe pair was scored as positive if (Abdelouas et al., 2000) the fluorescence intensity of the perfect match probe was at least 1.3 times greater than the intensity of the mismatch probe and (Anonymous, 1994) the difference between the perfect match and mismatch intensities were 130 times greater than the square of the background intensity. An OTU was identified as present if at least 92% of the probe pairs for a specific OTU were scored as positive (pf ≥ 0.92). An OTU was scored as positive for a soil layer if the OTU met these criteria for both replicate microarrays of each layer. ARB (Ludwig et al., 2004) version 08.07.08prv and the SILVA 04.10.08 reference database were used for the production of neighbor joining phylogenetic trees and MeV (Saeed et al., 2003) for the production of heat maps.
Statistical Analyses

Statistical differences between duplicate PhyloChips and triplicate clone libraries for each layer were evaluated by Unifrac (Lozupone and Knight, 2005). Unweighted Principal Coordinates Analysis (PCoA) and lineage specific analysis were performed using Unifrac software for both the clone library and PhyloChip NAST-aligned sequences. Before PCoA analysis, clone libraries were analyzed using DOTUR (Schloss and Handelsman, 2005) (www.plantpath.wisc.edu/fac/joh/dotur.html) in which a 97% cutoff was used to group sequences into OTUs. A single representative sequence from each OTU was included in analysis to eliminate phylogenetic weighting. Shannon’s and Simpson’s diversity indices as well as rarefaction curves (Appendix E) for both the clone library and PhyloChip data sets were also calculated using DOTUR.

Quantitative PCR

Family-specific primers for the Acidimicrobiaceae, Flexibacteriaceae, Streptomycetaceae and KSA Unclassified families were designed using the PROBE DESIGN and MATCH PROBE applications in ARB (Ludwig et al., 2004) version 08.07.08prv. Primers were designed and tested using an ARB neighbor joining phylogenetic tree with all sequences detected by both PhyloChip and clone library analyses. Each family-specific primer was paired with a general bacterial primer (Appendix E). All primer pairs were determined to be highly specific to the target family (data not shown). Triplicate DNA extracts of each soil layer were diluted to the concentration used for amplification in clone library analysis. Equal volumes of each of the diluted DNA extracts were pooled for each soil layer. A two-step amplification using
5 nanograms of template DNA from each soil layer was carried out using the Rotor-Gene™ SYBR® Green PCR Kit (QIAGEN, Inc., Valencia, CA). An initial activation step of 95°C for 5 minutes, 35 cycles of a denaturation at 95°C for 5 seconds and a combined annealing/extension step at 60°C for 10 seconds was performed when using the Acidimicrobiaceae, Flexibacteriaceae and KSA Unclassified specific primers. Analysis with the Streptomycetaceae specific primers had an increased combined annealing/extension temperature of 65°C. Triplicate samples were analyzed for each soil layer using each set of family-specific primers. Results are reported as 16S rRNA gene copy number per nanogram total DNA extracted.

Nucleotide Sequence Accession Numbers

All nucleotide sequences from clone library analyses were deposited in GenBank under accession numbers GQ262819 to GQ264537.

Results

Clone Library and PhyloChip Analyses

A total of 448, 431, 382, and 458 clones were obtained from the F, FW, WW, and WC layers, respectively, after sequences were trimmed, aligned and screened for chimeras. The complete clone library of the simulated LLW site contained 1719 clones. Analysis of sequences followed the “standard operating procedure for phylogenetic inference” (Peplies et al., 2008) regarding sequence alignment and phylogenetic tree building where applicable. The triplicate clone library results for each layer were evaluated using Unifrac and were determined not to be significantly different (p ≥ 0.2).
Therefore, the triplicate libraries for each layer were combined and considered as one complete library for this study.

Duplicate PhyloChip analyses performed for each layer were also evaluated using Unifrac, determined not to be significantly different (p ≥ 0.2), combined, and also reported as one data set for each layer. A total of 717, 1356, 1567, and 1582 unique OTUs were scored as positive in the F, FW, WW and WC layers, respectively.

**Bacterial Community Structure**

Both the clone library and PhyloChip results indicated that the bacterial community profile changed with depth when viewed at the phylum level. Clone library analysis revealed that *Proteobacteria* were dominant in all four layers accounting for 29, 28, 35, and 56% of the F, FW, WW, and WC layer total clones, respectively (Figure 3.2A). Twelve phyla were detected in the F layer by clone library analysis, with the *Proteobacteria, Actinobacteria* and *Gemmata monadetes* phyla comprising the majority of the total clones detected. These three phyla represented 332 of the 448 F layer clones or 74%. The FW layer contained clones from 10 different phyla, the least of any of the layers. The FW layer was comprised mostly of clones within the *Proteobacteria, Actinobacteria, and Bacteroidetes*. The *Actinobacteria* and *Bacteroidetes* combined represented 60% of the total FW layer clones. This was a significant increase in *Bacteroidetes* clones from the F layer as they were 34% of the total FW layer clones and only 1% of the total F layer clones. The WW layer contained clones from 13 different phyla, the most of any layer, and the WC layers contained clones from 12 different phyla. Additionally, both layers were comprised mainly of *Proteobacteria, Bacteroidetes*, and
Acidobacteria. These three phyla represented 286 clones, 74% of the total WW layer clones, and 379 clones, 83% of the total clones in the WC layer.

The PhyloChip data also indicated a change in community profile with depth and showed greater numbers of unique OTUs with increasing depth (Figure 3.2B). Though the number of unique OTUs changed with depth, four phyla were consistently dominant,

![Figure 3.2 The bacterial community viewed at the phylum level with depth at the CTPS. (A) Percent abundance of each phylum as determined by clone library analysis with the total number of clones for that layer listed at the top of each bar. (B) Number of unique OTUs identified within each phylum based on clone library (CL) and PhyloChip (PC) analyses. F, Fill; FW, Fill Waste interface; WW, Wood Waste; WC, Waste Clay interface.](image)
and in similar ratios to each other, in all soil layers. The *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* accounted for approximately 77, 84, 82, and 81% of the total OTUs detected by PhyloChip analysis in the F, FW, WW, and WC layers, respectively. In each layer, the *Proteobacteria*, *Firmicutes*, *Actinobacteria*, and *Bacteroidetes* comprised approximately 50%, 15%, 11%, and 6%, respectively, of the total OTUs detected by the PhyloChip in each soil layer.

A comparison at the OTU level between methods indicates that the PhyloChip detected significantly more OTUs than the clone libraries in all soil layers. Clone library analyses detected 191, 173, 217, and 252 unique OTUs in the F, FW, WW, and WC layers, respectively compared to the PhyloChip analyses which as previously mentioned detected 717, 1356, 1567, and 1582 unique OTUs in the same layers. A total of 2002 unique OTUs were detected by the entire study. Of these, only 10% were detected by both the clone library and PhyloChip (Appendix E). Another 10% were detected by the clone library only while the remaining 80% were detected by the PhyloChip only.

**Bacterial Community Diversity**

Shannon’s and Simpson’s indices both indicated greater diversity in all four soil layers by PhyloChip analysis than by clone library analysis (Table 3.1). The Simpson’s indices calculated for both methods demonstrated a similar trend in which overall the F and FW layers had the least diversity, while the WW and WC layers had the greatest diversity.

Shannon’s indices calculated using the clone library data indicated there was no significant difference in diversity between soil layers. Conversely, Shannon’s indices
calculated with the PhyloChip data suggested there were significant differences in
diversity between layers. Shannon’s indices based on PhyloChip data determined that the
FW layer had the least diversity, followed by the F layer, while the WW and WC had the
greatest diversity.

Soil Layer Stratification

PCoA was performed with both the clone library and PhyloChip community data
sets and the results suggest that there were significant differences between the bacterial
communities with depth (Figure 3.3). The clone library data (Figure 3.3A) and
PhyloChip data (Figure 3.3B) were first analyzed separately and yielded similar results.
Triplicate clone libraries and duplicate PhyloChips for each soil layer clustered with
themselves, again confirming the similarities between the replicates. When comparing
soil layers, the WW and WC layers grouped closely together, while the F and FW layers
clustered independently from the other layers. Not surprisingly, when the clone library
and PhyloChip data sets were combined and analyzed, the method used to identify the
community appeared to influence the clustering of the data more heavily than the soil

<table>
<thead>
<tr>
<th>Layer</th>
<th>Shannon’s Index</th>
<th>Simpson’s Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CL (95% CI)</td>
<td>PC (95% CI)</td>
</tr>
<tr>
<td>F</td>
<td>5.56 (±0.090)</td>
<td>6.21 (±0.062)</td>
</tr>
<tr>
<td>FW</td>
<td>5.61 (±0.093)</td>
<td>5.86 (±0.074)</td>
</tr>
<tr>
<td>WW</td>
<td>5.67 (±0.071)</td>
<td>7.10 (±0.040)</td>
</tr>
<tr>
<td>WC</td>
<td>5.72 (±0.084)</td>
<td>7.03 (±0.041)</td>
</tr>
</tbody>
</table>

CL, Clone Library; PC, PhyloChip; CI, Confidence Interval; F, Fill; FW, Fill Waste interface;
layer, since the PhyloChip data sets clustered together and independently of any of the clone library data (Figure 3.3C). The clone library data still demonstrated the same trend seen in Figure 3.3A: the F and FW layers each clustered by themselves, while the WW and WC layers clustered together.

Lineage specific analysis of the clone libraries was performed with Unifrac to determine which phyla were responsible for the differences between layers observed in the PCoA analysis. Multiple branch nodes were evaluated and it was determined that groups within the *Actinobacteria* and *Bacteroidetes* phyla were responsible for the majority of significant differences between layers (p value < 0.05). Unifrac could not support lineage specific analysis with the PhyloChip data, due to the large number of sequences. Because *Actinobacteria* and *Bacteroidetes* phyla are known to contain

![Figure 3.3 Principal Coordinates Analysis (PCoA) of the (A) combined clone libraries, (B) combined PhyloChip data, and (C) combined clone library and PhyloChip data. A 97% identity cutoff was used to remove replicate sequences from the clone libraries before analysis. F, Fill; FW, Fill Waste interface; WW, Wood Waste; WC, Waste Clay interface.](image)
cellulose degrading microorganisms (Lynd et al., 2002) and were identified as groups accounting for much of the change in bacterial community structure with depth, they were evaluated further to identify how they changed with depth. While the Proteobacteria also accounted for some of the changes identified by lineage specific analysis, the majority of these Proteobacteria clones were identified and categorized by Unifrac as only “suggestive” (p value 0.05-0.1) and thus less statistically significant.

**Actinobacteria and Bacteroidetes Phyla**

There were 123, 113, 10 and 27 clones identified as belonging to the Actinobacteria in the F, FW, WW, and WC layers, respectively, corresponding to 28, 26, 3, and 6% of the total clones detected in each layer. Results indicate a difference in the Actinobacteria community structure with depth when viewed at the family level. In particular, four families showed significant changes with depth based on clone abundance: Acidimicrobiaceae, Glycomycetaceae, Micromonosporaceae and Streptomycetaceae (Figure 3.4, Appendix E). Of these four families, two were chosen for additional quantitative analysis using 16S rRNA gene family-specific primers.

Lineage specific analysis identified Acidimicrobiaceae as responsible for some of the differences seen with the F layer when compared to the other three layers. The Acidimicrobiaceae family contributed 33% of the total Actinobacteria clones and 8.9% of the total clones detected in the F layer. An approximate 10-fold decrease in the percentage of Acidimicrobiaceae clones was observed between the F and FW layers (Figure 3.4A). No Acidimicrobiaceae clones were detected in the WW layer and only 3 were detected in the WC layer, accounting for less than 1% of the total clones detected.
The PhyloChip, however, detected the presence of *Acidimicrobiaceae* OTUs in all four soil layers suggesting they are present throughout. The quantitative PCR data confirm the trends observed based on clone library analysis and also supports the Phylochip results as it detected the presence of *Acidimicrobiaceae* in the WW layer where no clones were identified. The *Streptomycetaceae* family had an approximately 40-fold increase in clone abundance between the F and FW layers (Figure 3.4B). This increase was followed by significant decreases between the FW and WW layer. The quantitative PCR analysis also identified a significant increase between the F and FW layers in which approximately a 100-fold increase was observed in *Streptomycetaceae* 16S rRNA gene copy number per nanogram total DNA. This was also followed by a significant decrease between the FW and WW layer. However, between the WW and WC layers a decrease in *Streptomycetaceae* 16S rRNA gene copy number per nanogram total DNA was observed while the clone libraries detected no clones in the WW layer and only one clone in the WC layer. The PhyloChip detected a large increase in the number of unique OTUs between the F layer and all other layers.

In the *Bacteroidetes* phylum, 5, 146, 93, and 69 clones were detected in the F, FW, WW, and WC layers, respectively, contributing approximately 1, 34, 24, and 15% of the total clones detected in these layers. This significant increase in the number of *Bacteroidetes* clones between the F layer and the other three layers partially explains how this phylum contributes to the observed stratification between layers. Four families in particular showed significant changes in clone abundance with depth and were identified by lineage specific analysis as contributing to the stratification between layers:
Figure 3.4 (A) *Acidimicrobiaceae* and (B) *Streptomycetaceae* families within the *Actinobacteria* phylum and (C) *Flexibacteraceae* and (D) KSA Unclassified families within the *Bacteroidetes* phylum that had significant changes with depth as viewed by PhyloChip and clone library analyses. PhyloChip results are presented as a presence (black) absence (gray) heatmap for each OTU detected within the family. Each row, marked (*), represents a unique OTU. An OTU was determined present in a soil layer if the pf value was above or equal to 0.92 for both PhyloChips. Clone abundance of each family is reported as the percent of the total clones detected per soil layer. Quantitative PCR was performed using family-specific primers for amplification of the 16S rRNA gene. F, Fill; FW, Fill Waste interface; WW, Wood Waste; WC, Waste Clay interface.

*Crenotrichaceae*, *Flexibacteriaceae*, *Sphingobacteriaceae*, and KSA Unclassified clones (Figure 3.4, Appendix E). Of these four families, two were chosen for additional quantitative analysis using 16S rRNA gene family-specific primers.
No *Flexibacteriaceae* clones were detected in the F or FW layers, though the PhyloChip and quantitative PCR detected their presence in both layers. *Flexibacteriaceae* clones accounted for 5.5% and 5.0% of the WW and WC layer total clones, respectively (Figure 3.4C). Quantitative PCR analysis detected a decrease in *Flexibacteriaceae* 16S rRNA gene copy number per nanogram total DNA between the WW and WC layers, but a greater decrease than was observed by clone library analysis. The PhyloChip detected a greater number of unique OTUs within the WW and WC layers when compared to the other two layers.

KSA Unclassified clones detected in the F layer based on clone library analysis, accounted for only 0.9% of the total clones (Figure 3.4D). An approximate 7-fold increase in clone abundance was observed between the F and FW layers followed by a significant decrease in the WW and WC layers. Interestingly, the PhyloChip only detected one unique OTU that was present in all four soil layers. The quantitative data supports the trend observed by clone library analysis in which there was an increase in KSA Unclassified 16S rRNA gene copy number per nanogram total DNA in the FW layer followed by a significant decrease in the WW and WC layers. It also detected this family in all four soil layers which supports the PhyloChip results.

**Potential for Cellulose Degradation**

To gain a better understanding of the potential role of the *Actinobacteria* phylum in response to the presence of cellulose, families were evaluated based on whether or not they had at least one significant change between two soil layers. A significant change was defined as at least a 4-fold increase or decrease in clone numbers, which coincides
with approximately a 1% change in total clone abundance, between any two layers.

Thirteen families out of 33 detected met this criterion: *Acidimicrobiaceae*, *Microthrixineae*, *Frankiaceae*, *Glycomycetaceae*, *Kineosporaceae*, *Microbacteriaceae*, *Micromonosporaceae*, *Streptomycetaceae*, *Thermomonosporaceae*, *Rubrobacteraceae*, and three unclassified families. These families were then differentiated based on their potential capabilities to degrade cellulose. Those that had been reported in the literature to be known cellulose degraders, cellobiose utilizers, or suggested to be cellulose degraders were grouped together as reported and implied cellulose degraders (Labeda et al., 1985; Safo-Sampah and Torrey, 1988; Coughlan and Mayer, 1992; Lynd et al., 2002; Labeda and Kroppenstedt, 2004; Bayer et al., 2006; Evtushenko and Takeuchi, 2006; Kämpfer, 2006; Kroppenstedt and Goodfellow, 2006; Vobis, 2006; Bentley et al., 2008). Those families that have never been shown to degrade cellulose, utilize cellobiose nor suggested to be able to do so were also grouped together as non-cellulose degraders. These groups were then compared in terms of their abundance and relative diversity with depth.

The clone abundance of the non-cellulose degrading group was highest in the F layer, accounting for 18.3% of the total clones detected in this layer, and decreased approximately 5-fold between the F and FW layer (Figure 3.5A). There were only 3 clones from this group in the WW layer and 7 clones in the WC layer accounting for less than 2% of the total clones in both layers. Conversely, the number of clones of the reported and implied cellulose degrading group was highest in the FW layer increasing 6-fold in abundance between the F and FW layer. This group accounted for 17.9% of the
total clones detected in the FW layer, decreasing in abundance in the deeper layers accounting for 1.6% of the total clones in the WW layer and 3.5% of the total clones in the WC layer. The greatest relative diversity, identified by clone library analysis, also correlated with the soil layer in which the greatest clone abundance was detected (Figure 3.5B). This was the F layer for the non-cellulose degrading group and the FW layer for the reported and implied cellulose degrading group. The PhyloChip also detected the greatest number of unique OTUs in the F layer for the non-cellulose degrading group, and in the FW layer for the reported and implied cellulose degrading group (Figure 3.5C). However, the change in the number of unique OTUs detected by PhyloChip analysis and relative abundance between all four layers was not as great as indicated by the clone

![Figure 3.5](image-url)

**Figure 3.5** Focus group comparisons of *Actinobacteria* phylum. Families with a significant decrease in clone number between at least two layers (e.g. the significant change between F and FW layers) were categorized as either reported and implied cellulose degraders (families that are previously known to be cellulose degraders, cellobiose utilizers, or have been suggested to be potential cellulose degraders) or non-cellulose degraders (families that have not been shown in the literature to degrade cellulose, cellobiose nor has it been suggested that they can). These two groups were then compared based on (A) clone abundance and the number of OTUs detected by (B) Clone Library and (C) PhyloChip analyses. F, Fill; FW, Fill Waste interface; WW, Wood Waste; WC, Waste Clay interface.
libraries suggesting clone libraries may be more sensitive to significant changes in populations than the PhyloChip. Interestingly, the PhyloChip detected a greater number of unique OTUs within the reported and implied cellulose degrading group than the non-cellulose degrading group in all four layers. This may be due to an underestimate of the reported and implied cellulose degrading group’s presence and diversity by the clone libraries, or may be due to a larger number of probes for this group found on the PhyloChip therefore increasing its chance of detection.

Unlike the Actinobacteria, all of the families that showed significant differences between layers contain known cellulose degraders (Hreggvidsson et al., 1996; Johansen et al., 1999; Lynd et al., 2002; Holmes, 2006; Khan et al., 2007a; Khan et al., 2007b), except for the KSA Unclassified family of which no metabolic capabilities could be found in the literature. Regardless, the large number of reported and implied cellulose degrading Bacteroidetes families detected by clone abundance and PhyloChip analysis in the FW, WW, and WC layers suggests that there is potential for cellulose degradation in these layers.

Discussion

Clone Library and PhyloChip Comparison

Both the clone library and PhyloChip analyses yielded valuable information about the bacterial community structure and diversity at the CTPS. While 1719 clones is a substantial clone library data set, the results of the PhyloChip analyses demonstrate that even with a large number of clones, the results barely depict the total diversity that was found at the CTPS as almost 80% of the total OTUs observed were detected by the
PhyloChip only. The PhyloChip’s sensitivity to low abundance OTUs is useful in identifying rare members of the community that may play a key role in the environment but are not present in high numbers. Still, the clone libraries detected 203 OTUs that the PhyloChip did not detect, and also provide insight into the potential abundance and dominance of these organisms at the CTPS making it a valuable method to use as well.

Similar to previous studies in which both PhyloChips and clone libraries were used, the PhyloChip detected greater overall diversity and number of unique OTUs (Brodie et al., 2006; DeSantis et al., 2007; Flanagan et al., 2007; Sagaram et al., 2009; Sunagawa et al., 2009). As previously mentioned, there were OTUs and even entire families detected through clone library analysis that were not detected by the PhyloChip. This may be due to poor hybridization with the probe, a sequence having a stronger affinity to the mismatch probe, or the absence of these sequences in the database when the probes were designed. It is also important to point out that when comparing the presence or absence of a specific OTU between the four soil layers, there was a low percentage of matches between the two methods. While it was not surprising that a unique OTU was detected only by the PhyloChip in a soil layer, it was surprising to observe the number of unique OTUs detected in some layers by the clone libraries only and in other layers by the PhyloChip only. This further supports the value of using these two methods to complement each other to gain more information about the bacterial community and may be especially important in studies where one specific OTU or organism is focused on.
In addition to the molecular analyses discussed in this study, six bacterial isolates (members of the genera *Pseudomonas, Pedobacter, Streptomyces, Flavobacterium, Serratia, Cellulomonas*) were obtained from cellulose degrading enrichments inoculated with the soil from the FW, WW, and WC layers (Appendix E). The results of these cultivation studies can be compared to both the clone library and PhyloChip results to further demonstrate the differences between these two methods. All six isolates were detected at the family level by PhyloChip analysis in all three soil layers (Appendix E). As the PhyloChip detects a great amount of diversity and large number of community members, it is not surprising that it would detect all six families in all soil layers. Meanwhile, clone library analyses detected some of these families, such as *Enterobacteriaceae* containing the *Serratia* sp. isolate and *Sphingobacteriaceae* containing the *Pedobacter* sp. isolate, in layers from which they were not isolated. This suggests that either these organisms were present and we were unable to culture them, or a different member of the family was present. While it is not surprising that there are soil layers in which these organisms are present but we were unable to culture them, it is interesting that a few of the isolates were cultivated from soil layers in which clone library analyses did not detect the presence of their families. For example, in the WW layer the clone libraries did not detect any members of the family *Streptomycetaceae*. However, a *Streptomyces* sp. was isolated from the WW soil layer and the PhyloChip confirms the family’s presence. If only clone library analysis had been conducted, the results would suggest that there were no members of this family present in this layer.
These results further demonstrate limits of clone library analysis and its potential to miss much of the diversity present at the site.

The results of this study show that the PhyloChip detects greater diversity which provides a more complete picture of the community structure and is important in identifying rare members of the community that may play an important functional role at the site. However, it is limited by the fact that in its current state it is not a quantitative method. Therefore, it cannot be used to determine which members of the community are more abundant and will not detect changes in abundance between soil layers. Also, the PhyloChip does not appear to be as sensitive to small changes within the community as seen with the *Actinobacteria* and *Bacteroidetes* phyla.

The clone libraries are semi-quantitative and begin to address which members of the community are abundant. Quantitative analysis performed for select families within the *Actinobacteria* and *Bacteroidetes* phyla support the data obtained by clone library analysis suggesting that such a large clone library dataset provides better confidence in the quantitative aspect of the clone library results. Still, as some differences were seen in the results of the quantitative PCR and clone library analyses, there are biases in the construction and analysis of clone libraries that limit its ability to be truly quantitative. On the other hand, they are more sensitive to changes within the community structure than the PhyloChip which is an additional advantage to using clone library analyses.

**LLW Site Microbial Communities**

A total of 2002 unique OTUs were detected by both methods combined in all four soil layers and the dominant phyla observed (*Proteobacteria, Actinobacteria,*
Bacteroidetes, Acidobacteria, Firmicutes) were similar to those in other soil studies (Janssen, 2006; Schloss and Handelsman, 2006; Elshahed et al., 2008). Additionally, at least one of the methods used in this study detected the class, and in most cases, the family containing multiple genera identified in previous studies (including Bacillus, Pseudomonas, Citrobacter, Clostridia, Azospira, Quadricoccus, Brevundimonas and Trichococcus) focusing on LLW sites where culture techniques and small clone libraries were used to characterize the bacterial community thus confirming their results (Francis et al., 1980; Fox et al., 2006). The molecular techniques used in this study identified significantly more members of the bacterial community than previous studies. For example, Fox et al. (2006) identified 8 distinct RFLP sequences from 29 clones in their low-level waste microbial community batch studies and even in the enrichments established in parallel to this study only six isolates were cultured while 2002 unique OTUs were identified. While it is known that culture-based techniques only focus on a small fraction of the microbial community, the findings of this study put into perspective how small a fraction that may be.

Influence of Cellulose
on the Bacterial Community

Significant changes in the community structure and dominant phyla were observed with depth at the CTPS by both clone library and PhyloChip analyses suggesting the presence of cellulosic waste significantly influences the bacterial community at this site. PCoA analysis also supports this hypothesis as it showed a stratification of the bacterial community occurring within the CTPS between the F, FW, and WW layers. The similarities observed between the WW and WC layer bacterial
communities suggest that this part of the CTPS is not as stratified as in the shallower depths. This may be due to the presence of the clay lining in the bottom that allows for the retention of water at this depth decreasing stratification between the two soil layers.

The F layer had a low diversity overall, suggesting a more oligotrophic soil environment, most likely containing few carbon and energy sources likely supplied through downward transport during precipitation and snowmelt events. Additionally, the decrease in the number of phyla detected and low calculated diversity at the FW layer, suggests there may be a selective influence on the community at this depth where those bacteria with a certain metabolic advantage are dominant. The abundance of the _Actinobacteria_ and _Bacteroidetes_ in this layer as well as specific families within these phyla that contain known or potential cellulose degraders, suggests that cellulose may be the selective influence at this depth and cellulose degrading microorganisms may have a metabolic advantage.

The WW layer of the CTPS contains large quantities of cellulosic materials. Therefore, it was hypothesized that this layer would most likely enrich for cellulose degraders. In this layer, both the clone library and PhyloChip results indicate the presence of families containing known cellulose degraders, suggesting cellulose degradation may be occurring at this depth. However, increased diversity was also observed in this layer suggesting that cellulose is likely broken down and utilized by either cellulose degrading organisms themselves or by other bacteria that rely on these breakdown products for growth. These products, readily utilized by a wide variety of microorganisms, would support a greater diversity of microorganisms in this layer.
When compared to the WW and WC layers, the decreased diversity observed in the FW layer may be due to selective pressures on microorganisms in this layer, such as a lack of trace nutrients that may have been buried with the simulated waste, lack of retained water or retained breakdown products, which lead to the observed decrease in diversity in the FW layer. It is also important to note that while fungi were not studied here, we recognize that they may be catalyzing cellulose degradation at this site, and therefore may be influencing the activity and diversity of the bacterial community between the different soil layers.

While the presence of these microorganisms cannot be linked to metabolic function directly and there may be other environmental variables besides cellulose influencing the bacterial community structure, the results demonstrate the possibility of cellulose playing a role in the changes in community structure with depth.

We hypothesized that the Firmicutes would be dominant at this site since this phylum contains many known cellulose degraders (Lynd et al., 2002; Desvaux, 2005), are often dominant in soil environments (Janssen, 2006), and are spore-formers, which is likely advantageous when fluxes of water and nutrients into the system are minimal. The PhyloChip detected a large number of Firmicutes OTUs in all four layers demonstrating a large relative diversity of this phylum present; however, the clone libraries detected only 24 Firmicutes clones total in all four soil layers and overall the number decreased with depth. It is possible that members of this phylum are either not very abundant at this site, or the extraction and cloning method was not optimal for these organisms.
While all four layers were dominated by *Proteobacteria*, this was not surprising since the *Proteobacteria* is a large, well studied phylum containing many known members. Some members of the *Proteobacteria* such as *Pseudomonas spp.* can carry out aerobic cellulose degradation (Lynd et al., 2002) and while they may play a role in cellulose degradation at this site as they were detected by both methods, they did not change significantly with depth. Members of this phylum, as well as other phyla that did not change significantly with depth, may play important roles in other processes occurring in the soil such as metal cycling or the cycling of other nutrients. This may have significance in future studies which will focus on the interactions between the bacterial community and heavy metals and radionuclides found at this site.

**Significance and Future Studies**

The results of this study provide insight on how the presence of cellulosic waste influences the bacterial community. This is the most in-depth study to date of the bacterial community found at a LLW site. To the authors’ knowledge, this is also the most in-depth study to date using both clone libraries and PhyloChip analyses to identify the bacterial community found in any one soil environment due to the large clone library size, numerous PhyloChips analyzed and evaluation of the site at multiple depths. Multi-depth sampling, such as that performed in this study, can identify potentially important changes in the microbial community that may otherwise be overlooked. This will lead to the ability to better define and identify the potential roles different microorganisms have in metal mobility at these LLW sites and better design remediation processes that may be needed at these sites in the future.
Specifically, the results presented here will provide an extensive baseline for future studies investigating how bacterial community structure and function changes as a function of cellulose utilization. Column studies are being used to potentially identify which groups of organisms may be playing a key role in heavy metal and radionuclide mobility in simulated LLW environments. In these studies the bacterial community at both the DNA and RNA level will be evaluated and geochemical parameters will be monitored. These analyses will aid in linking the bacterial community structure with the community function. The results presented here are the first step in better understanding the interactions between the bacterial community, cellulosic waste, and contaminants at LLW sites.

Acknowledgements

The authors would like to thank Yvette Piceno, Todd DeSantis, Gary Andersen and Eoin Brodie at Lawrence Berkeley National Laboratory for their indispensible training in running G2 PhyloChips and help in acquiring and analyzing the PhyloChip data. Thanks to the Idaho State University Molecular Research Core Facility for sequencing and to those at Idaho National Laboratory who provided technical assistance in completing the clone libraries including Michelle Walton, Frank Roberto, Cody Permann, and John Aston. We would also like to extend our thanks to Steve Lopez for sampling access to the Cold Test Pit South as well as to Bill Smith and Joe Lord for operation of the PowerProbe sampling unit. Additional thanks to Peg Dirckx and Chantel Naylor for their graphical support.
The Montana State University portion of this work was supported by the U.S. Department of Energy, Office of Science, Environmental Remediation Science Program (ERSP), contract DE-FG02-06ER64206. The INL portion of the work was supported by the U.S. Department of Energy, Assistant Secretary for the Office of Science, ERSP, under DOE-NE Idaho Operations Office contract number DE-AC07-05ID14517.
References


Kashefi, K., Lovley, D., 2000. Reduction of Fe(III), Mn(IV), and toxic metals at 100°C by Pyrobaculum islandicum. Applied and Environmental Microbiology 66:1050-1056.


Khan, S., Nakagawa, Y., Harayama, S., 2007b. Sediminibacter furfurosus gen. nov., sp. nov. and Gilvibacter sediminis gen. nov., sp. nov., novel members of the family


Sani, R., Peyton, B., Smith, W., Apel, W., Petersen, J., 2002. Dissimilatory reduction of Cr(VI), Fe(III), and U(VI) by *Cellulomonas* isolates. Applied Microbiology and Biotechnology 60:192-199.


CHAPTER 4

THE INFLUENCE OF CARBON SOURCE ON HEXAVALENT CHROMIUM TOXICITY AND REDUCTION BY AN ENVIRONMENTAL ARTHROBACTER SP. ISOLATE

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Journal: Environmental Toxicology and Chemistry

Status of manuscript:
_x_ Prepared for submission to a peer-reviewed journal
___ Officially submitted to a peer-reviewed journal
___ Accepted by a peer-reviewed journal
___ Published in a peer-reviewed journal
Abstract

Hexavalent chromium is a common contaminant at numerous Department of Energy sites including low-level waste sites. Low-level waste sites typically contain cellulosic waste co-disposed with heavy metals and radionuclides. While there are many processes that could influence the fate of these contaminants in the subsurface, it is possible that as cellulose is degraded, its breakdown products can be utilized by metal-reducing microorganisms as carbon and energy sources. The purpose of this study was to determine the influence of potential cellulose degradation products as carbon and energy sources on the chromate toxicity and reduction capabilities of the environmental Arthrobacter sp. isolate EF01. Results indicate that the carbon source present influenced Cr(VI) toxicity and reduction by isolate EF01. When glucose was the sole carbon source in the presence of 50 µM Cr(VI), isolate EF01 had a decreased growth rate and maximum OD, but no decreased lag time. No significant difference in lag time, growth rate or maximum OD was observed in the presence and absence of 50 µM Cr(VI) when lactate was the sole carbon source. When either ethanol or butyrate was the sole carbon source available in the presence of 50 µM Cr(VI), isolate EF01 was unable to grow, but cells were still viable. Isolate EF01 was only capable of reducing Cr(VI) when either glucose or lactate were present while no significant decrease in Cr(VI) concentration was observed when either ethanol or butyrate were present. The addition of sulfate, a structural analog to chromate, significantly mitigated toxicity, but not completely. Overall, these results suggest that carbon metabolism by Arthrobacter sp. isolate EF01 plays a role in Cr(VI) toxicity and reduction. As cellulose degradation products may vary
in situ, so may the role that *Arthrobacter* sp. isolate EF01 plays in the fate of Cr(VI) in low-level waste environments.

**Introduction**

The Department of Energy (DOE) is responsible for a number of low-level waste (LLW) sites such as at the Idaho National Laboratory where cellulosic waste is contaminated with heavy metals and radionuclides (Anonymous, 1994; Saunders and Toran, 1995; Anonymous, 2000). The fate of metals such as chromium in these sites is largely unknown. This cellulosic waste, often in form of items such as cardboard, mop heads, and paper towels, can be degraded by microorganisms (Leschine, 1995; Chew et al., 2001; Lynd et al., 2002). This degradation could lead to the physical release of these contaminants potentially increasing their mobility. Additionally, cellulose degradation products such as organic acids have been shown to chelate metals and radionuclides which would also increase their mobility (Means et al., 1978; Humphreys et al., 1997; Read et al., 1998; Francis et al., 1999; Choy et al., 2006). On the other hand, degradation products including sugars and organic acids may also serve as carbon and energy sources for metal-reducing microorganisms (Abdelouas et al., 2000; Kashefi and Lovley, 2000; Chew et al., 2001; Liu et al., 2002; Sani et al., 2002; Viamajala et al., 2002; Fox et al., 2006; Viamajala et al., 2007). Specifically, the *in situ* reduction of the highly toxic and water soluble hexavalent chromium (Cr(VI)) to its trivalent form (Cr(III)) which is much less toxic and less water soluble would be advantageous (Yassi and Nieboer, 1988; Barceloux, 1999). This would be extremely beneficial in LLW environments as it would lead to decreased mobility of this contaminant and its potential to enter the groundwater.
Numerous microorganisms have been shown to have high Cr(VI)-resistance and Cr(VI) reduction capabilities (Turick et al., 1996; Chen and Hao, 1998; Fredrickson et al., 2000; Francisco et al., 2002; Liu et al., 2002; Sani et al., 2002; Viamajala et al., 2002). Typically this occurs through mechanisms such as efflux pumps, chromate transporters, chromate reductases, DNA repair machinery and biosorption (Cervantes et al., 2001; Cheung and Gu, 2007; Ramírez-Díaz et al., 2008). *Arthrobacter* spp. are well known for their high tolerance to many toxic compounds including Cr(VI) (Roane and Pepper, 1999; Benyehuda et al., 2003; Megharaj et al., 2003; Horton et al., 2006; Henne et al., 2009, Moberly et al., 2010) and have been shown to reduce Cr(VI) predominantly on the cell surface as well as mitigate toxicity through the use of efflux pumps (Asatiani et al., 2004, Henne et al., 2009). Subsequently, *Arthrobacter* spp. have been isolated from numerous DOE sites contaminated with Cr(VI), including the Hanford and Savannah River sites, and are often the most common genus isolated from these sites (Benyehuda et al., 2003; Fredrickson et al., 2004).

Previous studies have reported the varying Cr(VI)-resistance capabilities of *Arthrobacter* spp. grown on nutrient rich media with glucose being the most commonly used carbon source (Benyehuda et al., 2003; Megharaj et al., 2003). However, little has been reported about the influence of different carbon sources on both Cr(VI)-resistance and Cr(VI) reduction by these organisms. As it is well known that the carbon and energy source available can influence both the microbial community structure and the community’s ability to reduce Cr(VI) (Schutter and Dick, 2001; Nakatsu et al., 2005; Kourtev et al., 2006; Tekerlekopoulou et al., 2010; Thompson et al., 2010), it is likely
that a change in carbon source would influence a single organisms’ Cr(VI)-resistance and reduction capabilities as well. This may be especially important in environments where various carbon sources are added to stimulate chromate reducers and also in environments where the carbon source available may change such as at a LLW site where multiple cellulose breakdown products may be present.

*Arthrobacter* spp. have been isolated from a number of additional environments that suggest they may be an appropriate model organism for studying the interactions between carbon source and Cr(VI) in LLW environments. In addition to their prevalence in Cr(VI) contaminated DOE sites, an *Arthrobacter* sp. isolate was obtained from the termite hind gut in which mesquite wood from the gut and its fermentation breakdown products were the sole carbon sources available for growth (Thayer, 1976). Through liquid enrichments, an *Arthrobacter* sp. was isolated that was capable of depleting toxic compounds released during acid pretreatment of lignocellulosic biomass which typically inhibits further fermentation of the lignocellulosic material (Lopez et al., 2004). These examples suggest that *Arthrobacter* spp. have the potential to play a metabolic role in a chromate contaminated environment in which cellulose or its degradation products are the dominant carbon sources available such as a LLW site.

The purpose of this study was to determine the influence of potential cellulose degradation products supplied as carbon sources on Cr(VI) toxicity (defined as the influence on microbial growth through altered lag time, growth rate and maximum growth) and Cr(VI) reduction capabilities of an environmental *Arthrobacter* sp. isolate.
Isolation and Identification

*Arthrobacter* sp. isolate EF01 (hereafter referred to as isolate EF01) was isolated from soil obtained from a simulated low-level radioactive waste site at the Cold Test Pit South site at the Idaho National Laboratory (Field et al., 2010). Specifically, soil from the wood waste layer was used in column experiments in which the site conditions were mimicked. The sole carbon source provided was in the form of Whatman No. 1 Filter paper (0.25 g) saturated with 0.723 mg U in the form of UO$_2$Cl$_2$ and 0.41 mg Cr in the form of K$_2$CrO$_4$. Columns were saturated and a defined, carbon-free medium was run upwards at a flow rate of 0.074 mL/min (approximately 1 pore volume every 55 minutes) through the columns (media described in detail below). Isolate EF01 was obtained from the effluent which was spread plated on Tryptic Soy Agar (BD Difco, Franklin Lakes, NJ) plates and incubated aerobically at room temperature (22 ± 2°C). Repeated streaking of colonies was performed until a pure culture was obtained. The 16S rRNA gene was sequenced and isolate EF01 was identified using the BLAST function from the NCBI database (www.ncbi.nlm.nih.gov).

Growth Conditions

Isolate EF01 was grown aerobically from frozen glycerol stocks in Tryptic Soy Broth (BD Difco, Franklin Lakes, NJ) for 20 hours at room temperature (22 ± 2°C) and shaken at 150 rpm for 20 hours. The culture was centrifuged then washed and resuspended with defined, carbon-free medium three times. Isolate EF01 was grown aerobically in a defined, carbon-free medium which simulates the groundwater chemistry.
at the Idaho National Laboratory modified from VanEngelen et al. (2010). Carbon was added separately where indicated. This medium contained the following: 80 mg NaCl, 26 mg K$_2$HPO$_4$, 3.35 g PIPES Buffer, 0.6 mg KCl, 0.4 mg KOH, 12.7 mg Na$_2$SO$_4$, 3.5 mg CaO, and 7.5 mg MgCl$_2$$\cdot$6H$_2$O amended with 5 ml of Wolfe’s vitamin solution (Atlas, 2004), and 1 ml SL-4 trace elements solution (Atlas, 2004). The last resuspended culture was diluted with sterile medium in order to obtain an optical density of approximately 0.2 for the inocula. The inocula OD for all experiments ranged from 0.2 to 0.23. Treatments were all inoculated with 10% of the prepared culture.

**Batch Studies**

**Carbon Source Utilization Study:** All carbon source stocks were made in CSS medium at a concentration of 150 mM carbon. The following carbon sources, all representing potential cellulose degradation products, were used to assess the ability of isolate EF01 to utilize them for growth: Glucose (dextrose), fructose, maltose, mannose, sucrose, xylose, sodium acetate, sodium butyrate, sodium formate, sodium fumarate, sodium propionate, sodium succinate, sodium lactate (60% w/w) and 100% ethanol (EtOH). In sterile, 24-well microtiter plates, duplicate wells were set up for each carbon source in which wells contained a final concentration of 15 mM carbon, CSS medium and isolate EF01. Duplicate cell-free control wells were also set up. Two wells contained CSS medium only. Plates were shaken at 150 rpm at room temperature (22 ± 2°C) and after 72 hours removed to take absorbance measurements at 600 nm and samples were taken from each well for protein analysis.
Cr(VI) Toxicity Batch Tube Studies: Based on the results obtained in the 24-well plate carbon source screening study, four carbon sources were used for more detailed studies. Glucose, ethanol, lactate and butyrate, were used to assess the influence of Cr(VI) on aerobic growth and Cr(VI) reduction capabilities of isolate EF01 when utilizing each of these carbon sources. These studies were carried out aerobically in 25 mL screw cap test tubes with all treatments set up in triplicate. For each carbon source, treatments were set up containing CSS medium, 15 mM carbon and isolate EF01. Additional treatments were set up containing CSS medium, 15 mM carbon, 50 µm Cr(VI) and isolate EF01. Appropriate cell-free and carbon-free treatments were set up as well. Tubes were shaken at 150 rpm at room temperature (22 ± 2°C). Absorbance measurements at 600 nm were taken over time as well as samples for Cr(VI) and protein analyses.

Cell Viability Study: The cell viability study was conducted using glucose as the carbon source and the same treatments as those listed in the Cr(VI) toxicity batch tube studies. However, instead of using 24 mL screw cap test tubes, 250 mL Erlenmeyer flasks were used. Flasks were shaken at 150 rpm at room temperature (22 ± 2°C) and over time samples were taken for Cr(VI) analysis, absorbance at 600 nm, protein analysis and plate counts. Plate counts were conducted by serially diluting the sample in sterile phosphate buffered saline. Dilutions were used for drop plating in which 5 replicates of 10 µL were pipetted onto TSA plates for each dilution. These plates were incubated at room temperature (22 ± 2°C) and colonies were counted and averaged to calculate CFU/mL.
Sulfate Exclusion Study: The sulfate exclusion study was carried out with glucose as the carbon source and the same treatments as those listed in the Cr(VI) toxicity batch studies. In addition, treatments containing 5 mM sodium sulfate were set up. One treatment contained CSS medium, 15 mM carbon, 5 mM sodium sulfate and isolate EF01. Another treatment contained CSS medium, 15 mM carbon, 5 mM sodium sulfate, 50 µm Cr(VI) and isolate EF01. Appropriate cell-free and carbon-free treatments were set up as well. Tubes were shaken at 150 rpm at room temperature (22 ± 2°C). Absorbance measurements at 600 nm were taken over time. Samples were taken over time for Cr(VI) and protein analyses.

Analytical Methods

Cr(VI) Quantification: Cr(VI) concentrations were determined spectrophotometrically using the diphenylcarbazide assay as described by Nyman et al. (2002). 150 µL of the sample was added to 50 µL of 0.8N H₂SO₄ in a 96-well microtiter plate followed by the addition of 20 µL of 0.25% 1,5-diphenylcarbazide (Sigma-Aldrich, St. Louis, MO) in 100% acetone. Assay blanks were prepared by the addition of 20 µL of 100% acetone without 1,5-diphenylcarbazide. Absorbance was measured at 540 nm, adjusted with corresponding assay blanks and compared to standards made from a K₂CrO₄ stock solution (Ricca Chemical Company, Arlington, TX).

Protein Quantification: A colorimetric protein assay, modified from Sani et al. (2001), was used to estimate total protein. Briefly, 200 µL 1N NaOH was added to 200 µL of sample and digested in a hot water bath at 90°C for 10 minutes. After samples
cooled, 28 µL of a 6:10 v/v HCl solution was added. Triplicate technical replicates were then performed in which three 50 µL aliquots per sample were added to a 96-well plate. 150 µL of Coomassie reagent (Pierce, Rockford, IL) was added to each of the wells and allowed to react for 15 minutes after which absorbance readings were taken at 595 nm. Absorbance measurements were compared to those of bovine serum albumin standards which were analyzed using the same procedure to determine protein concentrations. The technical replicate protein concentrations were averaged for each sample to provide final protein concentrations for each sample.

**Total Soluble Cr Quantification:** Inductively coupled plasma mass spectrometry (ICP-MS) was used to quantify total Cr when isolate EF01 was grown on lactate in the presence of 50 µm Cr(VI). Samples were filtered with a 0.2 µm nylon syringe filter and diluted in 5% Trace Metal Grade HNO₃ before being analyzed on an Agilent Technologies 7500ce ICP Mass Spectrometer.

**Cr(VI) Reduction Rates:** First order reduction rates with respect to Cr(VI) concentration were calculated using the following model: \[ c_{\text{Cr(VI)}} = c_{\text{Cr(VI),o}} \times e^{-kt} \] in which \( c_{\text{Cr(VI),o}} \) is the initial Cr(VI) concentration at time zero, \( c_{\text{Cr(VI)}} \) is the Cr(VI) concentration at time \( t \) and \( k \) is the first order rate coefficient.

**Lag Time Calculations:** Lag time calculations were made using a modified Gompertz equation (Giotta et al., 2006). Using optical density data, the following equation can be used to estimate lag time \( \gamma \) in which \( N \) is the optical density when growth is first observed at time \( t \), \( N_0 \) is the optical density at time zero, \( \mu_{\text{max}} \) is the
maximum growth rate, and \( A \) is equal to \( \ln(N_{st}/N_0) \) where \( N_{st} \) is the optical density when the culture reaches steady state.

\[
\ln \frac{N}{N_0} = A \exp\left\{ - \exp\left[ \frac{\mu_{\text{max}} \cdot e}{A} (\gamma - t) + 1 \right] \right\}
\]

**Results and Discussion**

**Carbon Source Utilization Capabilities**

Isolate EF01 utilized 13 of the 14 potential cellulose degradation products for growth after 72 hours (Figure 4.1). The greatest amount of growth, as measured by an increase in protein, was on sucrose. The least amount of growth within this time period appeared to be on xylose and ethanol, while there was no measured growth when formate was the carbon source available. These results are consistent with previous findings of other *Arthrobacter* spp. in which a wide variety of carbon sources could be utilized for growth (Jones and Keddie, 1992). This suggests that this organism may be able to utilize a wide variety of potential cellulose degradation products for growth in situ.

**Influence of Carbon Source on Growth in the Presence of Cr(VI)**

Glucose, lactate, butyrate and ethanol were used as representative carbon sources from various types of potential cellulose degradation products including sugars, organic acids and alcohols. These four carbon sources were used to evaluate the effect of Cr(VI) on growth of isolate EF01.

Results indicate that in the presence of 50 \( \mu \text{M} \) Cr(VI), the carbon source present significantly influenced growth of isolate EF01 (Figure 4.2). If glucose was the carbon
Figure 4.1 Growth of *Arthrobacter* sp. isolate EF01 on potential cellulose degradation products after 72 hours. All carbon sources were normalized to 15 mM carbon. Error bars represent ± one standard deviation (n = 2).

When lactate was the carbon source available, the presence of 50 μM Cr(VI) significantly influenced the growth rate and maximum OD; however, the lag time was not affected (Table 4.1). The growth rate was significantly faster in the absence of Cr(VI), 0.14 hr⁻¹, compared to 0.09 hr⁻¹ when 50 μM Cr(VI) was present. Maximum OD was reached both in the presence and absence of Cr(VI) after 18 hours (Figure 4.2A). However, when Cr(VI) was present, the maximum OD was decreased by 66%.

When lactate was the carbon source available, isolate EF01 showed no significant difference in growth rate, maximum growth, or lag time whether 50 μM Cr(VI) was present or absent (Figure 4.2B, Table 4.1). Interestingly, the growth rate of isolate EF01 on lactate in the absence of Cr(VI), was slowest of all of the carbon sources tested.
Figure 4.2 Growth of *Arthrobacter* sp. isolate EF01 when glucose (A), lactate (B), ethanol (C) and butyrate (D) were the sole carbon sources in the presence (●) and absence (■) of 50 µm Cr(VI). Additional symbols: CSS media (▲), *Arthrobacter* sp. isolate EF01 (♦), Carbon source (x). Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.

When ethanol was the sole carbon source, isolate EF01 reached maximum growth after 60 hours with a lag time of approximately 12 hours (Figure 4.2C, Table 4.1). However, in the presence of 50 µM Cr(VI) there was no measurable growth, either by turbidity or protein, after 327 hours (approximately 14 days). Even after an extended period of time (38 days), there was still no measurable cell growth as assessed by turbidity or protein.
To determine the highest concentration of Cr(VI) that allows for measurable growth by isolate EF01, additional experiments were conducted. Results indicate that isolate EF01 could only grow when the Cr(VI) concentration was below 5 µM (Appendix F). This concentration is extremely low and is actually close to the U.S. EPA Drinking Water Standard of 0.1 mg/L (approximately 2 µM as K₂CrO₄) (Environmental Protection Agency, 2011).

Similar results to ethanol were found when butyrate was the sole carbon source available (Figure 4.2D, Table 4.1). No measurable growth was observed by isolate EF01 in the presence of 50 µM Cr(VI) over a period of 38 days. However, additional experiments demonstrated that isolate EF01 can grow in the presence of 5 µM Cr(VI) when grown on butyrate (Appendix F). These results demonstrated that 5 µM Cr(VI) was the highest concentration to reduce microbial growth significantly, but unlike ethanol, not completely inhibit growth.

Overall these results demonstrate that carbon metabolism by isolate EF01 plays a role in Cr(VI) toxicity. When a sugar such as glucose was provided the organism was capable of growing in the presence of 50 µM Cr(VI). This was repeated with sucrose and xylose, and growth of isolate EF01 was observed (Appendix F) suggesting this effect is not limited to glucose. When alcohols or some organic acids were present, Cr(VI) inhibited growth of isolate EF01. These results were not specific to ethanol and butyrate only as isopropanol, methanol, 1-butanol and succinate were also tested and no growth was observed in the presence of 50 µM Cr(VI) on these carbon sources (Appendix F). Lactate is also an organic acid; however, it is metabolized differently than butyrate and
Table 4.1 Growth Parameters of *Arthrobacter* sp. isolate EF01 in the Presence and Absence of 50 µM Cr(VI)

<table>
<thead>
<tr>
<th>Carbon Source</th>
<th>Growth Rate*</th>
<th>Lag Time†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon Source + Arthrobacter sp. isolate EF01 ± 50µM Cr(VI) [hr⁻¹]</td>
<td>Carbon Source + Arthrobacter sp. isolate EF01 ± 50µM Cr(VI) [hr]</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>0.140 ± 0.0060</td>
<td>0.091 ± 0.0055</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.013 ± 0.0005</td>
<td>0.013 ± 0.0005</td>
</tr>
<tr>
<td>Butyrate</td>
<td>0.044 ± 0.0021</td>
<td>NG</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.032 ± 0.0145</td>
<td>NG</td>
</tr>
</tbody>
</table>

*Error reported for at least three biological replicate separate studies
†Error reported for three biological replicates within one study
NG; No Growth Observed

succinate and so the opposing results are not surprising. Carbon metabolism will be discussed in more detail in Chapter 6 of this dissertation. Overall these results clearly demonstrate that carbon metabolism plays a role in Cr(VI) toxicity of isolate EF01. Interestingly, these results differ from those found for *Pseudomonas putida* F1 in which it was observed that Cr(VI) was most toxic when lactate was the carbon source present, followed by glucose, then acetate (Thompson et al., 2010). This may be due to differences through which these carbon sources are metabolized as well as the mechanisms through which Cr(VI) toxicity is mitigated by each organism.

Influence of Carbon Source on Cr(VI) Reduction

Similar to Cr(VI) toxicity results, these studies indicate that the carbon source present also influenced the ability of isolate EF01 to reduce Cr(VI). When glucose was the sole carbon source in the presence of 50 µM Cr(VI), isolate EF01 reduced Cr(VI) by
50% to 25 µM Cr(VI) after 144 hours (Figure 4.3A). The calculated first order reduction rate coefficient was 4.4E-03 ± 8.8E-04 hr⁻¹. Most of this reduction occurred while the cells were in stationary phase.

In the presence of lactate, isolate EF01 reduced Cr(VI) by 80% after 380 hours (Figure 4.3B). However, there was a significant decrease in measured Cr(VI) in controls containing lactate and Cr(VI). This may be due to the formation of a lactate-Cr(VI) complex or more likely the sodium lactate syrup may contain impurities that are acting as reductants. In these controls, approximately 40% of the Cr(VI) present was no longer measurable using the DPC assay. Therefore, the net reduction attributed to the presence of isolate EF01 was only 40% or 20 µM Cr(VI) over the 380 hour period, most of which occurred in the first 160 hours while the cells were in exponential phase. The calculated first order reduction rate coefficient was 5.0E-03 ± 1.2E-03 hr⁻¹ which is not significantly different than that calculated for glucose which suggests isolate EF01 reduces Cr(VI) with the two carbon sources at similar rates.

To determine whether the decrease in Cr(VI) concentrations over time was due to Cr(VI) reduction in the system and not through sorption to the cells, ICP-MS analysis was conducted on filtered samples when isolate EF01 was grown in the presence of lactate and 50 µM Cr(VI). There was no significant decrease in total Cr as measured through ICP-MS analysis over a period of 200 hours even though a significant decrease in Cr(VI) was measured using the diphenylcarbazide method when lactate was the carbon source present (Appendix F). These results indicate that the Cr(VI) was being changed to
Figure 4.3 Cr(VI) concentrations over time when glucose (A), lactate (B), ethanol (C) and butyrate (D) were the sole carbon sources available to *Arthrobacter* sp. isolate EF01 in the presence of 50 µM Cr(VI) (■). Additional symbols: CSS media only (▲), *Arthrobacter* sp. isolate EF01 and 50 µM Cr(VI) (●), carbon source and 50 µM Cr(VI) (♦), 50 µm Cr(VI) only (x). Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.

a different chromium redox state, likely Cr(III) and the decrease in Cr(VI) concentration observed is not due to sorption to the cells.

When either butyrate or ethanol were the sole carbon sources, no significant reduction in Cr(VI) was measured over time (Figures 4.3C and 4.3D). Even over a period of 38 days no Cr(VI) reduction was observed when butyrate was the carbon source provided. This correlated with the lack of growth in this treatment. Similarly,
when ethanol was the sole carbon source in the presence of 50 µM Cr(VI), there was no significant reduction in Cr(VI) over a period of 38 days, either (data not shown). This also correlated with no growth of isolate EF01 in the system.

Isolate EF01 could reduce Cr(VI) at 50 µM but not when butyrate or ethanol were the carbon sources provided in the presence of 50 µM Cr(VI). These results indicate that Cr(VI) reduction by isolate EF01 directly correlates with cell yield in the presence of 50 µM Cr(VI). Additional studies with sucrose and xylose also demonstrated isolate EF01’s ability to reduce Cr(VI) in the presence of 50 µM Cr(VI) (Appendix F). If ethanol or butyrate were provided in the presence of 50 µM Cr(VI), there was no significant decrease in Cr(VI) concentrations over time. These findings were supported by those of other alcohols and organic acids tested including methanol, isopropanol, 1-butanol and succinate (data not shown). These results further demonstrate that the carbon source available for growth influences Cr(VI) reduction capabilities of isolate EF01.

Cell Viability in the Presence of Cr(VI)

To determine if the lack of growth by isolate EF01 when ethanol or butyrate was the carbon source available was due to cell death or growth inhibition, a cell viability study was conducted using ethanol as the sole carbon source. These studies were carried out similarly to those described previously, except that plate counts were also performed to determine if chromate is bacteriocidal, killing the cells, or bacteriostatic, inhibiting growth.

After 187 hours, some cells from the treatment containing ethanol, isolate EF01 and 50 µM Cr(VI) were still viable (Figure 4.4). However, there was a significant
decrease in the number of cells that were viable compared to the chromate-free controls. An approximate five order of magnitude loss of cell viability was observed in which only 3.2x10^2 CFU/mL were detected after 187 hours, suggesting that the majority of the cells were no longer viable. These results suggest that in the presence of ethanol, Cr(VI) toxicity to isolate EF01 is both bacteriocidal and bacteriostatic.

It is important to note that there was no significant change in cell viability in the treatment containing just isolate EF01 and 50 µM Cr(VI). This indicates that the 5 order decrease in cell viability was caused by a combination of ethanol and 50 µM Cr(VI),

Figure 4.4 Cell viability of *Arthrobacter* sp. isolate EF01 when ethanol (EtOH) was the sole carbon source in the presence and absence of 50 µM Cr(VI). Plate count data representing cell viability are reported as log CFU/mL. Error bars represent ± one standard deviation (n = 5) and are smaller than the symbols where not visible.
further demonstrating the Cr(VI) toxicity mechanism in isolate EF01 is tied to carbon metabolism.

**Cr(VI) Toxicity Mitigation by Sulfate**

Cr(VI) in the form of CrO$_4^{2-}$ can enter the cell through the same transport system as sulfate (SO$_4^{2-}$) (Nies et al., 1998). To possibly mitigate toxicity through competitively excluding Cr(VI) from the cell, SO$_4^{2-}$ was added to the system in excess (100:1 SO$_4^{2-}$ to CrO$_4^{2-}$). Results suggest that the addition of excess sulfate mitigates toxicity to a small extent (Figure 4.5). While the maximum OD increased 18% from 0.407 to 0.473, this

![Figure 4.5 Growth of Arthrobacter sp. isolate EF01 on glucose when 50 µM Cr(VI) and 5 mM sulfate (SO$_4^{2-}$) were present. Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.](image-url)
Figure 4.6 Cr(VI) reduction by *Arthrobacter* sp. isolate EF01 when grown on glucose in the presence of 50 µM Cr(VI) and 5 mM sulfate (SO$_4^{2-}$) after 150 hours. Initial concentration of Cr(VI) was 50 µM. Error bars represent ± one standard deviation (n = 3).

was still significantly lower (approximately 50%) than the maximum growth OD in the absence of Cr(VI). The lag time and growth rates were not significantly different whether or not SO$_4^{2-}$ was present. It is important to point out that the addition of 5 mM SO$_4^{2-}$ did not significantly affect growth of isolate EF01 on glucose, therefore, it is unlikely that sulfate acted negatively as an additional toxicity mechanism. These results suggest that competitive exclusion by sulfate does not decrease Cr(VI) toxicity to isolate EF01.

Cr(VI) reduction by isolate EF01 when grown on glucose was not significantly influenced by the addition of 5 mM SO$_4^{2-}$ (Figure 4.6). Cr(VI) concentrations were 20 µM and 18 µM Cr(VI) after 150 hours in the presence and absence of 5 mM SO$_4^{2-}$,
respectively. Only end point Cr(VI) concentrations were measured and therefore Cr(VI) reduction rates were not calculated. The addition of excess SO$_4^{2-}$ led to an increase in microbial growth, but was unable to completely mitigate Cr(VI) toxicity and no significant difference in Cr(VI) reduction was observed. These results suggest that potential machinery in sulfate transport is not likely playing a role in Cr(VI) resistance by isolate EF01.

**Conclusions**

*Arthrobacter* spp. have been isolated from numerous contaminated sites including many DOE sites and may be playing an important role in the subsurface transformation of contaminants, including Cr(VI). Specifically at LLW sites where Cr(VI) is present and cellulose and its degradation products are the dominant carbon sources available, *Arthrobacter* spp. such as isolate EF01 may be playing an important role in the fate of chromium. The results of this study demonstrate the importance of the carbon and energy source available and the potential impact on Cr(VI) reduction in these environments by isolate EF01.

As biostimulation through the addition of carbon is an attractive remediation option, it is clear from this study that what carbon source is added has the potential to impact Cr(VI) reduction by organisms such as isolate EF01. For example, if carbon in the form of sugars (such as glucose) is added isolate EF01, and likely similar organisms, would be able to both grow and reduce Cr(VI). However, if some organic acids or alcohols were added, this organism would not be able to grow or reduce Cr(VI) and that role would have to be filled by other metal-reducers. This demonstrates the need to assess the
microorganisms of interest at a specific site and to design a treatment plan based on the overall goals.

In a complex subsurface environment such as a low-level waste site, there are most likely multiple carbon sources available. While monitoring the microbial community and the contaminant itself are necessary in the success of in situ reduction of Cr(VI), monitoring the carbon sources available in these sites may be just as valuable. These results also suggest that in the laboratory, evaluation of microbial toxicity and resistance should be performed on representative substrates rather than on one carbon source, such as glucose, as these results may be carbon source dependent. This would provide a better understanding of the fundamental interactions between contaminants and microorganisms.

Acknowledgements

The authors would like to sincerely thank Matthew Fields, Brady Lee, William A. Apel, Adie Phillips and the Bioprocess laboratory research group for their laboratory assistance and intellectual contributions to this research. The financial support for this research was provided by the U.S. Department of Energy, Office of Science, Subsurface Biogeochemical Research Program contract numbers DE-FG02-06ER64206 and DE-AC07-05ID14517.
References


Fredrickson, J., Kostandarithes, H., Li, S., Plymale, A., Daly, M., 2000. Reduction of Fe(III), Cr(VI), U(VI), and Tc(VII) by Deinococcus radiodurans R1. Applied and Environmental Microbiology 66:2006-2011.


Kashefi, K., Lovley, D., 2000. Reduction of Fe(III), Mn(IV), and toxic metals at 100°C by Pyrobaculum islandicum. Applied and Environmental Microbiology 66:1050-1056.


Sani, R., Peyton, B., Smith, W., Apel, W., Petersen, J., 2002. Dissimilatory reduction of Cr(VI), Fe(III), and U(VI) by Cellulomonas isolates. Applied Microbiology and Biotechnology 60:192-199.


CHAPTER 5

IDENTIFYING THE CHROMATE TOXICITY MECHANISM IN ARTHROBACTER SPP.

Introduction

Microorganisms are diverse in the ways in which they can be resistant to or reduce chromate. As discussed in previous chapters, these mechanisms include efflux pumps, the use of chromate reductases, DNA repair machinery and biosorption (Cervantes et al., 2001; Cervantes and Campos-García, 2007; Cheung and Gu, 2007; Ramírez-Díaz et al., 2008). Chromate resistance and reduction are not necessarily linked and therefore an organism may be chromate resistant, but not capable of reducing chromate (Ramírez-Díaz et al., 2008). In Chapter 4 it was observed that Arthrobacter sp. isolate EF01 was chromate resistant and capable of chromate reduction, but that the carbon source provided significantly affected these capabilities.

Chromate toxicity often occurs through oxidative damage to the organism when Cr(VI) is reduced within the cell to Cr(V), Cr(IV) or Cr(III) (Ramírez-Díaz et al., 2008; Nickens et al., 2010). While this may occur in isolate EF01, results of Chapter 4 indicate that chromate toxicity of isolate EF01 is also linked to carbon metabolism. In soil environments, there is likely more than one carbon source available to microorganisms. Identifying the specific mechanism through which carbon metabolism is affecting chromate toxicity within isolate EF01 may be valuable as this information would provide a better understanding of the interactions between isolate EF01, carbon and energy sources, and chromate in soil environments.
In this chapter, data from genome data mining and additional laboratory experiments provide a better understanding of carbon metabolism and attempt to identify the specific mechanism through which chromate is toxic to isolate EF01. However, as this organism was recently isolated, the genome has yet to be sequenced. Therefore, another *Arthrobacter* sp. that was isolated from a contaminated site, *Arthrobacter aurescens* TC1, was used in these studies as its genome has been sequenced previously (Mongodin et al., 2006). The goal of these studies was to attempt to identify the chromate toxicity mechanism in *Arthrobacter aurescens* TC1 and isolate EF01.

**Materials and Methods**

**Growth Conditions**

*Arthrobacter aurescens* TC1 was obtained from the American Type Culture Collection (ATCC# BAA-1386). Comparisons between the 16S rRNA genes of *A. aurescens* TC1 and isolate EF01 indicate that they are 96% similar. *A. aurescens* TC1 was grown aerobically in a defined, carbon-free medium that simulates the groundwater chemistry at the Idaho National Laboratory modified from VanEngelen et al. (2010). Carbon was added separately where indicated. Carbon Source Screening (CSS) medium, contained the following per liter: 80 mg NaCl, 26 mg K$_2$HPO$_4$, 3.35g PIPES Buffer, 0.6 mg KCl, 0.4 mg KOH, 12.7 mg Na$_2$SO$_4$, 3.5mg CaO, and 7.5 mg MgCl$_2*6$H$_2$O amended with 5 ml of Wolfe’s vitamin solution (Atlas, 2004), and 1 ml SL-4 trace elements solution (Atlas, 2004). Growth conditions for *Arthrobacter* sp. isolate EF01 (hereafter referred to as isolate EF01) were the same as for *A. aurescens* TC1. More detail can be found in Chapter 4 of this dissertation.
A. aurescens TC1 was grown aerobically from frozen glycerol stocks in Tryptic Soy Broth (BD Difco, Franklin Lakes, NJ) at room temperature (22 ± 2°C) and shaken at 150 rpm for 20 hours. The culture was centrifuged, washed and resuspended in CSS medium three times. The final resuspended culture was then diluted with sterile medium in order to obtain an optical density of approximately 0.2 for the inoculum. The inoculum OD for all experiments ranged from 0.2 to 0.23 and all treatments contained 10% inoculum using this culture.

Carbon Source Utilization Study

All carbon source stocks were made at a concentration of 150 mM carbon in CSS medium. The following carbon sources, all representing potential cellulose degradation products, were used to assess the ability of the isolate to utilize them for growth: Glucose (dextrose), fructose, maltose, mannose, sucrose, xylose, sodium acetate, sodium butyrate, sodium formate, sodium fumarate, sodium propionate, sodium succinate, sodium lactate (60% w/w) and 100% ethanol. This study was conducted in sterile, 24-well microtiter plates. Duplicate wells were set up for each carbon source in which wells contained a final concentration of 15 mM carbon, CSS medium and A. aurescens TC1. Duplicate control wells lacking A. aurescens TC1 were also set up. Two additional control wells contained CSS medium only. Plates were shaken at 150 rpm at room temperature (22 ± 2°C) for 72 hours after which time absorbance was measured at 600 nm and samples were taken from each well for protein analysis.
Cr(VI) Toxicity Studies

Four carbon sources (glucose, ethanol, lactate and butyrate) representing potential cellulose degradation products were used to assess the influence of Cr(VI) on aerobic growth and Cr(VI) reduction capabilities of *A. aurescens* TC1 in detail. These studies were carried out aerobically in 25 mL screw cap test tubes with all treatments set up in triplicate. For each carbon source, treatments were set up containing CSS medium, 15 mM carbon and *A. aurescens* TC1. Additional treatments were set up containing CSS medium, 15 mM carbon, 50 µM Cr(VI) and *A. aurescens* TC1. Appropriate cell-free and carbon-free treatments were set up as well.

Aerobic plate screen studies using sterile 24-well microtiter plates were carried out to determine if carbon sources representing metabolic pathway intermediates could be utilized for growth both in the presence and absence of 50 µM Cr(VI). These additional compounds were chosen as they were intermediates of pathways of interest and stable under study conditions. They included pyruvic acid, sodium citrate, sodium fumarate, sodium succinate and sodium acetate all normalized to 15 mM carbon. These studies were carried out with both *A. aurescens* TC1 and isolate EF01 to compare the results between organisms. Triplicate wells were set up for each treatment. For each carbon source, treatments were set up containing CSS medium, 15 mM carbon and *A. aurescens* TC1 or isolate EF01. Additional treatments were set up containing 50 µM Cr(VI) in addition to CSS medium, 15 mM carbon and *A. aurescens* TC1 or isolate EF01. Appropriate cell-free and carbon-free treatments were set up as well. Plates were shaken at 150 rpm at room temperature (22 ± 2°C) and absorbance measurements were taken.
over time at 600 nm. At the end of the study, samples were taken for Cr(VI) and protein analyses.

These carbon source studies were carried out twice for each organism. When results were not consistent between experiments, they were repeated in 25 mL screw-cap test tubes (following the procedure described above) as the larger volumes provide more accurate results and less chance for contamination.

Analytical Methods

**Cr(VI) Quantification:** Cr(VI) concentrations were determined spectrophotometrically using the diphenylcarbazide assay as described by Nyman et al. (2002). 150 µL of the sample was added to 50 µL of 0.8N H₂SO₄ in a 96-well microtiter plate followed by the addition of 20 µL of 0.25% 1,5-diphenylcarbazide (Sigma-Aldrich, St. Louis, MO) in 100% acetone. Assay blanks were prepared by the addition of 20 µL of 100% acetone without 1,5-diphenylcarbazide. Absorbance was measured at 540 nm, adjusted with corresponding assay blanks and compared to standards made from a K₂CrO₄ stock solution (Ricca Chemical Company, Arlington, TX).

**Protein Quantification:** A colorimetric protein assay, modified from Sani et al. (2001), was used to estimate total protein. Briefly, 200 µL 1N NaOH was added to 200 µL of sample and digested in a hot water bath at 90°C for 10 minutes. After samples had cooled, 28 µL of a 6:10 v/v HCl solution was added. Triplicate technical replicates were then performed in which three 50 µL aliquots per sample were added to a 96-well plate. 150 µL of Coomassie reagent (Pierce, Rockford, IL) was added to each of the wells and
allowed to react for 15 minutes after which time absorbance readings were taken at 595 nm. Absorbance measurements were compared to those of bovine serum albumin standards which were analyzed using the same procedure to determine protein concentrations. The technical replicate protein concentrations were averaged for each sample to provide final protein concentrations for each sample.

**Results and Discussion**

*Arthrobacter* spp. Comparisons

**Carbon Source Utilization:** To identify the chromate toxicity mechanism within *A. aurescens* TC1, first it had to be determined if the same carbon source dependent toxic effect of Cr(VI) was observed for *A. aurescens* TC1 as was for isolate EF01. *A. aurescens* TC1 was able to utilize the same 13 carbon sources for growth after 72 hours as isolate EF01 (Figure 5.1). Neither organism could utilize formate for growth. More growth was observed for *A. aurescens* TC1 on xylose and succinate compared to isolate EF01 while less growth was observed for *A. aurescens* TC1 on butyrate and sucrose compared to isolate EF01. Both organisms were capable of utilizing a variety of potential cellulose degradation products for growth.

**Influence of Carbon Source on Growth in the Presence of Cr(VI):** Results indicate that the carbon source present influenced Cr(VI) toxicity of *A. aurescens* TC1 as was observed for isolate EF01. When glucose was the carbon source available, a significant decrease in the maximum OD (approximately 45%) by *A. aurescens* TC1 was observed in the presence of 50 µM Cr(VI) (Figure 5.2). The growth rate of *A. aurescens*
Figure 5.1 Carbon source utilization by *A. aurescens* TC1 (black bars) and isolate EF01 (white bars) over 72 hours as measured by protein. Error bars represent ± one standard deviation (n = 2).

TC1 was calculated to be greater in the presence of 50 µM Cr(VI) than in its absence (0.11 ± 5.0E-03 hr⁻¹ and 0.09 ± 5.0E-03 hr⁻¹, respectively) while the opposite was observed for isolate EF01 (Appendix G). No lag time in growth was observed in the presence or absence of 50 µM Cr(VI) by *A. aurescens* TC1 which was similar to isolate EF01 (Appendix G).

When lactate was the sole carbon source, there was no significant difference in the growth rate, lag time or maximum OD of *A. aurescens* TC1 in the presence or absence of 50 µM Cr(VI) (Figure 5.3, Appendix G). These results are consistent with those found for isolate EF01 in which no effect on growth rate, lag time or maximum OD was observed when lactate was present. In general, a higher maximum OD was observed
for *A. aurescens* TC1 (0.65) when compared with isolate EF01 (0.43) which is consistent with the higher protein yields in the carbon source screening study. The growth rates were slower for *A. aurescens* TC1 compared to isolate EF01 (0.007 ± 1.6E-04 hr⁻¹ and 0.013 ± 5.0E-04 hr⁻¹, respectively).

*A. aurescens* TC1 was capable of growing in the absence of chromate when ethanol was the sole carbon source (Figure 5.4). No lag time was observed for *A. aurescens* TC1 when ethanol was present, while there was a lag time of approximately 12 hours for isolate EF01 (Appendix G); however, the growth rate was significantly slower, 0.007 ± 6.0E-05 hr⁻¹, compared to isolate EF01, 0.032 ± 0.015 hr⁻¹ (Appendix G). When 50 µM Cr(VI) was present, no growth was observed for *A. aurescens* TC1 after 168 hours. This is similar to isolate EF01 which was unable to grow in the presence of 50 µM Cr(VI) when ethanol was supplied.

*A. aurescens* TC1 had a significantly different growth pattern when butyrate was the sole carbon source compared to isolate EF01 (Figure 5.5). While *A. aurescens* TC1 was capable of growing when butyrate was provided, its growth rate was approximately 50% slower than isolate EF01 (0.024 ± 1.5E-02 hr⁻¹ and 0.044 ± 2.1E-03 hr⁻¹, respectively) (Appendix G). This correlated with *A. aurescens* TC1 reaching a maximum OD at 262 hours which was much later than isolate EF01 which reached its maximum OD after 60 hours. These results explain why significantly less protein was measured after 72 hours for *A. aurescens* TC1 on butyrate than isolate EF01 in the carbon source utilization study. Interestingly, for *A. aurescens* TC1 no lag time was observed while isolate EF01 had a lag time of approximately 16.5 hours (Appendix G).
Figure 5.2 Microbial growth comparison between *A. aurescens* TC1 and isolate EF01 when glucose was the sole carbon source in the presence and absence of 50 µM Cr(VI). Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.

In the presence of 50 µM Cr(VI), *A. aurescens* TC1 did not grow after 285 hours. These results are consistent with those for isolate EF01.

A significant difference in the growth pattern was observed between the two organisms when xylose was supplied (Appendix G). A greater maximum OD was observed for *A. aurescens* TC1 (1.06) when Cr(VI) was absent from the system compared to isolate EF01 (0.81). When Cr(VI) was absent from the system, *A. aurescens* TC1 had a higher growth rate than isolate EF01 (0.034 ± 1.0E-03 hr\(^{-1}\) and 0.017 ± 2.0E-04 hr\(^{-1}\), respectively). *A. aurescens* TC1 also had a higher growth rate than isolate EF01 when 50 µM Cr(VI) was present (0.02 ± 3.0E-04 hr\(^{-1}\) and 0.006 ± 8.0E-04 hr\(^{-1}\), respectively).

Compared to the other carbon sources discussed, xylose was the only carbon source on
which *A. aurescens* TC1 had a higher growth rate than isolate EF01. Interestingly, there was no lag time for isolate EF01 when xylose was present in the absence of Cr(VI), while there was a lag time of approximately 6 hours for *A. aurescens* TC1. When 50 µM Cr(VI) was present, the calculated lag time for *A. aurescens* TC1 was zero, which is actually less than in the absence of 50 µM Cr(VI). However, as the maximum OD was reached at 113 hours in the presence of Cr(VI) and at 89 hours in its absence, a 5 hour lag time is relatively short. In the presence of 50 µM Cr(VI) the maximum OD was decreased by approximately 20%. This was in direct contrast to isolate EF01 which had an increase in lag time of 235 hours and a significant decrease in growth rate (from 0.017
Figure 5.4 Microbial growth comparison between *A. aurescens* TC1 and isolate EF01 when ethanol was the sole carbon source in the presence and absence of 50 µM Cr(VI). Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible. EtOH; ethanol.

to 0.006 hr\(^{-1}\) in the presence of 50 µM Cr(VI). However, the overall trend is similar in that both organisms grew in the presence of 50 µM Cr(VI) when xylose was the sole carbon source.

These results suggest that while *A. aurescens* TC1 and isolate EF01 have varying growth rates, lag times and maximum ODs in the presence and absence of 50 µM Cr(VI), the overall trends are similar. The carbon source present influenced Cr(VI) toxicity to both isolate EF01 and *A. aurescens* TC1.

**Influence of Carbon Source on Cr(VI) Reduction:** Overall results indicate that the carbon source provided significantly influences Cr(VI) reduction by *A. aurescens* TC1 as
Figure 5.5 Microbial growth comparison between *A. aurescens* TC1 and isolate EF01 when butyrate was the sole carbon source in the presence and absence of 50 µM Cr(VI). Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.

was observed for isolate EF01. When glucose was utilized for growth, *A. aurescens* TC1 was capable of reducing 50% of the Cr(VI) after 146 hours (Figure 5.6). This is very similar to the 56% Cr(VI) reduced by isolate EF01 after the same amount of time. Cr(VI) reduction rate coefficients were also very similar between *A. aurescens* TC1 and isolate EF01 (4.7E-03 ± 2.5E-04 hr⁻¹ and 4.4E-03 ± 8.8E-04 hr⁻¹, respectively).

Similar to results observed for isolate EF01, when lactate was present, *A. aurescens* TC1 was capable of reducing Cr(VI) (Figure 5.7). It appeared that the majority of Cr(VI) was reduced when the culture was in early exponential phase (0-70 hours). Cr(VI) also decreased in the lactate and 50 µM Cr(VI) control suggesting that there may be an additional reducing agent in the sodium lactate syrup. This effect was
Figure 5.6 Cr(VI) concentration over time when glucose was the sole carbon source provided for \textit{A. aurescens} TC1. Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.

observed in five separate experiments. When accounting for the decrease in Cr(VI) concentration in the controls, the total Cr(VI) reduced biologically was estimated to be 20%, or 10 μM Cr(VI), after 300 hours. Isolate EF01 reduced 40%, or 20 μM Cr(VI), over 380 hours with the biological reduction occurring within the first 143 hours indicating that the difference between the two organisms was not due to the time frame of the study conducted. Cr(VI) reduction rate coefficients also demonstrated that \textit{A. aurescens} TC1 reduced Cr(VI) significantly slower than isolate EF01 (2.5E-03 ± 2.5E-04 hr\(^{-1}\) and 5.0E-03 ± 1.2E-03 hr\(^{-1}\), respectively). As observed for isolate EF01, when \textit{A.}
*aurescens* TC1 was grown in the presence of ethanol or butyrate, no Cr(VI) reduction was observed after 236 hours and 305 hours, respectively (Appendix G).

If xylose was present, Cr(VI) was reduced approximately 50% by *A. aurescens* TC1 over 250 hours (Appendix G). It appears as though isolate EF01 reduced Cr(VI) to a further extent, approximately 75% of the available Cr(VI), over a 527 hour period under the same conditions. The study with *A. aurescens* TC1 was not extended past 250 hours as the culture had entered cell death phase (where absorbance measurements are decreasing) so it is unknown if additional Cr(VI) would have been reduced if given more time.

![Figure 5.7 Cr(VI) concentration over time when lactate was provided as the sole carbon source to *A. aurescens* TC1 and isolate EF01. Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.](image_url)
These results indicate that while *A. aurescens* TC1 and isolate EF01 can reduce Cr(VI) at different rates and to different extents, the same trends were observed for both organisms. When glucose, lactate or xylose were present, both *Arthrobacter* spp. were capable of reducing Cr(VI) while there was no significant decrease in Cr(VI) concentrations over time when ethanol or butyrate were present.

**Potential Chromate Toxicity Mechanism**

The Cr(VI) toxicity and Cr(VI) reduction studies discussed have demonstrated that carbon metabolism plays a role in chromate toxicity and reduction of both isolate EF01 and *A. aurescens* TC1. This demonstrates that the chromate toxicity trends observed for isolate EF01 are not limited to this organism and may be common among *Arthrobacter* spp. Overall, when sugars (such as glucose, sucrose or xylose), and lactate were provided as a carbon and energy source these organisms were capable of growing in the presence of and reducing 50 µM Cr(VI). However, if alcohols (such as ethanol or methanol) and some organic acids (such as butyrate and acetate) were provided, neither organism was capable of growing or reducing 50 µM Cr(VI).

There are a number of potential interactions that could lead to the observed chromate toxicity in *A. aurescens* TC1 and isolate EF01. For the remainder of this chapter these potential interactions will be addressed in regards to their likelihood of occurring under the conditions tested and results of experiments that have been performed to test these interactions will be presented.

**General Oxidative Damage:** As previously discussed, chromate toxicity to microorganisms can occur through general oxidative damage to the cells including to
DNA and proteins (Ramírez-Díaz et al., 2008; Nickens et al., 2010). *Arthrobacter* spp. have been observed to have high chromate tolerances in part due to their ability to protect against oxidative damage. The genome of *A. aurescens* TC1 has been sequenced and it was found that about 10% of the identified genes have a top BLAST hit to other genes within its own genome (Mongodin et al., 2006). This demonstrates that a large number of genes are duplicated possibly providing functional redundancy which is believed to be a response to altered environments and stress on the cells. Additionally, the genome encodes for a large number of transcriptional factors (34 in total), all but one of which are found on the chromosome. It is hypothesized that an increased number of alternative transcriptional factors is a function of a complex lifestyle or fluctuations in the conditions of the surrounding environment (Mongodin et al., 2006). Most importantly, *A. aurescens* TC1 has more genes related to oxidative stress response than many other organisms exposed to extreme environmental conditions including *Pseudomonas putida* KT2440, *Arthrobacter* sp. FB24 and the intracellular plant pathogen *Leifsonia xyli* CTCB07 (Mongodin et al., 2006). It is clear that *A. aurescens* TC1 has many ways of protecting itself from general oxidative damage that may occur as a result of Cr(VI) exposure. Additionally, if general oxidative stress had caused Cr(VI) toxicity it should have been observed regardless of the carbon source supplied. Therefore, it is unlikely that this is the mechanism through which Cr(VI) is toxic to isolate EF01 and *A. aurescens* TC1.

**General Energy Limitations:** Under aerobic conditions, a microorganism will gain more energy from sugars than from organic acids or alcohols. Therefore, it could be possible that *Arthrobacter* spp. cannot generate enough energy from the carbon source
available to both grow and mitigate toxicity (through Cr(VI) reduction or other oxidative stress responses). This would support the observation that some of the cells were viable, but did not grow under the conditions presented. However, it is unlikely that a limit in energy generation was occurring under the conditions tested with both *Arthrobacter* spp. Carbon was added in excess in all experiments at a ratio of 100:1 carbon to Cr(VI). Therefore, there should have been more than enough carbon available both to generate energy for growth and the necessary 3 electrons for the reduction of Cr(VI), if that is how *Arthrobacter* spp. were mitigating toxicity. In fact, when ethanol was supplied in the presence of Cr(VI), isolate EF01 could only grow when the Cr(VI) concentration was below 5 µM. Similar results were observed for *A. aurescens* TC1. At 5 µM Cr(VI), isolate EF01 was unable to grow and the carbon to Cr(VI) ratio was then 1000:1 further suggesting that an energy limitation was not the cause of the observed chromate toxicity mechanism in these two organisms.

**Enzyme Interactions:** It is clear that the chromate toxicity mechanism in *A. aurescens* TC1 and isolate EF01 is linked to carbon metabolism. Therefore, it is possible that chromium, in some form, is interacting with one or more of the enzymes involved in carbon metabolism pathways in which alcohols and many organic acids are metabolized. This would lead to carbon metabolism stalling at some point in the pathway and not enough energy being generated in the presence of Cr(VI) for microbial growth or Cr(VI) reduction. This hypothesis supports the observation that cell growth was inhibited and no Cr(VI) reduction was observed, but some cells were still viable.
When reviewing the pathways involved in metabolism of alcohols and most organic acids, it was observed that the fatty acid metabolism, butanoate metabolism, pyruvate metabolism, acetyl-CoA biosynthesis and citrate cycle (TCA cycle) all contained at least 3 different oxidoreductases (EC class 1. enzymes). All of these pathways are involved in metabolizing carbon sources of which when supplied, *A. aurescens* TC1 and isolate EF01 were unable to grow in the presence of 50 µM Cr(VI). These carbon sources include ethanol, isopropanol, 1-butanol, butyrate and acetate.

Meanwhile, glycolysis, the pentose and glucuronate interconversions pathway (through which xylose is metabolized), fructose and mannose metabolism and starch and sucrose metabolism pathways contain only 1 or no oxidoreductases. These are pathways through which carbon sources were metabolized and growth was observed even in the presence of 50 µM Cr(VI). These carbon sources include glucose, sucrose and xylose. Therefore, it is reasonable that if chromium is interacting with the enzymes within the cell it is possible that it may be through the interaction of one or more of these oxidoreductases.

A total of sixteen oxidoreductases were identified in the fatty acid metabolism, butanoate metabolism, pyruvate metabolism, Acetyl-CoA biosynthesis and citrate cycle (TCA cycle) (Table 5.1). There appears to be no one commonality for all of these oxidoreductases; they have different active sites, they are located on different operons and are under the control of different transcriptional regulators and they utilize different electron transfer co-factors. It is likely that they are not all involved in chromate toxicity, but none can be ruled out without further analyses. The identification of these oxidoreductases and their potential involvement in chromium toxicity is similar to
previous studies by Henne et al. (2009b) in which proteomic analysis was performed for Arthrobacter sp. strain FB24 under chromate stress which would further indicate their potential role in chromate toxicity to A. aurescens TC1 and isolate EF01.

Henne et al. (2009b) found that many of the proteins that decreased in abundance under chromate stress conditions included those involved in carbon and energy metabolism. Specifically, they identified components of the pyruvate dehydrogenase complex and aldehyde dehydrogenase as proteins that decreased in abundance when chromate was present. The protein sequences of the pyruvate dehydrogenase and aldehyde dehydrogenase enzymes were 70% and 92% similar, respectively, to those in A. aurescens TC1, listed in Table 5.1. One protein that increased in abundance under chromate stress was a putative malate-quinone oxidoreductase which has been hypothesized to be involved in chromate reduction of strain FB24 (Henne et al., 2009a). This protein has an 85% sequence similarity to the malate-quinone oxidoreductase in A. aurescens TC1 listed in Table 5.1.

One specific oxidoreductase, the aldehyde dehydrogenase (NAD+) (EC 1.2.1.3), is involved in all five pathways (fatty acid metabolism, Acetyl-CoA biosynthesis, pyruvate metabolism, propanoate metabolism and butanoate metabolism) through which carbon is possibly metabolized when the presence of chromate inhibits growth (Table 5.1, Appendix G). As this demonstrates the versatility of this enzyme, its interaction with chromium should be explored further through enzyme assays in which the influence of chromium on the enzyme activity can be assessed.
Table 5.1 Oxidoreductases Involved in *A. aurescens* TC1 Carbon Metabolism Pathways of Interest.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>E.C. Number</th>
<th>Carbon Metabolism</th>
<th>Reaction</th>
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</thead>
<tbody>
<tr>
<td>3-hydroxyacyl-CoA dehydrogenase</td>
<td>1.1.1.35</td>
<td>Fatty acid metabolism</td>
<td>(S)-3-Hydroxyhexadecanoyl-coA ↔ 3-Oxohexadecanoyl-CoA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Butanoate metabolism</td>
<td>Acetoacetyl-CoA ↔ (S)-3-Hydroxybutanoyl-CoA</td>
</tr>
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<td>3-hydroxybutyrate dehydrogenase</td>
<td>1.1.1.30</td>
<td>Butanoate metabolism</td>
<td>(R)-3-Hydroxybutanoate ↔ Acetoacetate</td>
</tr>
<tr>
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<td>Acetyl-CoA biosynthesis</td>
<td>Ethanol ↔ Acetaldehyde</td>
</tr>
<tr>
<td>Alcohol dehydrogenase NADP(+)</td>
<td>1.1.1.2</td>
<td>Acetyl-CoA biosynthesis</td>
<td>Ethanol ↔ Acetaldehyde</td>
</tr>
<tr>
<td>Aldehyde dehydrogenase (NAD(+)</td>
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<td>Aldehyde metabolism</td>
<td>Aldehyde ↔ Fatty acid</td>
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<tr>
<td></td>
<td></td>
<td>Acetyl-CoA biosynthesis</td>
<td>Acetate ↔ Acetaldehyde</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pyruvate Metabolism</td>
<td>Acetate ↔ Acetaldehyde</td>
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<td></td>
<td></td>
<td>Propanoate Metabolism</td>
<td>Propionate ↔ 2-Propyn-1-ol</td>
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<tr>
<td></td>
<td></td>
<td>Butanoate metabolism</td>
<td>3-Butyn-1-al ↔ 3-Butynoate</td>
</tr>
<tr>
<td>Dihydrolipoyl dehydrogenase</td>
<td>1.8.1.4</td>
<td>Acetyl-CoA biosynthesis</td>
<td>Dihydrolipoamide-E ↔ Lipoamide-E</td>
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<td></td>
<td></td>
<td>Pyruvate metabolism</td>
<td>Lipomide-E ↔ Dihydrolipoamide-E</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Citrate cycle (TCA)</td>
<td>Dihydrolipoamide-E ↔ 5-Succinyl-dihydrolipoamide-E</td>
</tr>
<tr>
<td>Ferrodoxin NAD(+) reductase</td>
<td>1.18.1.3</td>
<td>Fatty acid metabolism</td>
<td>Alkane O ↔ 1-alcohol</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>1.1.1.37</td>
<td>Pyruvate metabolism</td>
<td>L-Malate ↔ Oxaloacetate</td>
</tr>
<tr>
<td>Malate quinone oxidoreductase</td>
<td>1.1.5.4</td>
<td>Pyruvate metabolism</td>
<td>L-Malate ↔ Oxaloacetate</td>
</tr>
<tr>
<td>Methylmalonate-semialdehyde dehydrogenase (acylating)</td>
<td>1.2.1.27</td>
<td>Propanoate metabolism</td>
<td>Propanoyl-CoA ↔ 5-S-Methylmalonate semialdehyde</td>
</tr>
<tr>
<td>Oxoglutarate dehydrogenase (succinyl-transferring)</td>
<td>1.2.4.2</td>
<td>Citrate cycle (TCA)</td>
<td>2-Oxoglutarate ↔ 3-Carboxyl-1-hydroxyproplyl-ThPP ↔ 5-Succinyl-dihydrolipoamide-E</td>
</tr>
<tr>
<td>Putative acyl-CoA dehydrogenase</td>
<td>1.3.99-</td>
<td>Fatty acid metabolism</td>
<td>Hexadecanoyl-CoA ↔ transHexadecan2-enol-CoA</td>
</tr>
<tr>
<td>Putative acyl-CoA oxidase</td>
<td>1.3.3.6</td>
<td>Fatty acid metabolism</td>
<td>Hexadecanoyl-CoA ↔ transHexadecan2-enol-CoA</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase (acyetyl-transferring)</td>
<td>1.2.4.1</td>
<td>Acetyl-CoA biosynthesis</td>
<td>Pyruvate → Hydroxyethyl-ThPP → 5-Acetyl-dehydrolipomide-E; or intermediate Lipomide-E</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pyruvate metabolism</td>
<td>pyruvate ↔ 2-Hydroxyethyl-ThPP</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>1.3.99.1</td>
<td>Butanoate metabolism</td>
<td>Succinate ↔ Fumarate</td>
</tr>
<tr>
<td>Succinate-semialdehyde dehydrogenase (NAD(P)(+)</td>
<td>1.2.1.16</td>
<td>Butanoate metabolism</td>
<td>Succinate ↔ Succinate-semialdehyde</td>
</tr>
</tbody>
</table>

To attempt to narrow down what enzymes may be involved in the potential interaction with chromium, a number of intermediates in the Acetyl-CoA biosynthesis, butanoate metabolism and TCA cycle pathways were used as carbon sources in the presence of 50 µM Cr(VI) including pyruvate, fumarate, succinate, and citrate (Table
5.2). The number of intermediates that could be tested was limited due to the fact that many of the intermediates in these pathways are unstable under our study conditions.

Overall, the results of the studies conducted with pathway intermediates did not lead to any conclusive findings. We were unable to identify any specific mechanism through which chromium may be interacting with the organisms’ carbon metabolism. However, these results did generate a few hypotheses that could be pursued further.

One area warranting further research is the potential interactions between chromium and enzymes involved in the Acetyl-CoA biosynthesis pathway (Figure 5.8).

Table 5.2 Growth and Cr(VI) Reduction of *Arthrobacter* spp. on Various Carbon Sources

<table>
<thead>
<tr>
<th>Carbon Source (15mM C)</th>
<th>Arthrobacter sp. Isolate EF01</th>
<th>Arthrobacter aurescens TC1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth on Carbon Source</td>
<td>Growth on Carbon Source in Presence of 50 µM Cr(VI)</td>
<td>Cr(VI) Reduction</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Xylose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lactate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ethanol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Methanol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1-Butanol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Butyrate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Propionate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Succinate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acetate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Citrate</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fumarate</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

(+) Significant growth or Cr(VI) reduction (Error ± standard deviation); (-) Shaded boxes no significant growth or Cr(VI) reduction.
Acetyl-CoA is an important intermediate in carbon metabolism, specifically as it enters the TCA cycle (Figure 5.9) but also in regards to biosynthesis of cell components such as fatty acids. When fatty acids are catabolized, Acetyl-CoA is produced and this enters the TCA cycle. Similarly, alcohols such as ethanol are ultimately converted to Acetyl-CoA through a three-step process of which is catalyzed by oxidoreductases (Figure 5.8, Table 5.1). Additionally, pyruvate can be converted to Acetyl-CoA by the pyruvate dehydrogenase complex. If pyruvate cannot be converted to Acetyl-CoA then it can be converted to oxaloacetate by the pyruvate carboxylase (EC 6.4.1.1) and enter the TCA cycle that way (Figure 5.9). If Acetyl-CoA biosynthesis is inhibited in the presence of 50 µM Cr(VI), then pyruvate may have to utilize the pyruvate carboxylase to ensure carbon enters the TCA cycle. The findings of Henne et al. (2009b) support the hypothesis that Acetyl-CoA biosynthesis is inhibited when Arthrobacter sp. FB24 is exposed to chromium as a decrease in abundance of the pyruvate dehydrogenase complex was observed as well as a slight increase in the abundance of the pyruvate carboxylase. Both Acetyl-CoA and oxaloacetate are necessary for the TCA cycle to run and for citrate to be formed. Therefore, Acetyl-CoA is still necessary in some quantities. It is possible that potential chromium interactions with the enzymes involved in Acetyl-CoA biosynthesis inhibits Acetyl-CoA production, but not completely stops it. This would decrease the amount of energy and cell components that the organism was generating, likely decreasing cell yields and/or growth rate. This is similar to the findings observed for both Arthrobacter spp. when they were provided a carbon source such as glucose in the presence of Cr(VI) of which a decrease in cell yield and growth rate was observed.
Figure 5.8 Glycolysis/Gluconeogenesis and Acetyl-CoA biosynthesis pathway annotated for the *A. aurescens* TC1 genome. Enzymes are presented as EC classifications and crossed out enzymes are not found or have not been predicted to be in the *A. aurescens* TC1 genome. (www.microbesonline.org)
Figure 5.9 Citrate (TCA) cycle pathway annotated for the *A. aurescens* TC1 genome. Enzymes are presented as EC classifications and crossed out enzymes are not found or have not been predicted to be in the *A. aurescens* TC1 genome. ([www.microbesonline.org](http://www.microbesonline.org))

It is also possible that *A. aurescens* TC1 converting pyruvate to oxaloacetate and running the left arm of the TCA cycle of which Succinyl-CoA would be the end product. This allows for the generation of some energy generation and necessary amino acids. A by-product of this process is acetate, from the conversion of excess Acetyl-CoA. Preliminary metabolite analyses indicate acetate is produced in large quantities in the presence of 50 µM Cr(VI) and not in its absence when isolate EF01 was supplied glucose.
as the sole carbon source (data not shown). These results suggest that the utilization of this alternative energy generation in the TCA cycle is possible.

Both of the scenarios discussed above suggest that the organisms need to convert pyruvate to oxaloacetate in order to still generate energy if Acetyl-CoA biosynthesis is inhibited. When alcohols or some organic acids are provided as sole carbon sources in the presence of 50 µM Cr(VI), it is possible that neither organisms is capable converting these carbon sources to pyruvate, which is often how organisms catabolize these carbon sources, or another metabolite that could be used for further energy generation. This may be due to an energy limitation or the organisms’ lack of enzymes necessary. This would lead to cell growth inhibition which is what was observed for both Arthrobacter spp. when grown in the presence of alcohols or some organic acids and 50 µM Cr(VI). Future studies could evaluate the possible interactions between chromium and the oxidoreductases involved in Acetyl-CoA biosynthesis including the pyruvate dehydrogenase complex, aldehyde dehydrogenase and alcohol dehydrogenase.

**Transcriptional Regulator Interactions:** As mentioned previously, *A. aurescens* TC1 has a large number of transcriptional regulators (34 in total) that have been hypothesized to be due to its exposure to changing environmental conditions and potential for stress-related responses (Mongodin et al., 2006). Five one-component systems have been identified within the genome of *A. aurescens* TC1 of which 122 proteins may be involved: the TetR, MarR, LysR IclR and AraC systems (Mongodin et al., 2006). When comparing metabolic pathways of the various carbon sources, no trends were observed in the transcriptional regulators identified for the enzymes involved in
these carbon metabolism pathways. However, it is important to note that the transcriptional regulator was not identified for each enzyme and some information may be missing. Regardless, there was no clear relationship between the transcriptional regulator and enzymes involved in carbon metabolism pathways in which alcohols or most organics are utilizing.

The interactions between transcriptional regulators and chromium cannot be ruled out as a potential mechanism. There may be structural similarities between some of these regulators through which chromium may interact. However, this is a complex question to address as many of these regulators function as multi-protein components and numerous interactions could be occurring. This warrants further investigation, but due to time was unable to be addressed further within these studies. Further studies should focus on identifying the similarities between these transcriptional regulators as no trend was observed based on the target enzymes of these regulators.

**Enzyme CoFactor Interactions:** The oxidoreductases discussed all utilize an electron transfer cofactor to help shuttle electrons. Many of these oxidoreductases of interest use NADH as a cofactor. It is possible that chromium is interacting with these cofactors and not the enzymes themselves. However, NADH has been shown to play a significant role in Cr(VI) reduction by microorganisms under aerobic conditions (Cervantes and Campos-Garcia, 2007). Therefore, exposure of NADH to chromium should not affect its ability to function as the two have interacted in many other systems. Also, as NADH is an important electron shuttle in many aspects of energy generation in cells, especially through the proton motive force, it is likely that if chromium was
interacting with NADH and inhibiting its ability to transfer electrons, then toxicity would be a general response regardless of the carbon source present. While the interactions between chromium and NADH cannot be ruled out, it is unlikely that this is the mechanism through which chromium is toxic to *A. aurescens* TC1 and isolate EF01. However, enzyme assays with NADH and chromium, in various forms, should be conducted in order to determine if chromium is inhibiting the activity of NADH.

**Conclusions and Future Studies**

Carbon source dependent Cr(VI) toxicity was demonstrated in both isolate EF01 and *A. aurescens* TC1. Differences were observed in microbial growth and Cr(VI) reduction between these two organisms which may have been due to genetic differences as they are only related at the genus level. However, the carbon source dependent Cr(VI) toxicity effects were similar between organisms suggesting this chromate toxicity is more general among *Arthrobacter* spp.

While the specific mechanism has not been identified, much progress has been made in developing hypotheses on what this mechanism may be. It is unlikely that Cr(VI) toxicity occurs through general oxidative damage to the cells or from general energy limitations. It is possible that chromium is interacting, in some form, with enzymes involved in the Acetyl-CoA biosynthesis pathway. However, the evaluation of a number of pathway intermediates as carbon sources in the presence of 50 µM Cr(VI), did not identify one single potential interaction. Rather, the results presented here along with those of previous studies suggest that additional studies should focus on the enzymes involved in Acetyl-CoA biosynthesis (including the aldehyde dehydrogenase,
alcohol dehydrogenase and pyruvate dehydrogenase complex) as it is possible that chromium is interacting with these enzymes. Enzyme assays focusing on these oxidoreductases should be conducted to better assess their potential interactions with chromium and its influence on enzyme activity.

As many of the enzymes can be involved in more than one pathway, it would be beneficial to carry out additional studies identifying metabolites that are produced in the presence and absence of 50 µM Cr(VI) to try and more clearly identify exactly how these carbon sources are being metabolized and potentially identify in which part of the pathway metabolism is stalled. Assessing the structural similarities between the transcriptional regulators should also be conducted as the interaction between chromium and transcriptional regulators cannot be ruled out as a potential toxicity mechanism. Overall the results of these studies demonstrate that there is a carbon source dependent chromate toxicity to *Arthrobacter* spp. and further studies need to be conducted in order to identify the specific mechanism(s).
References


Sani, R., Peyton, B., Smith, W., Apel, W., Petersen, J., 2002. Dissimilatory reduction of Cr(VI), Fe(III), and U(VI) by Cellulomonas isolates. Applied Microbiology and Biotechnology 60:192-199.

CHAPTER 6

INFLUENCE OF CARBON SOURCE, IRON MINERALS, AND ELECTRON SHUTTLING COMPOUNDS ON HEXAVALENT CHROMIUM REDUCTION BY CELLULOMONAS SP. STRAIN ES6

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Co-author: Sridhar Viamajala
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Co-author: Alfred B. Cunningham
Contributions: Data analysis, editing

Co-author: Brent M. Peyton
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Journal: Chemosphere

Status of manuscript:
- Prepared for submission to a peer-reviewed journal
- Officially submitted to a peer-reviewed journal
- Accepted by a peer-reviewed journal
- Published in a peer-reviewed journal

Publisher: Elsevier Limited

Submission Date: March 08, 2011
Abstract

The reduction of hexavalent chromium, Cr(VI), to trivalent chromium, Cr(III), can be an important aspect of remediation processes at Department of Energy (DOE) and other contaminated sites. *Cellulomonas* species are found at several Cr(VI) contaminated and uncontaminated locations at the DOE site in Hanford, Washington. Members of this genus have demonstrated the ability to effectively reduce Cr(VI) to Cr(III) fermentatively and therefore play a potential role in Cr(VI) remediation at this site. Batch studies were conducted with *Cellulomonas* sp. strain ES6 to assess the influence of various carbon sources, iron minerals, and electron shuttling compounds on Cr(VI) reduction. These chemical species are likely to be present in these terrestrial environments during *in situ* bioremediation. Results indicated there were a number of interactions between these compounds that influenced Cr(VI) reduction rates. The type of carbon source as well as the type of electron shuttle present influenced Cr(VI) reduction rates. When an electron shuttle, such as anthraquinone-2,6-disulfonate (AQDS), was present in the system, reduction rates increased significantly. Biologically reduced AQDS (referred to as ‘AHDS’) reduced Cr(VI) almost instantaneously. The presence of iron minerals did not significantly influence Cr(VI) reduction rates. However, strain ES6 or AHDS could directly reduce surface-associated Fe(III) to Fe(II), which was capable of reducing Cr(VI) at a near instantaneous rate. These results suggest the rate limiting step in these systems is the transfer of electrons from strain ES6 to the intermediate or terminal electron acceptor whether that is Cr(VI), Fe(III), or AQDS.
Introduction

Hexavalent chromium, Cr(VI), is a common groundwater contaminant in many locations including Department of Energy (DOE) sites. It has been used in numerous industrial processes including chromate plating, the manufacturing of dyes and pigments, leather tanning, wood preservation, and alloy production (Calder, 1988; James, 1994; Hayes, 1997; Barceloux, 1999). At many DOE sites, including DOE’s Hanford site in Washington, chromium was used in nuclear production processes and leached into the soil after storage tanks corroded (Anonymous, 2005). Cr(VI) is a known carcinogen, very water soluble and mobile (Yassi and Nieboer, 1988; Barceloux, 1999). Numerous subsurface microorganisms have been shown to reduce Cr(VI) to Cr(III), which is less toxic, less water soluble and less mobile (Turick et al., 1996; Chen and Hao, 1998; Fredrickson et al., 2000; Francisco et al., 2002; Liu et al., 2002; Sani et al., 2002; Viamajala et al., 2007).

*Cellulomonas* spp., such as strain ES6, have been shown to reduce Cr(VI) to Cr(III) (Francisco et al., 2002; Sani et al., 2002; Viamajala et al., 2007). In a previous effort, enrichments from chromium contaminated and uncontaminated areas of the DOE site in Hanford, Washington, resulted in nine bacterial isolates capable of Cr(VI) to Cr(III) reduction, eight of which were Gram-positive bacteria. Four of these isolates, including strain ES6, were of the *Cellulomonas* genus (Viamajala et al., 2007).

Compared to Gram-negative bacteria, relatively little research has been conducted on the ability of Gram-positive bacteria to reduce Cr(VI) and the factors that influence reduction. Additionally, as *Cellulomonas* sp. strain ES6 (henceforth referred to as strain
ES6) was isolated from the DOE Hanford site, it has the potential to play a role in the *in situ* remediation of Cr(VI) at this site.

Previous studies have shown that a variety of carbon sources, including sucrose and molasses, can stimulate Cr(VI) reduction (Chen and Hao, 1998; Smith et al., 2002; Tseng and Bielefeldt, 2002; Krishna and Philip, 2005; Viamajala et al., 2007). Sucrose and molasses are especially appealing as they not only stimulate a variety of microorganisms, but are also relatively inexpensive compared to other sugars (Lichtenhaler, 2002) and therefore, very likely more cost-effective. While strain ES6 can utilize both carbon sources for anaerobic growth in the absence of additional electron acceptors (Gerlach et al., 2011), little is known about Cr(VI) reduction kinetics during fermentative growth of subsurface-relevant bacteria.

Direct microbial Cr(VI) reduction through enzymatic mechanisms is a slow process when compared to chemical reduction (Nyman et al. 2002; Wielinga et al., 2001) and therefore, the presence of additional electron donors and shuttles may play an important role in Cr(VI) reduction rates *in situ*. Soil and groundwater systems can contain a variety of compounds including natural organic matter, such as humics, as well as iron minerals (Lovley and Phillips, 1986a,b). These compounds have been shown to influence the reduction of oxidized environmental contaminants such as reducible heavy metals and organics (Eary and Rai, 1988; Pettine et al., 1998; Seaman et al., 1999; Amonette et al., 2000; Wielinga et al., 2001; Nyman et al., 2002; Vázquez-Morillas et al., 2006; Okutman Tas and Pavlostathis, 2007; Kumpiene et al., 2008; Kwon and Finneran, 2009; Li et al., 2009; Liu et al., 2009), and it is important to determine their influence on
Cr(VI) reduction rates as well. Additionally, Fe(II)-mediated Cr(VI) reduction may not only be faster, but potentially more beneficial as it often results in the formation of insoluble and immobile Cr(III) hydroxides (Eary and Rai, 1988; Kumpiene et al., 2008).

Iron minerals are diverse in their Fe(II)/Fe(III) compositions, surface areas, and crystallinities, which have all been shown to influence the ability of microorganisms to reduce them (Nealson and Saffarini, 1994; Roden and Zachara, 1996; Lovley, 1997; Zachara et al., 1998; Fredrickson et al., 2000; Newman, 2001). As mentioned above, the microbial reduction of iron is considered especially important in Cr(VI) contaminated aquifers as it has been shown that Fe(II) can directly reduce Cr(VI), often at rates much higher than enzymatic processes (Buerge and Hug, 1997; Pettine et al., 1998; Seaman et al., 1999; Wielinga et al., 2001; Nyman et al., 2002; Vázquez-Morillas et al., 2006; Viamajala et al., 2008; Li et al., 2009). However, in these Cr(VI)-contaminated environments, iron minerals may also act as competing electron acceptors when in an oxidized state. Therefore, it is not surprising that studies have shown that the type of iron oxide present can influence both the extent and rate of microbial Cr(VI) reduction (Seaman et al., 1999; Fredrickson et al., 2000; Wielinga et al., 2001; Xu et al., 2005; Jung et al., 2007). As strain ES6 has the capability to reduce Fe(III) to Fe(II) (Viamajala et al., 2008; Gerlach et al., 2011), the production of surface-associated and dissolved Fe(II) from different iron minerals by strain ES6 may influence the extent and reduction rate of Cr(VI) when present in the same system.

Electron shuttles such as humics and quinones can decrease or eliminate the need for direct transfer of electrons by microbes to electron acceptors such as solid phase iron
oxides and Cr(VI). Studies have shown that the presence of electron shuttles can increase the rate and extent of both Cr(VI) and Fe(III) reduction by microorganisms (Lovley and Phillips, 1986b; Lovley, 1997; Fredrickson et al., 1998; Lovley et al., 1998; Zachara et al., 1998; Lovley and Blunt-Harris, 1999; Nevin and Lovley, 2000; Liu et al., 2002; Royer et al., 2002; Turick et al., 2002). Gerlach et al. (2011) demonstrated that the addition of the electron shuttle, anthraquinone-2,6-disulfonate (AQDS), led to an increased rate of Fe(III) reduction by strain ES6, but little is known about how AQDS affects Cr(VI) reduction.

To better design in situ Cr(VI) bioremediation systems, the interactions between the soil chemistry, the microorganisms present in these soils, and Cr(VI) have to be well understood. The purpose of this study was to determine the combined influence of the carbon source present (electron donor), electron shuttling compounds, and different iron mineral phases and concentrations on reduction rates of Cr(VI) by strain ES6.

Materials and Methods

Culture Inoculum

*Cellulomonas* sp. strain ES6 was maintained in frozen stock cultures containing tryptic soy broth (TSB, 30 g/L, Difco Laboratories) with 20% glycerol at –70 °C. Cells were pre-cultured in TSB for 24 hours on a horizontal shaker at 150 rpm and 30 °C, transferred into fresh TSB, and grown again for 18 hours in TSB (each 1% initial inoculum). This transferred culture was grown for 16 hours, after which time cells were centrifuged, washed and resuspended in a synthetic groundwater medium (SGW) as described by Borch et al. (2005). The resuspended culture was used to inoculate the
batch experiments described below. To determine the initial concentrations of viable cells (measured as colony forming units, CFU), serial dilutions were performed in phosphate buffered saline, aliquots spread on tryptic soy agar (TSA, Difco Laboratories) plates incubated for 48 hours at 30 °C, and colonies were counted.

**Batch Studies**

Anaerobic batch experiments were set up in 30-mL anaerobic culture tubes (Bellco Glass®, Vineland, NJ) with an 80% N\textsubscript{2}/20% CO\textsubscript{2} mixed gas headspace. The tubes were sealed with butyl rubber stoppers and aluminum crimp seals. Each treatment was set up in triplicate. Treatments were inoculated with strain ES6 (prepared as described above) and, at the start of each experiment, K\textsubscript{2}CrO\textsubscript{4} was added to a final concentration of 5 mg/L Cr(VI) (96 µM Cr(VI)) per vial.

**Influence of Carbon Sources on Cr(VI) Reduction by Strain ES6:** Two treatments contained SGW and either 10 mM sucrose or 3.42 g/L molasses (Grandma’s Molasses, Mott’s Inc., Stamford, CT). Two more treatments contained SGW, 10mM of either sucrose or 3.42 g/L molasses, and 100 µM AQDS. Two control treatments contained no carbon source; one also contained 100 µM AQDS while the other contained no AQDS. The initial cell concentration for all carbon source treatments was determined to be 7.63 x 10\textsuperscript{7} ± 3.79 x 10\textsuperscript{6} CFU/mL.

**Influence of Electron Shuttles on Cr(VI) Reduction by Strain ES6:** Treatments containing different electron shuttles were established using 100 µM 2-hydroxy-1,4-naphthaquinone (HNQ), 100 µM AQDS, 100 µM Vitamin B\textsubscript{12}, and 29.5 mg/L Elliot soil
humic acid standard (International Humic Substances Society (IHSS), MN, USA).

Vitamin B₁₂ is not classified as an electron shuttle but has been shown to enhance microbial reduction of carbon tetrachloride by *Shewanella alga* strain BrY (Workman et al., 1997) and therefore was added to determine if it could act in a similar capacity in this study. Initial cell concentration for all biotic electron shuttle treatments was determined to be $6.60 \times 10^8 \pm 8.18 \times 10^7$ CFU/mL.

**Influence of Electron Shuttle-to-Cr(VI) Ratio on Cr(VI) Reduction Rates by Strain ES6:** These treatments contained SGW, 10 mM sucrose, and either 0, 10, 50, 100, 500, 1000, or 8900 µM AQDS. Initial cell concentration for these biotic treatments was determined to be $1.47 \times 10^8 \pm 4.16 \times 10^7$ CFU/mL.

**Influence of Iron Minerals on Cr(VI) Reduction by Strain ES6:** Five treatments contained SGW, 100 µM AQDS, 10 mM sucrose, and 6 mM Fe(III) in the form of one of the following five minerals: hydrous ferric oxide (HFO), hematite, goethite, maghemite, and magnetite (see Gerlach et al., 2011 for details). Additional treatments were set up using hematite, goethite, and magnetite without AQDS. Two control treatments were included in which either no iron mineral was present or no cells were added. Iron minerals were pasteurized at 80°C for five hours prior to being used in batch studies. Pasteurization was used instead of autoclaving to minimize the potential impact of autoclaving on mineralogy. Initial cell concentrations for these biotic treatments except those with HFO were $4.73 \times 10^8 \pm 9.29 \times 10^7$ CFU/mL and for treatments containing HFO $3.87 \times 10^8 \pm 7.77 \times 10^7$ CFU/mL.
Influence of Fe-to-Cr Ratio on Cr(VI) Reduction by Strain ES6: Five treatments contained SGW, 10 mM sucrose and either 9, 6, 3, 1.5 or 0 mM Fe(III) as HFO. Five equivalent treatments were set up containing 100 µM AQDS. Cell-free and iron mineral-free control treatments were set up as well. Initial cell concentration for these biotic treatments was determined to be 2.00 x 10^8 ± 3.61 x 10^7 CFU/mL.

Influence of Microbially Produced Fe(II) on Cr(VI) Reduction: Six treatments contained SGW, 10 mM sucrose and 6 mM Fe(III) in the form of HFO. Four of these treatments were inoculated with strain ES6 (as described above) of which two treatments also contained 100 µM AQDS. Two other treatments were cell-free controls, one of which also contained 100 µM AQDS. After 32 days, when Fe(III) and AQDS were reduced in the treatments containing strain ES6, two of these treatments (one with AQDS and one without) and both of the cell-free controls were sterilized by autoclaving so any further reduction in the systems would be abiotic. Cell-free controls were autoclaved so that any potential changes in mineralogy would be reflected in all control treatments. Cr(VI) was added to all six treatments after cooling to evaluate the effect of non-biological Cr(VI) reduction in the presence of biologically reduced HFO.

Analytical Methods

Cr(VI) Quantification: Cr(VI) concentrations were determined spectrophotometrically using the diphenylcarbazide assay as described by Nyman et al. (2002). 150µL of the sample was added to 50µL of 0.8N H₂SO₄ in a 96-well microtiter plate followed by the addition of 20µL of 0.25% 1,5-diphenylcarbazide (DPC, Sigma-
Aldrich, St. Louis, MO) in 100% acetone. Assay blanks were prepared by the addition of 20µL of 100% acetone without DPC. Absorbance was measured at 540nm, adjusted to corresponding assay blanks and compared to standards made from a K$_2$CrO$_4$ stock solution (Ricca Chemical Company, Arlington, TX).

**Iron Analysis:** Fe(II) quantification was performed using the procedure described by Lovely & Phillips (1987). Absorbance was measured at 540 nm. Total-Fe was determined using the same procedure replacing 0.5N HCl with a mixture of 0.25N HCl and 0.25N NH$_2$OH-HCl.

**Data Analysis:** First order rate coefficients (k) for the reactions were calculated based on the following model: $c_{Cr(VI)} = c_{Cr(VI)_0}e^{-kt}$ through the use of TableCurve™ 2D v. 4 (AISN Software, Inc.) in which $c_{Cr(VI)_0}$ is the initial Cr(VI) concentration at time zero and $c_{Cr(VI)}$ is the Cr(VI) concentration at time (t). First order rate coefficients were normalized to the initial cell concentrations of strain ES6. A first order kinetic model was used to account for the potential influence of Cr(VI) concentration on the rate of Cr(VI) reduction, which is analogous to a previously published model describing Cr(VI) reduction by *Shewanella oneidensis* (Viamajala et al. 2002). Additionally, the correlation coefficients for the first order model were consistently greater than those for a zero order model, suggesting that a first order model better represented the data. Standard deviations of the experimental replicates are reported as these errors are commonly greater than the standard deviations associated with the first order fits. It is important to note that the model fits were used merely as an aid to interpreting the relative effects (and magnitudes of the effects) of various environmentally relevant...
factors (such as the presence of humics, Fe types, and concentrations) and not to describe
the fundamental kinetic process(es) associated with Cr(VI) reduction by strain ES6.

Results and Discussion

Influences of Carbon Sources on Cr(VI) Reduction by Strain ES6

Previous studies have shown that strain ES6 can reduce Cr(VI) to Cr(III) in the
presence of a variety of carbon sources (Sani et al., 2002; Viamajala et al., 2007). Since
sucrose (especially as molasses) is relatively inexpensive, we chose to focus on these
carbon sources.

Results indicate the rate of Cr(VI) reduction was in general greater in the presence
of molasses compared to sucrose (Figure 6.1, Appendix H). First order rate coefficients
normalized to cell numbers were calculated to be $4.64 \times 10^{-9} \pm 6.55 \times 10^{-10}$ mL/(CFU*hr)
and $1.06 \times 10^{-10} \pm 7.34 \times 10^{-12}$ mL/(CFU*hr), respectively. Molasses has a high sucrose
content (Prescott and Dunn, 1983), but as it contains other sugars (e.g. glucose and
fructose) as well as other compounds, it is possible that strain ES6 was able to utilize
these other compounds to increase Cr(VI) reduction rates over the treatments containing
sucrose only.

Cr(VI) reduction rates were enhanced in the presence of AQDS. First order rate
coefficients for molasses and sucrose in the presence of AQDS were calculated to be
$1.53 \times 10^{-8} \pm 3.10 \times 10^{-10}$ mL/(CFU*hr) and $8.63 \times 10^{-10} \pm 1.82 \times 10^{-10}$ mL/(CFU*hr),
respectively. The carbon-free and AQDS-free controls containing strain ES6 showed no
statistically significant Cr(VI) reduction after 7 days. Cr(VI) reduction was not
Cr(VI) reduction was fastest when molasses was the carbon source present. AQDS increased Cr(VI) reduction rates significantly regardless of the carbon source present. All treatments contained strain ES6 (7.63 x 10^7 ± 3.79 x 10^8 CFU/mL). Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.

Figure 6.1 Cr(VI) reduction by strain ES6 in the presence of different carbon sources and in the presence or absence of anthraquinone-2,6-disulfonate (AQDS), an electron shuttle. Cr(VI) was reduced fastest when molasses was the carbon source present. AQDS increased Cr(VI) reduction rates significantly regardless of the carbon source present. All treatments contained strain ES6 (7.63 x 10^7 ± 3.79 x 10^8 CFU/mL). Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.

detectable until 18 days into the experiment (data not shown) and first order Cr(VI) reduction rates were calculated to be 4.96 x 10^{-12} ± 1.01 x 10^{-12} mL/(CFU*hr) for that time frame. The addition of AQDS increased the rate of Cr(VI) reduction to 1.97 x 10^{-10} ± 3.29 x 10^{-11} mL/(CFU*hr) even though a carbon source was absent suggesting that storage compounds within the cells can act as the electron donor for Cr(VI) reduction.

These results are consistent with previous studies with strain ES6, which suggest that in the absence of an external electron donor endogenous electron reserves can be utilized for
the reduction of Cr(VI), Fe(III), and U(VI) in batch and column experiments (Viamajala et al., 2007; Viamajala et al., 2008, Gerlach et al. 2011). Interestingly, Cr(VI) reduction rates were significantly greater in the carbon-free treatment containing AQDS compared to the sucrose treatment without AQDS suggesting that the ability of cells to transfer electrons had a greater influence on Cr(VI) reduction rates than the presence of an easily utilizable carbon source.

Influence of Electron Shuttles on Cr(VI) Reduction by Strain ES6

Since the presence of AQDS enhanced Cr(VI) reduction rates (Figure 6.1), other electron shuttle compounds were also tested to determine if the type of electron shuttle present influenced Cr(VI) reduction.

In the presence of sucrose, each of the electron shuttles tested showed rapid Cr(VI) reduction and reached below detection levels (0.01 mg/L) within 22 hours (Figure 6.2, Appendix H). The quinones, AQDS and HNQ, enhanced Cr(VI) reduction rates by strain ES6 more than the humic acid standard (HAS). First order rate coefficients for AQDS and HNQ normalized to cell numbers were calculated to be $1.20 \times 10^{-9} \pm 6.78 \times 10^{-11}$ mL/(CFU*hr) and $1.68 \times 10^{-8} \pm 4.03 \times 10^{-10}$ mL/(CFU*hr), respectively.

Due to the greater initial cell concentration in these treatments ($6.60 \times 10^8 \pm 8.18 \times 10^9$ CFU/mL), complete reduction of Cr(VI) occurred faster than in the treatments described above, which had an initial cell concentration of $7.63 \times 10^7 \pm 3.79 \times 10^6$ CFU/mL. There is no statistically significant difference in the cell concentration-normalized first order Cr(VI) reduction rate coefficients ($k$) for any of the replicate
Figure 6.2 Cr(VI) reduction by strain ES6 in the presence of different electron shuttles. Cr(VI) was reduced fastest in the presence of the quinones (HNQ or AQDS) when compared to other potential electron shuttles. All treatments contained 10 mM sucrose and strain ES6 (6.60 x 10^8 ± 8.18 x 10^7 CFU/mL). HNQ: 2-hydroxy-1,4-naphthaquinone (100 μM), AQDS: anthraquinone-2,6-disulfonate (100 μM), HAS: Elliot soil humic acid standard (29.5 mg/L), Vit B12: Vitamin B_{12} (100 μM). Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.

While Vitamin B_{12} was shown previously to enhance the transformation of carbon tetrachloride by *Shewanella alga* strain BrY (Workman et al., 1997), a similar effect was not observed in our studies. In the presence of Vitamin B_{12}, Cr(VI) reduction rates were approximately equal to electron shuttle-free controls. The results suggest that while external electron shuttles are not necessary for strain ES6 to reduce Cr(VI), their presence increases Cr(VI) reduction rates significantly, and the presence of low-molecular weight
quinones appears to increase Cr(VI) reduction rates more than high-molecular weight humic acids.

**Influence of Electron Shuttle-to-Cr(VI) Ratio on Cr(VI) Reduction Rates by Strain ES6**

Experiments were performed to determine if Cr(VI) reduction rates were influenced by the concentration of an electron shuttle. Results showed a direct correlation between AQDS concentration and Cr(VI) reduction rates with greater concentrations of AQDS leading to faster reduction of Cr(VI) (Figure 6.3, Appendix H). In fact, there was close to a 50-fold increase in Cr(VI) reduction rates between the lowest (10 µM) and the highest (8900 µM) AQDS concentration tested. First order rate coefficients for the lowest and highest AQDS concentrations were calculated to be $9.82 \times 10^{-10} \pm 5.98 \times 10^{-11}$ mL/(CFU*hr) and $5.22 \times 10^{-8} \pm 6.24 \times 10^{-9}$ mL/(CFU*hr), respectively (Figure 3 Inset, Appendix H). As the addition of an electron shuttle greatly enhanced Cr(VI) reduction rates, it suggests that the rate limiting step in a system containing a carbon source, strain ES6, and Cr(VI) is the transfer of electrons from strain ES6 to Cr(VI).

**Influence of Iron Minerals on Cr(VI) Reduction by Strain ES6**

Previous studies have shown that the type of iron mineral present in a system influences Cr(VI) reduction rates (Wielinga et al., 2001; Xu et al., 2005). However, in this study the presence and type of iron mineral in the system did not appear to influence the rate of Cr(VI) reduction by strain ES6 (Figure 6.4, Appendix H).
Figure 6.3 Cr(VI) reduction by strain ES6 as a function of AQDS concentration. A direct correlation between AQDS concentration and Cr(VI) reduction rates can be observed. Inset: Averaged cell concentration-normalized first order rate coefficients for Cr(VI) reduction with increasing AQDS concentration. All treatments contained 10 mM sucrose and strain ES6 (1.47 x 10^8 ± 4.16 x 10^7 CFU/mL). AQDS: anthraquinone-2,6-disulfonate. Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.

In all treatments containing AQDS, except for the cell-free control, complete Cr(VI) reduction occurred by 18 hours. There was no statistically significant difference in Cr(VI) reduction rates (t-test, p > 0.05) between these treatments demonstrating that the type of iron mineral present did not influence strain ES6’s ability to reduce Cr(VI). Additionally, the presence of iron minerals in the system did not affect Cr(VI) reduction rates when AQDS was also present as there was no significant difference in Cr(VI)
reduction rates with treatments that contained an iron mineral and AQDS or just AQDS without an iron mineral present. While it appears that Cr(VI) was reduced slightly more slowly when HFO was present, the calculated first order rate constant was not significantly different from the rate constants calculated for the other iron minerals or the iron mineral-free treatment (t-test, p value > 0.05).

The same trend was observed when AQDS was absent from the system (Appendix H). No significant difference in Cr(VI) reduction rates was observed (t-test, p > 0.05) regardless of which iron mineral was present. However, complete reduction of Cr(VI) in the absence of AQDS took longer in all treatments containing iron minerals (approximately 42 hours) compared to treatments that contained AQDS.

These results suggest that the presence and type of iron minerals neither enhance nor inhibit Cr(VI) reduction rates by strain ES6, which does not support the findings of several previous studies with other iron-reducing organisms (Seaman et al., 1999; Fredrickson et al., 2000; Wielinga et al., 2001; Xu et al., 2005; Jung et al., 2007). It has been demonstrated that strain ES6 can reduce numerous iron minerals (Gerlach et al., 2011), although at rates slower than other iron-reducing microorganisms. Hence, it is possible that strain ES6 is not capable of reducing these iron minerals at a rate that is comparable to the rate of Cr(VI) reduction and therefore Cr(VI) reduction is carried out preferentially. It is also possible that strain ES6 indeed reduces at least some of the iron minerals, but that Cr(VI) reduction by microbially produced Fe(II) is so fast that competition of Fe(III) with Cr(VI) for electrons is negligible overall (see “Influence of
microbially produced Fe(II) on Cr(VI) reduction” below for results supporting this explanation).

Influence of Fe-to-Cr Ratio on Cr(VI) Reduction by Strain ES6

Additional treatments were established to further evaluate the possible influence of Fe(III) on Cr(VI) reduction by strain ES6. Varying amounts of the most easily

Figure 6.4 Cr(VI) reduction by strain ES6 in the presence of different iron minerals. Calculated first order rate coefficients determined that the presence of different iron mineral phases did not influence Cr(VI) reduction rates. All treatments contained 10 mM sucrose, 6 mM Fe(III), and strain ES6 unless indicated otherwise (initial cell concentrations $3.87 \times 10^8 \pm 7.77 \times 10^7$ CFU/mL for HFO containing treatments, $4.73 \times 10^8 \pm 9.29 \times 10^7$ CFU/mL for all other iron minerals). HFO: hydrous ferric oxide, HEM: hematite, GOE: goethite, MHM: maghemite, MGN: magnetite, AQDS: anthraquinone-2,6-disulfonate (concentration 100 μM where indicated). Error bars represent ± one standard deviation ($n = 3$) and are smaller than the symbols where not visible.
reducible iron mineral, HFO, were used to determine a possible influence on Cr(VI) reduction rates by strain ES6. Results suggested increased concentrations of HFO did not lead to a change in Cr(VI) reduction rates (Figure 6.5, Appendix H). However, there was a significant difference in reduction rates between treatments that contained AQDS and those that did not, regardless of the iron concentration (Appendix H). These results suggest that the presence of AQDS plays a greater role in Cr(VI) reduction rates by strain ES6 than the amount of Fe(III) mineral present.

HFO has been shown to be easily reduced by strain ES6 (Gerlach et al. 2011) and therefore, as discussed above, could either compete with Cr(VI) as electron acceptor, thus potentially decreasing Cr(VI) reduction rates, or in its reduced state act as an electron donor to Cr(VI), potentially increasing Cr(VI) reduction rates. The results showed complete Cr(VI) reduction in all treatments containing HFO and AQDS by 12 hours and no significant difference in Cr(VI) reduction rates with varying HFO concentrations, suggesting HFO is acting in either both capacities or neither capacity. Fe(II) analysis in these treatments showed that a significant increase in Fe(II) concentrations was not observed until after Cr(VI) was completely reduced (Appendix H). Hence, it is possible that Cr(VI) in these treatments is reduced directly by strain ES6 or through the shuttling of electrons by AQDS and Fe(III) reduction occurs only after Cr(VI) has been reduced completely. It is also possible that Fe(II), if produced throughout the entire duration of this experiment, is rapidly re-oxidized by Cr(VI) as long as it is present. Once Cr(VI) is
Figure 6.5 Cr(VI) reduction by strain ES6 as a function of Fe(III) concentration in the form of HFO. Fe(III) concentration did not significantly influence Cr(VI) reduction rates. All treatments contained 10 mM sucrose and strain ES6 (2.00 x 10^8 ± 3.61 x 10^7 CFU/mL) unless indicated otherwise. AQDS: anthraquinone-2,6-disulfonate (concentration 100 μM where indicated). Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.

completely reduced, Fe(II) can no longer be re-oxidized by Cr(VI) and Fe(II) concentrations begin to increase. Both scenarios would support our findings.

Influence of Microbially Produced Fe(II) on Cr(VI) Reduction

As it was possible that microbially produced Fe(II) may be directly reducing Cr(VI), treatments were established to determine the rate and extent at which microbially produced Fe(II) could reduce Cr(VI). The results indicate that microbially produced Fe(II) drastically enhanced Cr(VI) reduction rates (Figure 6.6). Complete reduction in all treatments containing microbially produced Fe(II) occurred within minutes and
Figure 6.6 Cr(VI) reduction by microbially produced Fe(II). Strain ES6 was allowed to reduce Fe(III) in the form of HFO in samples for a period of 18 days after which some treatments were autoclaved to kill cells. Control treatment did not contain microbially produced Fe(II), strain ES6 or AQDS (100 μM where indicated). Significant Cr(VI) reduction by microbially produced Fe(II) occurred within minutes in all treatments except the controls. Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.

calculated apparent first order rate coefficients were ≥ 263 hr$^{-1}$ indicating that Fe(II) chemically reduced Cr(VI) at an almost instantaneous rate. Additionally, Cr(VI) was added to treatments containing pre-reduced AQDS (referred to as ‘AHDS’, which was produced as described by Borch et al., 2005). Calculated apparent first order Cr(VI) reduction rate coefficients in the presence of 100 μM AHDS were > 400 hr$^{-1}$.
Combined Influence of Carbon Source, Electron Shuttle and Iron Minerals on Cr(VI) Reduction by Strain ES6

The results reported here suggest that Cr(VI) reduction by strain ES6 is a complex process in which the carbon source, electron shuttle, and iron minerals can all play a role. The relative influence of the different compounds on reduction rates are summarized in Figure 6.7. The addition of an external electron donor increased Cr(VI) reduction rates, as it was observed that molasses and sucrose increased reduction rates when compared to treatments without an external electron donor.

In addition, molasses increased Cr(VI) reduction rates to above the rates observed in the presence of pure sucrose, most likely due to the presence of other (more easily utilized) sugars or the presence of electron shuttling compounds within molasses. However, as Cr(VI) was reduced even in carbon-free controls, the presence of a carbon source does not appear to be necessary for Cr(VI) reduction by strain ES6 within the short time frame evaluated in these studies, and it is likely that strain ES6 utilizes endogenous electron reserves as an electron donor (Sani et al. 2002, Viamajala et al., 2007; Viamajala et al., 2008; Sivaswamy et al., 2011; Gerlach et al. 2011). This may be especially useful for in situ Cr(VI) reduction where it may be costly or difficult to continually add an electron donor. Instead, in the field, an electron donor could be added in the form of a concentrated pulse and the organisms could build up electron storage compounds, which could be utilized for Cr(VI) reduction later on.

The addition of an electron shuttle increased Cr(VI) reduction rates by approximately one order of magnitude in the presence of a carbon source and significantly increased Cr(VI) reduction rates, even in the absence of an external carbon
Figure 6.7 Summary of Cr(VI) reduction by strain ES6 in the presence of a carbon source, electron shuttle and iron mineral as shown by average first order reduction rate coefficients normalized to cell number. Results suggest the rate limiting step is the transfer of electrons from the cell to an initial electron acceptor. Rate coefficients are reported for treatment conditions containing 10 mM sucrose or molasses, 100 µM AQDS, 6 mM Fe(III) in the form of HFO, and 5 mg/L Cr(VI). 

source, suggesting that the transfer of electrons from strain ES6 to Cr(VI) was the rate limiting step in this study. The type of electron shuttle present also was found to play a role in Cr(VI) reduction rates with the highly soluble, low-molecular weight quinones, AQDS and HNQ, being more effective than the less soluble, high-molecular weight humic acid substances.

Interestingly, the presence of different types and amounts of Fe(III) did not significantly influence Cr(VI) reduction rates, suggesting that surface-associated Fe(III) ultimately does not act as a competing electron acceptor. Our results suggest that iron mineral reduction might occur in the presence of Cr(VI), but that reduced iron minerals
very quickly reduce Cr(VI), ultimately not significantly decreasing the overall rate of Cr(VI) reduction. The final product of Cr(VI) reduction was identified as Cr(III) in all treatments using X-ray absorption near edge structure (XANES) analysis (Appendix H).

It appears that the initial transfer of electrons from strain ES6 to an electron acceptor, whether it is an intermediate or terminal acceptor, is the rate limiting step in Cr(VI) reduction in the systems studied here. For example, when an electron shuttle such as AQDS was present, strain ES6 reduced Cr(VI) approximately an order of magnitude faster than in the absence of AQDS, presumably because the presence of an electron acceptor eliminates the need for direct contact between the cells and Cr(VI).

Additionally, if strain ES6 reduced AQDS, the resulting AHDS could abiotically reduce Cr(VI) at extremely high rates. When iron minerals were present in the system they did not significantly affect the rate of Cr(VI) reduction by strain ES6, regardless of their type or concentration, unless they were already reduced to a significant amount and abiotically reduced Cr(VI) which occurred at an extremely fast rate.

Conclusions

Hexavalent chromium remains a contaminant of interest at many DOE and industrial sites and biological reduction of this contaminant in situ may be an important part of its remediation. As numerous Cellulomonas spp. isolates, including strain ES6, have been obtained from DOE’s Hanford site, they may play an important role in the in situ remediation of Cr(VI) at this and other DOE sites, especially as they are capable of growing and carrying out Cr(VI) reduction fermentatively and thus do not require the addition of an external electron acceptor. The research presented here addresses how the
interactions of compounds that may be present in these soil systems influence Cr(VI) reduction rates by strain ES6.

The results of this study demonstrate that electron shuttles, such as AQDS, greatly enhance the reduction rates of Cr(VI) to Cr(III) and influence reduction rates more than the other factors investigated here. Microorganisms, such as Cellulomonas sp. strain ES6, can reduce AQDS, which can itself reduce Cr(VI) at an almost instantaneous rate. The presence of Fe(III) minerals at various concentrations did not influence Cr(VI) reduction rates by strain ES6 suggesting Cr(VI) may be preferentially reduced over Fe(III) by strain ES6 in these systems or the produced Fe(II) reduces Cr(VI) at a basically instantaneous rate. Oxidized iron minerals can be reduced by subsurface microorganisms, including strain ES6, producing surface-associated Fe(II), which in turn can reduce Cr(VI) to Cr(III) at a near instantaneous rate. It appears that the rate limiting step of Cr(VI) reduction in these subsurface relevant systems is the transfer of electrons from strain ES6 to the electron acceptor (intermediate or terminal), either Cr(VI), Fe(III), or AQDS.

Acknowledgements

The authors would like to thank Lindsey Hopper, Kristy Weaver, Nicholas Ballor, Thomas Borch and Crystal Russell for their various contributions in the laboratory. This research was supported by the U.S. Department of Energy, Office of Science, Environmental Management Science Program, under Grant No. DE-FG02-03ER63582 and DOE-NE Idaho Operations Office Contract DE-AC07-05ID14517. Partial financial support was provided by a grant from the Inland Northwest Research Alliance (INRA)
under contract MSU 002. We thank Matthew Marcus (ALS) and Matthew Ginder-Vogel for their help in synchrotron-based acquisition. The Advanced Light Source is supported by the Director, Office of Science, Office of Basic Energy Sciences, of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231.
References


Fredrickson, J., Kostandarithes, H., Li, S., Plymale, A., Daly, M., 2000. Reduction of Fe(III), Cr(VI), U(VI), and Tc(VII) by Deinococcus radiodurans R1. Applied and Environmental Microbiology 66:2006-2011.


Sani, R., Peyton, B., Smith, W., Apel, W., Petersen, J., 2002. Dissimilatory reduction of Cr(VI), Fe(III), and U(VI) by Cellulomonas isolates. Applied Microbiology and Biotechnology 60:192-199.


CHAPTER 7

CONCLUSIONS AND FUTURE STUDIES

Conclusions

The results of the research presented in this dissertation suggest that a number of factors influence the fate of chromium in soil systems including the carbon source available, the presence of electron shuttles and iron minerals. The in-depth analysis of the bacterial community at a simulated low-level waste site indicated the presence of cellulosic waste influenced the bacterial community structure. Specifically, the Actinobacteria and Bacteroidetes phyla were identified as accounting for the majority of changes with soil depth at the site. Both of these phyla contain members that are known for their cellulose-degrading capabilities. Multiple isolation techniques led to the acquisition of a variety of microorganisms some of which were capable of cellulose degradation and/or chromium reduction. Nine of twelve bacterial isolates were members of the Actinobacteria and Bacteroidetes phyla including Arthrobacter sp. isolate EF01. Isolate EF01 was found to be capable of utilizing numerous potential cellulose degradation products provided as carbon sources for growth. Results indicated that the carbon source present influenced Cr(VI) toxicity and reduction capabilities. Isolate EF01 was capable of growing in the presence of and reducing Cr(VI) when lactate or sugars such as glucose and sucrose were provided. However, if alcohols or some organic acids, such as ethanol and butyrate, were supplied isolate EF01 was unable to grow or reduce Cr(VI). This carbon metabolism-linked toxicity was also observed for Arthrobacter
"aurescens" TC1 suggesting this may be a more common toxicity mechanism among *Arthrobacter* spp. While the specific mechanism(s) through which chromium interacts with these organisms has yet to be identified, it is possible that chromium may interact with enzymes involved in carbon metabolism, such as the oxidoreductases involved in Acetyl-CoA biosynthesis, the transcriptional regulators of these enzymes or the enzyme cofactors. Studies performed with another environmental isolate, *Cellulomonas* sp. strain ES6, also indicated that the carbon source available influenced Cr(VI) reduction rates. Other constituents of soil systems such as humics and iron minerals, influenced Cr(VI) reduction rates by strain ES6 as well.

The interactions between microorganisms, soil constituents and chromium are complex in the subsurface. The results presented in this dissertation further demonstrate the complexity of these interactions. Studies assessed the interactions between environmentally relevant organisms, chromium and potential soil constituents at two DOE sites: the RWMC site at Idaho National Laboratory and the Hanford Site. Multiple approaches were taken to assess these interactions of which all provided insight on what factors influence the fate of chromium in these two soil systems. Bench-scale laboratory physiological and metal transformation studies assessed the fundamental interactions between soil constituents such as carbon source, a pure culture isolate and chromium. Molecular analyses assessed the bacterial community of a simulated low-level waste site on a larger-scale of which results generated hypotheses about the interactions between the bacterial community and the cellulosic waste present at the site.
The influence of carbon source on the fate of chromium in soils received the most in-depth analyses in this dissertation and results suggested that carbon metabolism by environmental *Arthrobacter* spp., plays a significant role in their ability to grow in the presence of and reduce Cr(VI). As *Arthrobacter* spp. are commonly isolated from DOE contaminated sites, it has been proposed that further attention should be placed on their role in biological remediation of contaminants at these sites (Henne et al., 2009a). To date, it is not known whether *Arthrobacter* spp. (including isolate EF01) are dominant or are playing a significant metabolic role especially in regards to metal reduction at the INL or Hanford sites. However, as isolate EF01 was obtained from the INL site, and other related organisms such as *Arthrobacter* sp. FB 24 have been obtained from other chromium contaminated sites (Henne et al., 2009a), it is possible that they are involved in chromium transformations at these sites. The results from the studies presented in chapters 4 and 5 provide insight into their potential role in the biological *in situ* remediation of hexavalent chromium. The results from the studies in chapters 4 and 5 also demonstrate the importance of characterizing an environmentally derived organism’s metabolic capabilities under environmentally relevant conditions. For example, metal toxicity and reduction studies are typically carried out through the use of a complex media or one single carbon source such as glucose. The utilization of nutrient rich media does not simulate the site characteristics from which a specific organism was obtained and may provide misleading information regarding its potential role in the environment. Evaluation of chromate toxicity to *Arthrobacter* sp. isolate EF01 when a commonly provided carbon source such as glucose, would suggest the organism is capable of
growing in the presence of and reducing Cr(VI). While this would not be incorrect, these results do not address the carbon dependent toxicity and reduction capabilities observed in the studies presented. Unfortunately, if a carbon source such as ethanol was added to stimulate microbial growth and metal reduction in situ, *Arthrobacter* sp. isolate EF01, and likely other *Arthrobacter* spp., would not be stimulated nor participate in Cr(VI) reduction. Therefore, in order to better understand the fate of chromium in soil systems, it is necessary to address the interactions between microorganisms and chromate under site-specific conditions.

**Recommendations for Future Studies**

**Characterization of Cold Test Pit South Site Isolates**

The characterization work presented in Chapter 2 focused on three of the facultative bacterial isolates obtained from the Cold Test Pit South site at Idaho National Laboratory: *Cellulomonas parahominis*, *Serratia* sp. and *Paenibacillus* sp. Cellulose degradation capability of insoluble cellulose sources were difficult to perform. Mostly due to the low biomass yields and high detection limits of the methods used (which include protein assays, HPLC analysis, and DNA stains) the interference of cellulose with the detection method and the difficulties with the reliable and consistent removal of attached cells from the cellulose source. In the future, degradation capabilities should be evaluated not by biomass, but rather by the change in cellulose crystallinity, measuring products formed over time or production of cellulase enzymes (Lynd et al., 2002; Bayer et al., 2006). Each of these methods presents its own challenges and optimization of
these methods could not be completed in time for the completion of this dissertation.

Specifically, the optimization of HPLC-based methods for measuring degradation products including organic acids are worth pursuing further as even when biomass was unable to be measured, product formation was detectable, but not quantifiable. *C. parahominis* was the only isolate capable of anaerobic degradation of filter paper on its own, though it was unable to do so aerobically. Preliminary HPLC analysis results indicated that potential products observed were acetate, fumarate and lactate under these conditions. However, biomass and product yields were low and degradation occurred very slow (approximately 2 months). This is still a unique quality and deserves further investigation; therefore, additional studies could be conducted to optimize growth and product yields. *Cellulomonas* spp. have been shown to be capable of performing cellulose degradation under both aerobic and anaerobic conditions (Lynd et al., 2002). However, *C. parahominis* isolated from the Cold Test Pit South site was only capable of carrying out cellulose degradation anaerobically. This is surprising as there is more potential energy through the degradation of cellulose utilizing oxygen as the terminal electron acceptor than performing this fermentatively under anaerobic conditions. As discussed in Chapter 1, aerobic degradation and anaerobic degradation are carried out using different enzyme “machinery”. Lacking the ability to degrade cellulose aerobically suggests it does not need to do so in the simulated low-level waste environment from which it was isolated. This is interesting both in respects to the cellulose degradation capabilities of this genus as well as the role this organism may be playing in the environment and could be further investigated.
For all three isolates, microbial growth and Cr(VI) reduction capabilities were different when grown aerobically or anaerobically. These capabilities were originally evaluated using glucose as the sole carbon source. Besides *Arthrobacter* sp. isolate EF01, *Serratia* sp. was the only other isolate that was evaluated based on its ability to utilize potential cellulose degradation products for growth in the presence and absence of 50 µM Cr(VI) (Chapter 2). This should be repeated for the other isolates as these carbon sources represent more environmentally relevant energy sources. Additionally, they could be conducted both aerobically and anaerobically in order to gain a better understanding how the presence and absence of oxygen influences the role carbon source utilization plays on Cr(VI) toxicity and reduction by the isolates. As soil environments are heterogeneous, including with respect to oxygen, the changes in these interactions will be important in evaluating their potential role in the environment. Interestingly, the results of *Serratia* sp. grown in the presence and absence of 50 µM Cr(VI) aerobically were similar to the findings observed for *Arthrobacter* sp. isolate EF01. The carbon source present for growth influenced Cr(VI) toxicity and reduction by the isolate. Specifically, when either isolate EF01 or *Serratia* sp. grew in the presence of glucose or lactate, they were capable of both growing in the presence of and reducing Cr(VI). If ethanol or an organic acid such as acetate or butyrate were present, no significant growth or Cr(VI) reduction was observed for either organism. As isolate EF01 and *Serratia* sp. are not closely related (isolate EF01 is an *Actinobacteria* while *Serratia* sp. is a *Gammaproteobacteria*), this suggests the chromate toxicity mechanism may potentially be more wide-spread among bacteria. This should be further explored through
experiments similar to those discussed in Chapters 4 and 5 with *Arthrobacter* spp. These studies should also be conducted both aerobically and anaerobically to evaluate how oxygen influences these interactions as *Serratia* sp. is a facultative anaerobe.

**Bacterial Community Interactions with Cellulosic Waste**

Results of the molecular analysis of the bacterial community within the simulated low-level waste environment suggested the presence of the cellulosic waste influenced the bacterial community structure (Chapter 3). These results provide an in-depth analysis of which bacteria were present at the site, but as they were DNA-based they do not address which bacteria were metabolically active. Future studies should aim to link the metabolic activities, specifically metal reduction and cellulose degradation, to the bacterial community members present. If additional soil cores were obtained, RNA-based analyses should be performed for each of the layers. Simultaneous extraction of DNA & RNA from the samples could be performed using a modified procedure from (Hurt et al., 2001) which has been successful for extraction of DNA and RNA from these soils in previous preliminary studies (procedure in Appendix I). DNA and RNA (converted to cDNA) could be used for various 16S rRNA gene analyses such as PhyloChip or pyrosequencing. This will allow for a comparison between the organisms that are present with those that are metabolically active. Additionally, GeoChip (a functional gene microarray) analyses could be used to identify the cellulases and metal reducing genes present and metabolically active in the system. The GeoChip has probes for 292 functional groups which include carbon cycling and metal resistance with probes specific to genes involved in cellulose, hemicelluloses and lignin degradation as well as
chromium resistance (He et al., 2010). The simultaneous DNA and RNA extraction procedure as well as the preliminary results from the RNA-based GeoChip analyses can be found in Appendix I. Lastly, as results suggested that the Actinobacteria and Bacteroidetes phyla were responsible for most of the changes observed with depth at this site, primers specific for these phyla and possibly families of interest within these phyla, should be used to determine in which layers they are active and through the use of quantitative PCR their abundance could be determined.

The molecular analyses could also be strengthened through the addition of biogeochemical data. For each layer soil cores should be evaluated for parameters such as pH, oxygen, water content, iron, cellulose degradation products (such as sugars and organic acids) and cellulose content. The results of these biogeochemical analyses could be compared with the microbial community results through multivariate analysis such as Canoco (Van Nostrand et al., 2009; Xie et al. 2011). Overall, these analyses would provide valuable information regarding the interactions between the metabolically active bacteria, cellulosic waste and biogeochemical parameters of the environment. Results will provide a more comprehensive analysis of how these interactions may affect metal mobility at this site and potentially other low-level waste sites.

Carbon Source Dependent Chromate Toxicity to Arthrobacter spp.

The research presented in chapters 4 and 5 indicated that the carbon source present influenced growth of Arthrobacter sp. isolate EF01 and Arthrobacter aurescens TC1 as well as their reduction capabilities in the presence of Cr(VI). The specific mechanism through which chromium is interacting with carbon metabolism in
Arthrobacter spp. has not been identified even though progress was made in developing testable hypotheses regarding these interactions such as those discussed in chapter 5. It is possible that chromium, in some form, is interacting with enzymes involved in carbon metabolism, transcriptional regulators of these enzymes or enzyme cofactors.

Specifically of interest for further investigation are those enzymes involved in Acetyl-CoA biosynthesis including the pyruvate dehydrogenase complex enzymes, aldehyde dehydrogenase and alcohol dehydrogenase. Enzyme assays could be performed to determine if chromium as Cr(VI) or Cr(III) affects the activity of these oxidoreductases. Additionally, protein analyses could be conducted in the presence and absence of Cr(VI) or Cr(III) through the use of HPLC, 2-D Gels or NMR similar to the studies conducted by Henne et al. (2009b). If Acetyl-CoA biosynthesis is inhibited and A. aurescens TC1 needs to convert pyruvate to oxaloacetate, additional studies could be performed assessing the expression levels and protein abundances of the pyruvate carboxylase as this enzyme catalyzes the reaction between pyruvate and oxaloacetate. The aldehyde dehydrogenase warrants further investigations as it is the only enzyme that plays a role in five of the carbon metabolism pathways through which carbon and energy flow is likely stalled in the presence of Cr(VI). Lastly, in order to narrow down where carbon metabolism may be stalled when alcohols or some organic acids are the sole carbon sources provided, metabolite identification could be conducted using HPLC analysis.

Other potential chromium toxicity mechanisms that could not be ruled out were the interactions with electron transfer cofactors, such as NADH, and the interaction between chromium and transcriptional regulators of the oxidoreductases. Enzyme assays
with NADH could be conducted to determine if chromium inhibits activity. While no common transcriptional regulator was found for all of the oxidoreductases of interest, future studies could look for similarities between the regulators themselves, such as structural similarities as it possible that more than one may be affected.

Besides the identification of the chromate toxicity mechanism, additional Cr(VI) toxicity studies should be conducted in which multiple carbon sources are provided at once to determine how this influences Cr(VI) toxicity. As cellulose is often broken down into more than one product this would better simulate what would occur in the environment. Preliminary studies were conducted with *Arthrobacter* sp. isolate EF01 in which ethanol and glucose were provided as carbon sources. The results suggest that the presence of more than one carbon source does influence Cr(VI) toxicity and reduction (Appendix F). Experiments similar to those conducted in chapters 4 and 5 should be conducted. In addition to measuring microbial growth and Cr(VI) concentrations over time, the carbon sources should be monitored (most likely through HPLC analysis or enzyme assays) in order to determine how the organism is metabolizing the carbon sources available and how this affects Cr(VI) toxicity.

**Combined Influence of Carbon Source, Electron Shuttles and Iron Minerals on Cr(VI) Reduction**

The results presented in Chapter 6, recently submitted for publication in *Chemosphere*, demonstrate that many soil constituents including the carbon source, iron minerals and electron shuttles such as humics can influence Cr(VI) reduction by an environmental isolate, *Cellulomonas* sp. strain ES6. One unique aspect of these
experiments was that they focused on the combined influence of these constituents which simulates environmental conditions more closely. This study provided valuable insight into the combined influence of a carbon source, iron mineral and electron shuttle on Cr(VI) reduction of strain ES6 and which of these more significantly influenced Cr(VI) reduction rates. These fundamental interactions could now be expanded on. For example, mixed systems containing more than one iron mineral or multiple electron shuttles could be evaluated. The experimental setup would be similar to those conducted in Chapter 6. Growth should be evaluated over time as well either through direct counts or plate counts as absorbance measurements will not be due to the presence of solid-phase iron minerals. Also, similar to iron, chromium should be measured over time as Cr(VI) and total Cr in solution. This data would be helpful in better understanding the fate of chromium in these systems and would support the findings in the XANES analysis (presented in Chapter 6). Lastly, a Cellulomonas parahominis isolate was obtained from the Cold Test Pit South site. These experiments could be repeated with isolate to determine if the same trends are observed and to better assess how the presence of the carbon sources, iron minerals and electron shuttles used in the study influence Cr(VI) reduction rates by this organism as well.
References


APPENDIX A

VANENGELEN ET AL. (2010)
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**UO_2^{2+}** SPECIATION DETERMINES URANIUM TOXICITY AND BIOACUMULATION IN AN ENVIRONMENTAL PSEUDOMONAS SP. ISOLATE

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(Submitted 8 July 2009; Returned for Revision 10 August 2009; Accepted 23 October 2009)

Abstract—In the present study, experiments were performed to investigate how representative cellulosic breakdown products, when serving as growth substrates under aerobic conditions, affect hexavalent uranyl cation (UO_2^{2+}) toxicity and bioaccumulation within a *Pseudomonas* sp. isolate (designated isolate A). Isolate A taken from the Cold Test Pit South (CTPS) region of the Idaho National Laboratory (INL), Idaho Falls, ID, USA. The INL houses low-level uranium-contaminated cellulosic material and understanding how this material, and specifically its breakdown products, affect U-bacterial interactions is important for understanding UO_2^{2+} fate and mobility. Toxicity was modeled using a generalized Monod expression. Bovine, dextrose, ethanol, and lactate served as growth substrates. The potential contribution of bicarbonate species present in high concentrations was also investigated and compared with toxicity and bioaccumulation patterns seen in low-bicarbonate conditions. Isolate A was significantly more sensitive to UO_2^{2+} and accumulated significantly more UO_2^{2+} in low-bicarbonate concentrations. In addition, UO_2^{2+} growth inhibition and bioaccumulation varied depending on the growth substrate. In the presence of high bicarbonate concentrations, sensitivity to UO_2^{2+} inhibition was greatly mitigated, and did not vary between the four substrates tested. The extent of UO_2^{2+} accumulation was also diminished. The observed patterns were related to UO_2^{2+} aqueous complexation, as predicted by MENDOQ (ver. 2.52) (Eason, PA, USA). In the low-bicarbonate medium, the presence of positively charged and unstable UO_2^{2+}-hydroxide complexes explained both the greater sensitivity of isolate A to UO_2^{2+} and the ability of isolate A to accumulate significant amounts of UO_2^{2+}. The exclusive presence of negatively charged and stable UO_2^{2+}-carbonate complexes in the high bis-carbonate medium explained the diminished sensitivity of isolate A to UO_2^{2+} toxicity, and limited ability of isolate A to accumulate UO_2^{2+}. Environ. Toxicol. Chem. 2010;29:763–769. © 2010 SETAC

Keywords—Uranium Low-level waste Speciation Toxicity Bioaccumulation

INTRODUCTION

Uranium (U) is a widely distributed subsurface contaminant at several Department of Energy (DOE) sites [1], and across the globe [2]. A sites where molecular oxygen is present, most U in the aqueous phase will be in the form of the hexavalent uranyl cation (UO_2^{2+}). Low-level waste (LLW) sites, including the Idaho National Laboratory (Idaho Falls, ID, USA) [3] and the Drigg site (Cumbria, NW England, UK) [4], are among U-contaminated sites housing buried contaminated cellulosic material, such as paper towels, cardboard, and lab coats. These materials, and specifically their breakdown products, can potentially contribute to both UO_2^{2+} immobilization and mobilization. For example, these breakdown products include short-chain fatty acids that can potentially enhance UO_2^{2+} mobility through chelation [5–7]. Mechanisms of UO_2^{2+} immobilization include UO_2^{2+} accumulation within biomass metabolizing the cellulose and cellulosic breakdown products [8–10]. In its reduced valence state (U(IV)), uranium can readily precipitate as UO_2, which exhibits limited mobility and toxicity [11,12]. The ability of iron- and sulfate-reducing bacteria to couple oxidation of organic substrates to U(IV) reduction under anaerobic conditions plays a prominent role in many uranium bioremediation schemes [13]. However, many LLW sites are aerobic, including the INL and Drigg sites [3,4], and, therefore, UO_2^{2+} reduction, an anaerobic process, is not expected to play a large role in decreasing uranium mobility in these systems.

Bacterial bioaccumulation, which could potentially contribute to UO_2^{2+} immobilization, has been studied extensively in laboratory settings as a means of immobilizing toxic metals, including Cd, Pb, Cu, Zn, and UO_2^{2+} [14]. A considerable amount of research has investigated this phenomenon as a potential component of bioremediation schemes in aerobic systems [15–17]. Among the factors that have been shown to influence bioaccumulation are the conditions under which the bacteria are grown, including choice of buffer and carbon source [18,19].

Understanding the impact such growth conditions have on UO_2^{2+} bacterial bioaccumulation processes in LLW sites is important, given the range of carbon and energy sources made available during cellulosic breakdown [20]. In addition, understanding how these conditions affect UO_2^{2+} toxicity is equally important because bioaccumulation will affect UO_2^{2+} mobility.
significantly only if native bacteria are able to reach appreciable numbers in the U-contaminated system. While specific mechanisms of UO$_2^{2-}$ toxicity to bacteria are yet to be described, UO$_2^{2-}$ is known to inhibit bacterial growth generally through oxidative stress [21]. The purpose of this study was to investigate how model cellulose breakdown products, including butyrate, dextran, ethanol, and lactate, when used as primary carbon and energy sources, affect UO$_2^{2-}$ bioaccumulation and toxicity in a Pseudomonas sp. isolate (hereafter referred to as isolate A) cultured from the Cold Test Pit South (CTPS) at the INL. Previous studies have concluded that UO$_2^{2-}$ toxicity and bioaccumulation potential is strongly influenced by complexation and speciation of the UO$_2^{2-}$ [22-23], and, therefore, when possible, differences in toxicity and bioaccumulation patterns were associated with changes in UO$_2^{2-}$ speciation.

**MATERIALS AND METHODS**

**Media composition and growth conditions**

Isolate A, the only Pseudomonas sp. collected, was isolated from the CTPS at the INL by repeated re-streaking of a non-fat liquid enrichment on agar methylcellulose (0.1%) plates. Cells were grown from frozen (−80°C) glycerol stocks (20% v/v) at 20°±0.5°C in chemically defined liquid media (pH = 7.0) containing of simulated INL groundwater [24] containing the following (per L): 1.0 mg KCl, 12.7 mg Na$_2$SO$_4$, 3.5 mg CaCl, 1.1 mg MgSO$_4$, 0.825 g NH$_4$Cl, 0.261 g K$_2$HPO$_4$, amended with 5 mL of Wolfe’s vitamin solution [25], and 1 mL SL-4 trace elements solution (25]. Unamended medium was sterilized by autoclaving at 121°C for 25 min, and the vitamins and trace-metal solutions were filter sterilized (0.2 μm). Because cellulose breakdown can release a variety of organic molecules which can potentially serve as carbon and electron sources for native bacteria [20], representative carbon substrates were chosen from four general categories: butyrate as a model carboxylate, ethanol as an alcohol, dextran as a sugar, and lactate, a fatty acid commonly used in studies involving UO$_2^{2-}$-microbe interactions. Carbon sources were added to a concentration of 15 mM carbon. Carbon dioxide (CO$_2$) is the ultimate cellulosic breakdown product, which in contaminated systems would exist in equilibrium with several bicarbonate species in the aqueous phase. While the presence of bicarbonates is expected regardless of metabolic activity, the equilibrium between the aqueous phase and atmospheric CO$_2$, the CO$_2$ generated by the activity of the native bacteria could potentially lead to an excess of bicarbonate species in the system. The influence of excess bicarbonate was therefore investigated by repeating each growth experiment with 10 mM NaHCO$_3$ added to the media, making eight possible growth conditions. Media with added NaHCO$_3$ is referred to as high-bicarbonate media, and media in equilibrium with atmospheric CO$_2$ as low-bicarbonate media. Media pH maintained at pH = 7.0 and was buffered with 10 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPS).

**U bioaccumulation experiments**

The ability of isolate A to accumulate UO$_2^{2-}$ was measured in each of the medium combinations described above. Cells were grown, harvested by centrifugation (6,000 g for 20 min), washed three times in fresh media, then added to Teflon screw-cap vials to give a concentration of 80±5 mg protein/L (measured using the Bradford assay [26]). The cells were allowed to incubate for 1 h (allowing the cells to resume growth, as measured by optical density at 600 nm). 50 μL of depleted UO$_2^{2-}$ (as UO$_2$Cl$_2$, International Bio-Analytical Industries) was then added to the cell cultures. Preliminary experimentation carried out in our lab demonstrated the UO$_2^{2-}$ bioaccumulation reaches equilibrium after 10 min, at which point UO$_2^{2-}$ concentrations in filtered samples (0.2 μm) were measured with a kinetic phosphorescence analyzer (KPA, ChemChek Instruments). Cells were then centrifuged (6,000 g for 5 min) and washed five times with a 100 μM EDTA (pH = 5.5) solution to remove loosely bound UO$_2^{2-}$ [27]. Cells were then digested in 50% HNO$_3$ and UO$_2^{2-}$ concentrations were measured with a KPA. Cell-free, heat-killed cells (exposed to 80°C for 15 min), UO$_2^{2-}$ free, and carbon-free controls served as comparisons.

**U toxicity experiments and modeling**

The sensitivity of isolate A to UO$_2^{2-}$ under each of eight growth conditions was investigated. Cells were grown in the same medium to be tested and allowed to reach late exponential growth phase. Cells were washed (three cycles of centrifugation at 6,000 g for 20 min) and suspended in 50 mL of fresh medium. Growth in high-bicarbonate media took place in 125 mL serum bottles sealed with butyl stoppers and crimped with aluminum seals. Growth in low-bicarbonate media took place in 250 mL baffled shaker flasks. UO$_2^{2-}$ concentrations (as uranyl chloride, UO$_2$Cl$_2$, International Bio-Analytical Industries) ranged from 0 to 250 μM. Cultures were shaken at 100 rpm at 20°±0.5°C. Liquid samples were periodically removed to measure protein concentrations using the Bradford assay [26]. UO$_2^{2-}$ inhibition of growth was modeled using the following generalized Michaelis-Menten expression demonstrated in Levenspiel Equation 1 [24]:

$$\frac{\mu}{\mu_0} = \left(1 - \frac{[\text{UO}_2^{2-}]}{[\text{UO}_2^{2-}]_0}\right)^{-n}$$

where $\mu$ is the first order growth rate in the presence of UO$_2^{2-}$, $\mu_0$ is the growth rate in UO$_2^{2-}$ free medium, and [UO$_2^{2-}$] is the concentration in μM in the medium. The [UO$_2^{2-}$]$_{100}$ value corresponds to the theoretical minimum [UO$_2^{2-}$] that completely inhibits growth. The exponent, n, is referred to as the toxic power. The quotient on the left side of Equation 1, hereafter referred to as the relative inhibition, was plotted against the UO$_2^{2-}$ concentration to generate toxicity curves. Cell-free and carbon-free controls were performed in parallel. The [UO$_2^{2-}$]$_{100}$ and n values were calculated according to Levenspiel [28]. The calculated [UO$_2^{2-}$]$_{100}$ values were confirmed experimentally (data not shown). Experiments were performed in triplicate. Cell-free and carbon-free controls were performed in parallel.

**U speciation modeling**

Uranium speciation in substrate-free media was determined using Visual MINTEQ, ver. 2.52. The partial pressure of atmospheric CO$_2$ was incorporated into the model and assumed to stay constant at 38.5 kPa. Due to a lack of published thermodynamic data, PIPES buffer was not included in the modeling. Incorporation of the four substrates was also
excluded, again due to a lack of published thermodynamic data for all substrates. Thermodynamic data were available for butyrate and lactate, but those substrates were not predicted to alter UO$_2^{2-}$ speciation significantly (data not shown).

Statistical analysis

All reported values represent the mean of triplicate experiments, and error bars correspond to 95% confidence intervals. Single factor ANOVA was performed on the calculated parameters. Results were determined to be significantly different if $p < 0.05$.

RESULTS AND DISCUSSION

UO$_2^{2-}$ toxicity and modeling results

Figure 1 shows the growth curves of isolate A on each of the four substrates over a range of [UO$_2^{2-}$] (0–150 μM) in low-bicarbonate media. With the exception of ethanol as a substrate, cells tolerated [UO$_2^{2-}$] up to 50 μM without significant inhibition, and when grown on either butyrate or dextrose, [UO$_2^{2-}$] up to 100 μM showed only moderate inhibition both in terms of growth rate and cell yield. Cultures grown on lactate were significantly inhibited by 100 μM UO$_2^{2-}$. Remarkably, cells grown on ethanol were sensitive to [UO$_2^{2-}$] as low as 0.1 μM, a concentration which falls below the United States Environmental Protection Agency (U.S. EPA) prescribed drinking water limit of 0.126 μM. To our knowledge, this is the first reported example of submicromolar UO$_2^{2-}$ concentrations inhibiting microbial growth.

Equation 1 was used to model the results obtained for each substrate, the results of which are shown graphically in Figure 2. Important parameters are summarized in Table 1. The toxicity of UO$_2^{2-}$ to isolate A was dependent on the carbon source tested, as indicated by the significantly different $n$ terms. However, the three [UO$_2^{2-}$]$_{50}$ values associated with growth on butyrate, dextrose, and lactate were not found to be statistically different ($p < 0.05$) and averaged 154 μM. Growth on ethanol produced a dramatically different [UO$_2^{2-}$]$_{50}$ value of 1.0 ± 0.22 μM. The impact of the toxic power can be seen when the IC$_{50}$ values are compared between the four substrates (Table 1). As $n$ approaches zero, the inhibition curve becomes more sharply concave downward and the IC$_{50}$ approaches UO$_2^{2-}$$_{\text{add}}$. As $n$ approaches 1, the inhibition curve becomes more linear, and IC$_{50}$ approaches 5 [UO$_2^{2-}$]$_{\text{add}}$. For $n > 1$, which is the case for the ethanol system, the corresponding inhibition curve becomes concave upward as IC$_{50}$ approaches zero. For this reason, the IC$_{50}$ values did vary significantly between all four substrates. Cells grown on butyrate had the highest IC$_{50}$ (138 ± 8 μM) followed by dextrose (103 ± 3 μM), lactate (91 ± 6 μM) and ethanol (0.19 ± 0.04 μM) (Table 1). Butyrate,

![Graph of growth curves](image)

Fig. 1. (a-c): Growth curves of isolate A in low carbonate media measured in hours on butyrate (a), dextrose (b), and lactate (c) over a range of UO$_2^{2-}$ concentrations (○ = UO$_2^{2-}$ free; □ = 50 μM UO$_2^{2-}$; △ = 100 μM UO$_2^{2-}$; ◇ = 150 μM UO$_2^{2-}$; ▲ = 200 μM UO$_2^{2-}$). Panel (d) shows growth curves of isolate A in low carbonate media on ethanol (○ = UO$_2^{2-}$ free; □ = 0.10 μM UO$_2^{2-}$; △ = 0.15 μM UO$_2^{2-}$; ◇ = 0.25 μM UO$_2^{2-}$; ▲ = 0.50 μM UO$_2^{2-}$; ▲ = 1.00 μM UO$_2^{2-}$). Carbon source had a significant effect on UO$_2^{2-}$ toxicity, particularly when ethanol served as carbon source, which led to growth inhibition at [UO$_2^{2-}$] concentrations as low as 0.10 μM UO$_2^{2-}$. Error bars represent 95% confidence intervals of triplicate measurements.


**UO^{2+}** speciation modeling results

The significant impact of bicarbonate on UO^{2+} toxicity to isolate A can be partially explained using MINEQ speciation modeling (ver. 2.52) (Table 2). These results are similar to previous studies (23,29-31), where UO^{2+} toxicity was mitigated by the presence of tightly binding UO^{2+} ligands, including bicarbonate species (23,30), and dissolved organic carbon (DOC) (28), both of which can form stable UO^{2+} complexes (29). Stability constants for UO^{2+}-bicarbonate complexes range from 8.0 to 20.0, compared with the stability constants of UO^{2+}-hydroxide complexes which range from 3.0 to 5.0 (32). The bioavailability of UO^{2+}, and thus toxicity, appears to correlate with stability of the UO^{2+} complexes present in solution. It is expected that UO^{2+} toxicity will depend heavily on the abundance of weakly bound UO^{2+}-hydroxide complexes which appear to interact more readily with important cellular functions.

In high-bicarbonate media, MINEQ predicted that UO^{2+} would be most present as UO_{2}CO_{3}^{2-} (45% of total component concentration), UO_{2}CO_{3} (35% of total component concentration), and CO_{3}UO_{2}CO_{3} (41% of total component concentration). The balance of the UO^{2+} (14% of total component concentration) was present as either phosphate complexes (UO_{2}HPO_{4} and UO_{2}PO_{4}) or minor bicarbonate complexes (UO_{2}CO_{3}OH, UO_{2}CO_{3}^{2-}, and CO_{3}UO_{2}CO_{3}^{2-}) (Table 2). In low-bicarbonate media, MINEQ predicted that UO^{2+} aqueous equilibrium speciation would be dominated by UO_{2}CO_{3}CO_{3}^{2-}, accounting for 84% of the total component concentration, followed by UO_{2}CO_{3} (16% of total component concentration), and UO_{2}HPO_{4} (2.5% of total component concentration). However, 25% of the total component concentration was predicted to be present as unbound hydroxide complexes, which were not present in the high-bicarbonate system. Consistent with previous studies, isolate A appears less sensitive to aqueous UO^{2+} ligand binding to bicarbonate and phosphite complexes than to UO^{2+} present as less stable hydroxide complexes. Even though a relatively small amount of the UO^{2+} present was weakly bound by hydroxides, the difference in associated bioavailability appears to cause the overall UO^{2+} toxicity to be significantly enhanced, particularly to the cells growing on ethanol.

**UO^{2+} bioaccumulation results**

Parallel experiments were performed to investigate the influence of carbon source and added bicarbonate on UO^{2+} bioaccumulation (Fig. 4). Previous studies have demonstrated the stability of Pseudomonas spp. to rapidly accumulate

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**Table 1**: Summary of the kinetic parameters describing UO^{2+} toxicity toward isolate A in each medium combination

<table>
<thead>
<tr>
<th>Bicarbonate</th>
<th>Carbon source</th>
<th>Total power (a)</th>
<th>UO^{2+}_{Inh} (μM)</th>
<th>Calculated OS (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>Bicarbonate</td>
<td>6.13 ± 0.10</td>
<td>128 ± 11</td>
<td>138 ± 8</td>
</tr>
<tr>
<td></td>
<td>Bicarbonate</td>
<td>6.01 ± 0.09</td>
<td>123 ± 5</td>
<td>163 ± 5</td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>6.75 ± 0.07</td>
<td>121 ± 6</td>
<td>91 ± 6</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>5.1 ± 0.20</td>
<td>126 ± 23</td>
<td>106 ± 6</td>
</tr>
<tr>
<td>High</td>
<td>All sources</td>
<td>6.57 ± 0.16</td>
<td>237 ± 20</td>
<td>152 ± 14</td>
</tr>
</tbody>
</table>

*The UO^{2+}_{Inh} values correspond to the theoretical maximum (UO^{2+}) that completely inhibits growth. The exponent, a, is referred to as the total power. Analysis of variance was carried out on all calculated a values for both the low and high carbonate systems. Between the different substrates, the a values associated with the low carbonate systems were found to be significantly different (a = 0.05). Using the same a value, all a values associated with the high carbonate systems were not significantly different, and thus the average of all a values are reported.*
significant amounts of UO$_2^{2+}$ [18,19]. The results of the present study showed that between the filtered samples and the digested cells, virtually all the UO$_2^{2+}$ originally added could be recovered. This suggested that a minimal amount of UO$_2^{2+}$ was being washed off the cell surface, and that virtually all UO$_2^{2+}$ was accumulated within the cell. Figure 4 shows the concentration of UO$_2^{2+}$ accumulated (μmol UO$_2^{2+}$/mg-protein) where, consistent with previous results [33], the results showed the type of carbon source had a significant impact on UO$_2^{2+}$ accumulation. In low-bicarbonate media, actively metabolizing cells of isolate A accumulated between 56 and 88% of the UO$_2^{2+}$ present. Cells metabolizing dextrose accumulated the most UO$_2^{2+}$ (0.56 μmol/mg-protein).

Table 2. MINTEQ modeling results showing the expected U(VI) species present in the carbonate limited and excess carbonate systems.

<table>
<thead>
<tr>
<th>Species</th>
<th>Low bicarbonate</th>
<th>High bicarbonate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of total component</td>
<td></td>
</tr>
<tr>
<td></td>
<td>concentration</td>
<td>% of total component</td>
</tr>
<tr>
<td>UO$_2$(OH)$_2^{+}$</td>
<td>0.20</td>
<td>NP</td>
</tr>
<tr>
<td>UO$_2$(OH)$_2^{+}$</td>
<td>1.4</td>
<td>NP</td>
</tr>
<tr>
<td>UO$_2$(OH)$_2^{+}$</td>
<td>0.65</td>
<td>NP</td>
</tr>
<tr>
<td>UO$_2$(OH)$_2^{+}$</td>
<td>0.02</td>
<td>NP</td>
</tr>
<tr>
<td>UO$_2$(OH)$_2^{+}$</td>
<td>0.23</td>
<td>NP</td>
</tr>
<tr>
<td>UO$_2$(OH)$_2^{+}$</td>
<td>2.4</td>
<td>0.03</td>
</tr>
<tr>
<td>UO$_2$(OH)$_2^{+}$</td>
<td>10.2</td>
<td>0.12</td>
</tr>
<tr>
<td>UO$_2$(OH)$_2^{+}$</td>
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*NP* denotes species not present. In the presence of excess carbonate, UO$_2^{2+}$ is mostly present as tightly bound carbonate or phosphate complexes, which might explain the apparent lower bioavailability of UO$_2^{2+}$. By contrast, the carbonate limited media is predicted to contain a number of loosely bound UO$_2^{2+}$-carbonate complexes, which might account for the relatively higher bioavailability of UO$_2^{2+}$ in these systems.

while the lactate system accumulated the least (0.35 μmol/mg-protein). Cells metabolizing butyrate and ethanol accumulated 0.44 μmol UO$_2^{2+}$/mg-protein and 0.40 μmol UO$_2^{2+}$/mg-protein, respectively. Carbon-free and heat-killed controls accumulated the least amount of UO$_2^{2+}$ (0.17 and 0.36 μmol UO$_2^{2+}$/mg-protein, respectively). This suggests that actively metabolizing cells accumulate significantly more UO$_2^{2+}$, which is consistent with a previous study which used granular biomass for UO$_2^{2+}$ removal from aqueous systems [9].

Bioaccumulation of UO$_2^{2+}$ by isolate A in the high-bicarbonate system was significantly affected by carbon source as well (Fig. 4). Among the metabolizing cells, those in the dextrose medium accumulated the most UO$_2^{2+}$ (0.12 μmol UO$_2^{2+}$/mg-protein), while cells metabolizing lactate accumulated the least (0.066 μmol UO$_2^{2+}$/mg-protein). However, on average, metabolizing cells only accumulated 20% as much UO$_2^{2+}$ compared with cells in low-bicarbonate media. The carbon-free and heat-killed controls accumulated the least amount of UO$_2^{2+}$ (0.054 and 0.062 μmol UO$_2^{2+}$/mg-protein, respectively).

The limited ability of isolate A to accumulate UO$_2^{2+}$ in high-bicarbonate media can be explained using speciation modeling results obtained by MINTEQ (ver. 2.52) (Table 2). In both systems, MINTEQ predicted that UO$_2^{2+}$ aqueous equilibrium speciation would be dominated by neutrally or negatively charged bicarbonate and phosphate complexes. As described earlier, in low-bicarbonate media, MINTEQ predicted that UO$_2^{2+}$ aqueous equilibrium speciation would include uranyl-hydroxide complexes, absent in high-bicarbonate media. These complexes included neutral species (UO$_2$(OH)$_2^{+}$), negatively charged species (UO$_2$(OH)$_2^{+}$), and, further unique to this system, positively charged species, including UO$_2$(OH)$_3^{+}$, (UO$_2$(OH)$_2^{+}$), and (UO$_2$(OH)$_2^{+}$) (Table 2). While only predicted to be a small percentage of the total UO$_2^{2+}$ in the system at equilibrium (2.25%), the presence of these positively charged species could explain the increased potential for bioaccumulation when the negative zeta potential of Pseudomonas spp. isolates is taken into consideration [34,35]. Positively charged UO$_2^{2+}$ complexes will be electrostatically attracted to the surface of the cells. This is in contrast to the high-bicarbonate media, in which none of the UO$_2^{2+}$ is predicted to be present as a positive complex and therefore not electrostatically attracted to the cells.
In addition, electrostatic considerations can explain the relatively minimal accumulation observed for heat-killed and carbon-limited cells. Recent experiments with *Pseudomonas aeruginosa* (ATCC 10145) showed that starved and dead cells had no negative zeta potentials compared with metabolically active cells [34]. This difference may partially explain why starved and killed cells would accumulate less positively charged species. Similar reasoning explains why starved and heat-killed cells accumulated relatively more 

$\text{UO}_2^{2-}$ in the presence of high bicarbonate compared to 

$\text{UO}_2^{2-}$ accumulated in the absence of added bicarbonate (Fig. 4). On average, heat-killed and carbon-limited cells accumulated only $74 \pm 17\%$ and $65 \pm 26\%$ of the amount of 

$\text{UO}_2^{2-}$ accumulated by metabolizing cells, respectively, in high-bicarbonate media. In the low-bicarbonate media, heat-killed and carbon-limited cells accumulated only $37 \pm 2\%$ and $40 \pm 2\%$ of the amount of 

$\text{UO}_2^{2-}$ accumulated by metabolizing cells, respectively. Positively charged 

$\text{UO}_2^{2+}$ species are less electrostatically attracted to killed and starved cells, as will negatively charged species be less electrostatically repulsed from killed and starved cells.

Electrostatic considerations do not explain the influence of carbon source on 

$\text{UO}_2^{2-}$ accumulation, because in both high- and low-bicarbonate media cells, metabolizing dextrose accumulated the most 

$\text{UO}_2^{2-}$. Substrate binding to 

$\text{UO}_2^{2-}$ also does not appear to explain the observed accumulation patterns, as there is no correlation between the extent of accumulation in cells metabolizing a particular substrate, and the ability of that substrate to form stable complexes with 

$\text{UO}_2^{2-}$. For example, of the four substrates only lactate and butyrate form complexes with 

$\text{UO}_2^{2-}$, in low-carbonate media cells metabolizing these substrates accumulated the least and second most 

$\text{UO}_2^{2-}$. However, metabolism does appear to impact 

$\text{UO}_2^{2-}$ bioaccumulation in isolate A.

The 

$\text{UO}_2^{2-}$ speciation modeling results explained many of the toxicity and bioaccumulation patterns observed between high- and low-bicarbonate systems. Our results show between the two systems an 80% decrease in 

$\text{UO}_2^{2-}$ bioaccumulation potential corresponded with a 40% increase in 

$\text{UO}_2^{2-}$ tolerance. However, this trend was not conserved across sub- strates within either the high- or low-bicarbonate systems. If a strong correlation between accumulation and toxicity existed, it would be expected that conditions that lead to more 

$\text{UO}_2^{2-}$ accumulation also lead to proportionately greater 

$\text{UO}_2^{2-}$ toxicity. However, in low-bicarbonate media, cells metabolizing ethanol accumulated only 24% less 

$\text{UO}_2^{2-}$ than cells metabolizing dextrose, but the IC$_50$ of cells growing on ethanol was only 0.2% that of cells growing on dextrose. In high-bicarbonate media, bioaccumulation depended on the carbon sources tested, but toxicity did not, indicating that 

$\text{UO}_2^{2-}$ accumulation in the presence of 10 mM bicarbonate has a negligible effect on toxicity. While adding bicarbonate reduced both bioaccumulation and toxicity, it did not do so proportionately (the twofold increase in accumulation led to a less than twofold reduction in 

$\text{UO}_2^{2-}$ toxicity). To our knowledge, no studies, in which correlations between 

$\text{UO}_2^{2-}$ toxicity and accumulation in bacterial cells have been published. The results of the present study are in contradiction to a study of 

$\text{UO}_2^{2-}$ and a *Chlorella* sp. which found that a twofold increase in intracellular 

$\text{UO}_2^{2-}$ resulted in a roughly twofold increase in 

$\text{UO}_2^{2-}$ toxicity [31].

**CONCLUSIONS**

The effect of cellulose breakdown products on 

$\text{UO}_2^{2-}$ toxicity and bioaccumulation within an environmental *Pseudomonas* sp. isolate (isolate A) under aerobic conditions was investigated. Among the breakdown products considered, the presence of high bicarbonate concentrations was found to have the most significant impact on both 

$\text{UO}_2^{2-}$ toxicity and bioaccumulation. 

$\text{UO}_2^{2-}$ was found to be more toxic in systems with low bicarbonate species concentrations, a result consistent with the predicted presence of unstable 

$\text{UO}_2^{2-}$-hydroxide complexes. This was especially true when cells were grown on ethanol. Under these conditions, cells were inhibited by 

$\text{UO}_2^{2-}$ concentrations which fell below the U.S. EPA drinking water limits. In media with high bicarbonate concentrations, 

$\text{UO}_2^{2-}$ was predicted to be present mostly as stable 

$\text{UO}_2^{2-}$-bicarbonate complexes, which were found to be less toxic due to their limited bioavailability. Cells in low-bicarbonate media were found to yield more 

$\text{UO}_2^{2-}$ bioaccumulation. This was largely attributed to electrostatic effects predicted based on 

$\text{UO}_2^{2-}$ speciation data. Given the potential impact of 

$\text{UO}_2^{2-}$ speciation on bacterial – 

$\text{UO}_2^{2-}$ interactions, efforts aimed at controlling 

$\text{UO}_2^{2-}$ speciation might prove to be worthwhile when developing 

$\text{UO}_2^{2-}$ bioaccumulation strategies. While 

$\text{UO}_2^{2-}$ toxicity and bioaccumulation patterns could largely be explained through MINTEQ speciation modeling results, the final relationship between 

$\text{UO}_2^{2-}$ toxicity and bioaccumulation remains unclear and is the subject of ongoing research.

**Acknowledgments**—The authors at Montana State University gratefully acknowledge the financial support provided by the U.S. Department of Energy, Office of Science, Environmental Remediation Science Program (ERSP) contract DE-FG02-06ER64576. The INL portion of the work was supported by the U.S. Department of Energy, Assistant Secretary for the Office of Science, ERSP, under DOE-NE Idaho Operations Office contract number DE-AC07-05ID14517. Laboratory facilities and support were provided by the Chemical and Biological Engineering Department and the Center for Biofilms Engineering at Montana State University.

**REFERENCES**


4992.
11. Rodion EB, Schlieph TE. 2005. Conceptual and numerical model of uranium(VI) reductive immobilization in fractured subsurface sedi-
12. Sani RK, Peyton BM, Smith WA, Apel WA, Petersen JN. 2002. Denitrification reduction of Cr(VI), Fe(III), and U(VI) by Cellulosamx
overproduction of alkaline phosphatase in three subsurface Famaean-
ma isolates. FEMS Microbiol Ecol 41:115–123.
19. Steenweg GW, Sivamurthi JL, Stirling EE, Parrett JR. 1981. Microbial cells as biosorbent for heavy metal accumulation of uranium by
Streptomyces coelicarina and Pseudomonas aeruginosa. Appl Environ
21. Harvey B, Beshah AI, Saez Y, McAlpine HH, Anderson GL. 2005. Whole-
1238.
91:397–401.
Raton, FL, USA.
31. Franklin NM, Storber IL, Markelk S, Lim JP. 2000. pH-dependent toxicity of copper and uranium to a tropical freshwater alga (Chlorococcum
sp.). Aquatic Toxicol 46:275–289.
chemical thermodynamics of Uranium, Neptunium, Plutonium, Ameti-
33. Malekandalo F, Lattik AM, Shukumari M, Levin M, Colwell RR. 2002. Effects of selected physical and chemical parameters on uranium uptake
34. Smos BE, Gneso D, Engwall MA, Macchini B. 1999. Surface physicochemical properties of Pseudomonas fluorescens and impact on
APPENDIX B

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Influence of carbon sources and electron shuttles on ferric iron reduction by *Cellulomonas* sp. strain ES6

Robin Gerlach · Erin K. Field · Sridhar Viamajala · Brent M. Peyton · William A. Apel · Al B. Cunningham

Received: 18 September 2010/Accepted: 31 January 2011
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Abstract Microbially reduced iron minerals can reductively transform a variety of contaminants including heavy metals, radionuclides, chlorinated aliphatics, and nitroaromatics. A number of *Cellulomonas* spp. strains, including strain ES6, isolated from aquifer samples obtained at the U.S. Department of Energy’s Hanford site in Washington, have been shown to be capable of reducing Cr(VI), TNT, natural organic matter, and soluble ferric iron (Fe(III)). This research investigated the ability of *Cellulomonas* sp. strain ES6 to reduce solid phase and dissolved Fe(III) utilizing different carbon sources and various electron shuttling compounds. Results suggest that Fe(III) reduction by and growth of strain ES6 was dependent upon the type of electron donor, the form of iron present, and the presence of synthetic or natural organic matter, such as anthraquinone-2,6-disulfonate (AQDS) or humic substances. This research suggests that *Cellulomonas* sp. strain ES6 could play a significant role in metal reduction in the Hanford subsurface and that the choice of carbon source and organic matter addition can allow for independent control of growth and iron reduction activity.

Keywords Fermenters · Anthraquinone-2,6-disulfonate (AQDS) · Humics · HFO · Ferrhydrite · Goethite · Magnetite · Maghemite · Hematite

Introduction

The reductive transformation of oxidized contaminants by ferrous iron, Fe(II), has received increasing interest in the recent years. Electron transfer from Fe(II) usually occurs fast and is non-specific so that a broad spectrum of compounds can react with surface-associated and soluble Fe(II). This makes Fe(II)-based remediation technologies an attractive alternative or addition to traditional cleanup strategies. Potentially treatable contaminants include heavy metals such as
Cr(VI), radionuclides such as U(VI), chlorinated aliphatics such as carbon tetrachloride (CT) and trichloroethylene (TCE) as well as nitroaromatics such as 2,4,6-trinitrotoluene (TNT) (Eary and Rai 1988; Erbs et al. 1999; Amonette et al. 2000; Fredrickson et al. 2000b; Lee and Batchelor 2002a, b; Holstetter et al. 2003; Borch et al. 2005).

In subsurface environments, Fe(II) can be produced chemically or biologically and engineered in situ remediation schemes utilizing the reactivity of subsurface Fe(II) have been demonstrated (Heijman et al. 1995; Yin and Allen 1999; Chilikapiti et al. 2000). Remediation based on the biological reduction of ferric [Fe(III)] minerals has been suggested as a low-cost technology for heavy metal and radionuclide immobilization at US Department of Energy (DOE) and other sites (Istok et al. 1999). It has been shown that a wide variety of bacteria, including but not limited to iron-respiring, fermenting, sulfate-reducing, halorespiring, and methanogenic bacteria, are able to release dissolved and solid phase ferric iron, either directly or indirectly, in the presence of electron shuttling compounds (Lovley et al. 1991; Benz et al. 1998; Cervantes et al. 2002).

Past research regarding biological Fe(III) reduction has focused mainly on Gram-negative metal-reducers such as Geobacter, Shewanella and Desulfuromonas spp. (Nealson and Saffarini 1994; Roden and Zachara 1996; Zachara et al. 1998; Lovley and Blunt-Harris 1999; Dong et al. 2000; Newman 2001; Liu et al. 2002; Royer et al. 2002a; Saffarini et al. 2002). In general, Gram-positive fermenters have received much less attention for their potential role in Fe(III) reduction and contaminant remediation. Previously, isolates were obtained from contaminated and uncontaminated subsurface locations at the DOE site in Hanford, Washington that were capable of Cr(VI) reduction (Smith et al. 2002; Viamajala et al. 2007). Of the nine isolates obtained, eight were Gram-positive of which four were from the Cellulomonas genus. Additional studies with three of the Cellulomonas isolates—strain ES5, strain WS01 and strain WS18, demonstrated their ability to reduce U(VI) as well as soluble Fe(III) using lactate as the electron donor (Sani et al. 2002). Finally, bench scale column experiments using Cellulomonas sp. strain ES6 indicated prolonged periods of Cr(VI) reduction in columns containing microbially reduced hydrous ferric oxide (HFO) via abiotic reduction of Cr(VI) by surface associated Fe(II) and regeneration of Fe(II) through microbial reduction by strain ES6 (Viamajala et al. 2008). This study continued to focus on strain ES6 in order to further investigate relevant microbial reduction mechanisms. In specific, the direct and indirect reduction of Fe(III) minerals by strain ES6 was assessed in order to better understand the potential for Fe(II)-based remediation through stimulation of indigenous bacteria at the Hanford site.

The choice of electron donor for Fe(III) reduction will likely determine the growth of a particular group of organisms and is important in order to achieve effective Fe(II) production to maintain electron flow necessary for biologically mediated contaminant reduction. The electron donor that is ultimately selected for field use will likely depend on regulatory approval, cost, availability, and ability to sustain growth of the microorganisms of interest.

While microorganisms can directly reduce Fe(III) through the addition of an electron donor, Fe(III) can also be reduced indirectly through the use of electron shuttles such as naturally occurring organic matter or the addition of quinone moieties. Previous studies have already shown that the presence of an electron shuttle can increase Fe(III) reduction rates and extent (Fredrickson et al. 1998, 2000a; Lovley et al. 1998; Lovley and Blunt-Harris 1999; Nevin and Lovley 2000; Royer et al. 2002b; Jiang and Kappler 2003; Bauer and Kappler 2009; Wolf et al. 2009). To design and implement effective Fe-based bioremediation strategies, it is important to clearly understand interactions of site-specific bacteria, such as strain ES6, with the numerous types of iron minerals that might be present (Lovley and Phillips 1986a; Lovley 1997).

The goal of these studies was to determine the influence of various carbon sources (electron donors) and electron shuttling compounds, such as synthetic and natural organic matter, on the growth and Fe(III) reduction capabilities of Cellulomonas sp. strain ES6 (henceforth referred to as strain ES6) of dissolved and solid phase Fe(III).

Materials and methods

Experimental

Organism and culture conditions

Strain ES6 was maintained in frozen stock cultures containing tryptic soy broth (TSB, 30 g/l). Difco
Laboratories) with 20% glycerol at -70°C. Cells were pre-cultured in TSB for 24 h on a horizontal shaker at 150 rpm and 30°C, transferred into fresh TSB, and grown again for 18 h in TSB (each 1% initial inoculum). Cultures were then harvested via centrifugation (5,860 x g, 20 min, 4°C), washed in oxygen-free synthetic groundwater (SGW), and re-suspended in SGW to the desired cell concentration.

**Media composition**

Synthetic groundwater (SGW) modified from Petersen et al. (1994) was used for all experiments. The final concentrations of the constituents are listed in Table 1. Sodium metalosate, sodium carbonate, sodium sulfate, yeast extract, and casamino acids were dissolved in deionized (DI) water and autoclaved at 121°C. The autoclaved solution was boiled under an oxygen-free atmosphere of N₂/CO₂ (80:20) for 10 min and cooled to room temperature under the same oxygen-free atmosphere to avoid re-dissolution of atmospheric oxygen. Sterile, oxygen-free stock solutions of sodium bicarbonate, potassium chloride, calcium chloride, magnesium hydroxide, and the trace minerals were added. The medium was dispensed into Balch tubes (used as experimental reactors as described below) under the N₂/CO₂ atmosphere to maintain oxygen-free conditions.

**Iron minerals**

Goethite (α-FeOOH, BET specific surface area (SSA) 16.7 m²/g), magnetite (γ-Fe₂O₃, SSA 43.9 m²/g), hematite (α-Fe₂O₃, SSA 7.8 m²/g), and maghemite (Fe₃O₄, SSA 6.6 m³/g) were obtained from Sigma Aldrich (Ward Hill, MA) with purities of 97% or greater. Hydrous ferric oxide (HFO, SSA 34.5 m²/g) was synthesized using the procedure of Lovley and Phillips (1986b). In brief, 13.8 g FeCl₃ (Fisher Scientific) was dissolved in 400 ml of deionized water and the solution pH was slowly adjusted to 7.0 using NaOH. The resulting HFO suspension was diluted in SGW and used for the experiments within 1 month. The concentrations of total iron in the experiments were between 1.74 and 3.58 mM depending on iron mineral and experimental goal. Stock solutions of Fe(III)-citrate (50 mM) and Fe(III)-NTA (100 mM) were prepared by dissolving 1.225 g of Fe(III)-citrate (Sigma, St. Louis, MO) and 1.62 g FeCl₃ with 1.91 g NTA in 70 ml of deionized water, respectively. The Fe(III)-citrate solution was heated until dissolved and the pH of both solutions was slowly adjusted to 6.5 using NaHCO₃. Both solutions were sparged with oxygen-free nitrogen gas, transferred into an oxygen-free glovebag, stirred overnight, filled to a total volume of 100 ml each, and finally filtered sterilized. It was determined that less than 1% of the Fe present in these solutions and suspensions was Fe(II).

**Batch experiments, Fe(III) reduction**

Oxygen-free SGW in Balch tubes containing iron minerals was amended with a carbon source, anthraquinone-2,6-disulfonate (AQDS, Sigma, St. Louis, MO), Elliott soil humic acid standard (International Humic Substances Society, MN, USA), and finally inoculated with strain ES6 before the tubes were crimp sealed using thick butyl rubber stops.
(Belco Glass, Vineland, NJ). All manipulations were performed under an oxygen-free N₂/CO₂ atmosphere, and all additions were made using sterile, purged syringes and needles. The carbon sources investigated were added from oxygen-free stock solutions and included acetate, lactate, glycerol, xylene, sucrose, and molasses (“Brer Rabbit Molasses—Unsulphured”, B&G Foods Inc., Roseland, NJ). All carbon sources were added from 1 M oxygen-free stock solutions to a final concentration of 10 mM. Molasses, which has a high content of sucrose (Prescott and Dunn 1983) was added from a concentrated stock solution to a final molasses concentration of 3.12 g/l. The initial bacterial concentrations were between $2.23 \times 10^7 \pm 2.08 \times 10^7$ and $6.73 \times 10^7 \pm 2.48 \times 10^7$ colony forming units (CFU) per ml (equivalent to between 25 and 770 mg per litre of protein) depending on the experiment. Experiments were performed in triplicate with appropriate controls lacking bacteria, carbon source, AQDS, or humic substances. The vials were incubated statically at ambient temperature in the dark, sampled periodically using purged, sterile syringes and needles, and analyzed to determine the concentration of ferrous and total iron, protein, CFU, substrate, and metabolites as described below.

**Analytical**

**Iron quantification**

Total iron (Fe$_{tot}$) and Fe(II) were determined using the ferrozine method. For Fe(II) analysis, sample aliquots were extracted with 0.5 or 2.5 N HCl. Extractions for Fe$_{tot}$ were performed in the additional presence of 0.25 M NH$_4$OH as a reductant. Commonly, the extraction time was 2 h but in some cases longer extraction times were used to verify complete extraction (Cooper et al. 2000; Royer et al. 2002b). Aliquots of acid extracts were added to a solution of 1 g ferrozine (Sigma, St. Louis, MO) in 11 HEPES buffer at pH 7 and the absorbance was determined spectrophotometrically at 540 nm (EL 808 Microplate Reader, BIO-TEK Instruments, Winooski, VT).

**Colony forming units (CFU) and protein**

The concentration of CFU was determined after serial dilution of 0.1 ml aliquots in phosphate buffered saline solution (pH 7). Aliquots were spread onto tryptic soy agar (TSA, 40 g/l, Difco Laboratories), incubated for 48 h at 30°C, and colonies were counted. No statistically significantly different counts were observed if plates were incubated for 72 h or longer. Total protein was measured by a modification of the Coomassie method (Bradford 1976). Cells were disrupted by mixing 0.5 ml of sample with 0.5 ml of 2 N NaOH. The mixture was heated for 30 min at 95°C. After cooling the sample to ambient temperature, the pH was lowered to approximately 2 by adding 0.167 ml of 6 N HCl. To this solution, 1 ml of Coomassie Plus reagent (Pierce, Rockford, IL) was added and the absorbance was measured at 595 nm on a UV-vis spectrophotometer (Milton Roy Company Spectronic® GENESYS 5™, Rochester, NY). The assay was calibrated using 2 g/l bovine serum albumin fraction V protein standard (Pierce, Rockford, IL) diluted to a concentration range of 0–30 mg/l protein.

**Carbohydrate and fatty acid analysis**

Saccharose, glucose, fructose, glycerol, and lactate were quantified using a Dionex DX-300 ion chromatograph equipped with a Dionex PA10 column and a pulsed amperometric detector. Samples (10 µl) were injected into a mobile phase consisting of 52 mM NaOH pumped at a flow rate of 1.2 ml/min. Lactate, acetate, formate, propionate, iso-butyrate, and n-butyrate were analyzed using the same ion chromatograph equipped with a AS10 column, 25 µl sample loop, and conductivity detector. The eluent consisted of a 3.5 mM potassium tetraborate solution pumped at a flow rate of 1 ml/min.

**Results**

Carbon source and electron acceptor influence on growth

Figure 1 shows the extent of bacterial growth [measured as change in protein (Δ protein)] for lactate-, xylene-, and sucrose-amended ES6 cultures in the presence of several potential electron acceptors including Fe(III)-NTA, Fe(III)-citrate, HFO, fumarate, and oxygen. Protein data suggest that after 21 days xylene supported more bacterial growth than
**Fig. 1** Change in protein after 21 days of incubation of strain ES6 in the presence of lactate, xylose, sucrose, and several different potential electron acceptors. The electron acceptors tested included Fe(III)-NTA, Fe(III)-citrate, hydrous ferric oxide (HFO), fumarate, and 

sucrose regardless of the terminal electron acceptor used, while lactate supported bacterial growth only if oxygen was the terminal electron acceptor. For all three carbon sources tested, the greatest increase in protein was observed when oxygen was the terminal electron acceptor. When any of the other electron acceptors were used, no significant differences in growth were observed between the electron acceptor-free control and the other potential electron acceptors. These results suggest that both the electron donor and acceptor influence bacterial growth.

**Carbon source influence on Fe(II) production**

Both, xylose and sucrose, as electron donors supported some, though very little, reduction of hydrous ferric oxide (HFO) or Fe(III)-citrate by strain ES6 as compared to uninoculated controls after 14 days (Fig. 2). The presence of 100 μM AQDS significantly increased both the rate and extent of HFO reduction regardless of which carbon source was present. After 14 days, approximately 90% of the Fe(III) present had been reduced to Fe(II) by strain ES6 when AQDS was present and xylose was the carbon source utilized; only 7% had been reduced to Fe(II) in the absence of AQDS. When sucrose was the carbon source 61% of the available Fe was reduced to Fe(II) in the presence of AQDS compared to only 4% when AQDS was absent. The slight reduction of HFO after longer incubation times might be due to the production and release of compounds by strain ES6, which allowed for some reduction of solid phase Fe(III) even in the absence of AQDS.

While the most Fe(II) was produced in the treatment containing xylose and AQDS after 14 days, significant amounts of Fe(II) were also produced when molasses, glycerol or sucrose were present as well as AQDS (Fig. 3). When acetate was utilized as a carbon source, minimal Fe(II) was produced regardless of whether or not AQDS was present. Significantly lower Fe(II) production was observed in AQDS-free treatments. Interestingly, if AQDS was present, xylose-amended treatments showed the greatest Fe(II) production (2.9 mM Fe(II)) but if AQDS was absent, molasses-amended treatments
showed the greatest Fe(II) production [0.92 mM Fe(II)] compared to the other carbon sources. When AQDS was absent, no significant amounts of Fe(II) were produced except when molasses was the carbon source utilized.

Neither lactate, glycerol, nor acetate appeared to support bacterial growth (protein, CFU, and optical density, data for lactate in the presence of 3 mM HFO can be seen in Fig. 1, other data not shown), but HFO reduction was observed in the presence of glycerol and lactate if 100 μM of AQDS were present. Sterile controls with each carbon source ± AQDS did not show significant Fe(III) reduction suggesting the reduction observed was not abiotic. Results suggest that bacterial growth was not necessary for Fe(III) reduction to occur. For each of the electron donors tested, Fe(II) production was lower in the absence of AQDS than in its presence. No statistically significant differences (t-test, P > 0.05) in biomass production were observed between AQDS amended and not AQDS amended treatments (data not shown). Hence, the increased production of Fe(II) in AQDS amended treatments was not the result of increased biomass production but was likely due to enhanced electron transfer from the bacterial cells to HFO.

These observations were reconfirmed in studies in which sucrose metabolism by strain ES6 was monitored in the presence and absence of HFO and AQDS. Substrate and metabolite measurements using ion chromatography indicated that lactate concentrations increased by approximately 25% in the presence of HFO and 40% in the presence of HFO with 100 μM AQDS (data not shown). Formate concentrations also increased approximately 10% in these treatments indicating a more complete oxidation of sucrose in the presence of HFO and HFO plus AQDS. The presence of 100 μM AQDS in the absence of HFO did not lead to any significant differences in metabolite patterns.

Electron shuttle influence on Fe(II) production

As AQDS was shown to increase HFO reduction rates, increasing AQDS concentrations were tested to determine if an increased concentration further increased HFO reduction rates. Results suggest increasing AQDS concentrations increased the rate of HFO reduction by strain ES6 in the presence of sucrose (Fig. 4). Initial Fe(III) reduction rates (Inset to Fig. 4), normalized by the cell concentration (CFU), increased approximately tenfold from 2.27 × 10^{-12} ± 2.23 × 10^{-13} μmol Fe(II)/CFU · h in the absence of AQDS to 3.31 × 10^{-11} ± 3.34 × 10^{-12} μmol Fe(III)/CFU · h in the presence of 500 μM AQDS. Cell-free control treatments containing the highest AQDS concentration tested in this research (500 μM) did not lead to significant HFO reduction again suggesting this was not an abiotic reaction.

Fe(II) reduction of different iron mineral phases

In the presence of 100 μM AQDS and sucrose as the electron donor, HFO was the most readily reducible iron mineral among the ones tested as at least 10 times more Fe(II) was produced when HFO was the iron mineral present compared to any others (Fig. 5). No significant differences in Fe(II) produced were observed between the other iron minerals until day

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Fig. 4 Influence of AQDS concentration on Fe(II) production from hydrous ferric oxide (HFO) by strain ES6 over time in the presence of sucrose. Inset: Zero order reaction rates normalized to cell number. Error bars represent ±one standard deviation (n = 3). Error bars are smaller than markers if not visible. Total amount of Fe(III) available in these experiments was 1.74 μmol/l.
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14. Results indicate 1.56 mM Fe(II) was produced from HFO after 14 days while only 0.23 and 0.15 mM Fe(II) was produced by maghemite and magnetite, respectively. Goethite and hematite were not significantly reduced over the 14 days period although slight reduction of these minerals was observed in long term experiments (>50 days) in the presence of AQDS (data not shown). It is possible that some of the Fe(II) produced in these experiments is originating from less crystalline Fe(III) phases present as impurities (up to 3%) in the purchased iron mineral phases.

Since magnetite (Fe₃O₄) already contains Fe(II) as part of its mineral structure, an increase in acid-extractable Fe(II) could have been due to an increase in extractability over time. However, controls containing magnetite but lacking strain ES6 or AQDS did not show an increase in acid-extractable Fe(II) over the duration of the experiment. Thus, results indicate the increase in 0.5 N HCl-extractable Fe(II) observed for magnetite is due to microbial reduction of ferric iron.

Dissolved Fe(III) reduction in the presence of an electron shuttle

While the enhancement of Fe(III) mineral reduction in the presence of AQDS has been described and attributed to enhanced electron transport between the bacterial cell and solid phase Fe(III) (Lovley et al. 1998; Newman 2001; Royer et al. 2002b), reports of electron shuttling compound-enhanced dissolved phase Fe(III) reduction appear to be rare. The results of these studies indicate that the presence of the electron shuttle AQDS drastically enhanced the reduction of Fe(III)-citrate by strain ES6 in the presence of sucrose as electron donor (Fig. 6). Reduction rates of Fe(III)-citrate were generally higher than for HFO, ranging from $9.85 \times 10^{-12} \pm 3.10 \times 10^{-13}$ μmol Fe(III)/CFU/h without AQDS to $7.34 \times 10^{-11} \pm 6.19 \times 10^{-12}$ μmol Fe(III)/CFU/h for systems containing 500 μM AQDS (inset to Fig. 6). These results indicate that soluble quinones, such as AQDS, can enhance the electron transport to solid phases and also to dissolved phase electron acceptors by strain ES6.

Fe(III) reduction in the presence of humic substances

While the addition of synthetic electron shuttling compounds such as AQDS to the environment might lack regulatory approval, the addition of natural organic matter such as humic and fulvic acids or humic substances should not encounter significant regulatory resistance. Hence, the influence of humic substances obtained from the International Humic Substance Society on the ability of strain ES6 to reduce HFO was also tested. Similar to the studies with AQDS, results demonstrate that the addition of the humic substances lead to an increase in Fe(II) production compared to controls lacking humic substances or lacking strain ES6 after 21 days (Fig. 7).

Discussion

Reductive transformation reactions for enhanced in situ bioremediation of oxidized environmental contaminants typically require an external electron donor. In most subsurface remediation scenarios, carbon sources such as those used in this study are considered the most convenient electron donor. The injection of readily available carbon can lead to biofuelling of the injection wells or the surrounding formation (Roberts et al. 1991; Sempriñi et al. 1991; Shouche et al. 1993). Hence, it is important to be able to control the permeability of injection wells and the surrounding formation while maximizing microbial
Fig. 6. Fe(II) production from Fe(III)-citrate by strain E56 in the presence of sucrose and different AQDS concentrations over time. Inset: Zero order reduction rates of Fe(II) production normalized to cell number. Error bars represent ± one standard deviation (n = 3). Error bars are smaller than markers if not visible. Total amount of Fe(III) available: 4.26 mmol/l.

Fig. 7. Fe(II) production over time from hydrous ferric oxide by E56 in the presence and absence of humic substances with sucrose as electron donor. Error bars represent ± one standard deviation (n = 3).

activity. In this case, maximizing Fe(III) reduction while limiting microbial growth. The choice of electron donor will depend on which indigenous organisms are present and the type of electron acceptor(s) present. Faculative anaerobes, such as the strain E56 investigated in this study, capable of respiration in the presence of oxygen and fermentation under anaerobic conditions can be selectively controlled by adding substrates that either do or do not support growth under the existing conditions.

Control of growth and Fe(III) reduction

Under anaerobic conditions, xylose appeared to support more growth than sucrose, while lactate and other short chain organic compounds supported little if any growth. Cellulomonas species are known to be capable of electron transport chain phosphorylation in the presence of oxygen (aerobic respiration) and substrate level phosphorylation (fermentation) in its absence (Stackebrandt et al. 2006). The energy yield is several times greater during aerobic respiration than during fermentation, thus explaining the lower amount of biomass produced in the absence of oxygen (Fig. 1). Lactate and other short chain organic compounds are not easily fermented explaining the lack of biomass production in their presence. In regards to lactate, these findings are consistent with Sani et al. (2002) who reported that lactate supported the reduction of NTA-chelated Fe(III) by Cellulomonas spp. under non-growth conditions, but anaerobic growth on Fe(III) as the terminal electron acceptor was not observed.

In the presence of oxygen, relatively inexpensive substrates such as lactate or glycerol as well as more complex substrates such as glucose, sucrose, or molasses can be used to build up biomass and consume oxygen. Once oxygen is consumed and no more biomass growth is desired, substrates can be added, which support reductive transformation reactions but no bacterial growth (e.g., lactate or glycerol). If additional growth of biomass is desired after oxygen has been consumed, sugars such as xylose or sucrose, or complex fermentable carbon sources such as molasses can be injected again.

Purified carbon sources, such as sucrose and xylose, are likely to be significantly more expensive than molasses, which often has sucrose as its main constituent (Prescott and Dunn 1983). Hence, molasses was tested in this study in order to include a fermentable carbon source with a high potential to be used in the field. In the absence of the electron shuttle
AQDS, molasses supported the greatest amount of Fe(III) reduction from HFO after 14 days (Fig. 3), which is important as the addition of AQDS may not be approved for in situ applications or be far too expensive. Molasses also supported significantly more growth, based on viable cell counts, than any of the other carbon sources tested with no significant difference between AQDS and non-AQDS supplemented treatments (data not shown). The greater Fe(II) production in the molasses amended treatments could thus potentially be explained by the observed increased biomass production. Based on our research, it is however more likely that some of the molasses constituents other than sucrose either more readily provided electrons for HFO reduction or enhanced the electron transfer from strain ES6 to HFO, i.e., functioned as an electron shuttle. Separate experiments conducted in our laboratories investigated the reductive transformation of Cr(VI) (manuscript in preparation) by strain ES6. These experiments provided evidence that the increased reduction rates in the presence of molasses were due to molasses constituents that increase electron transfer rates from the bacterial cells and not due to increased biomass production since the addition of molasses in these treatments significantly increased reduction rates without increasing biomass production rates over the duration of these experiments.

AQDS-enhanced Fe(III) reduction

AQDS and other synthetic quinones are often used as model compounds for the quinone moieties of humic substances and have been shown to enhance the reduction of amorphous and crystalline solid phase Fe(III) by a wide range of bacteria (Benz et al. 1998; Lovley et al. 1998; Cervantes et al. 2002; Dong et al. 2003; Kappler et al. 2004; Lutjens et al. 2004; Wolf et al. 2005). Our results demonstrate that the addition of AQDS and humic substances can significantly enhance the ability of strain ES6 to reduce solid phase Fe(III). The same is true for other Cellulomonas strains isolated from the Hanford site (data not shown).

The ability of strain ES6 to reduce HFO and Fe(III)-citrate increased with increasing AQDS concentrations, which indicates that the addition of compounds rich in quinone moieties could be used to enhance electron transfer processes in subsurface environments containing microbial communities capable of fermentation. The influence of iron chelating or electron shuttling compounds on the reduction of solid phase Fe(III) has been investigated by other researchers (Zachara et al. 1998; Lovley and Blunt-Harris 1999; Royer et al. 2002a; b; Saffarini et al. 2002; Turick et al. 2002) and the results of this study are similar to their findings.

The results of this study suggest that strain ES6 was capable of reducing solid phase Fe(III) to some extent and the amount of Fe(II) produced increased with the addition of an electron shuttle. While overall these findings are similar to those studies performed with other microorganisms, the rate and extent of Fe(II) produced from these solid phase iron minerals is lower than some well-studied iron reducing microorganisms including Shewanella spp. (Kostka and Nealson 1995; Roden and Zachara 1996; Zachara et al. 1998; Dong et al. 2000; Royer et al. 2002b). However, it was observed that all of these solid phase iron minerals were reduced to some extent by strain ES6 even in the absence of an electron shuttle such as AQDS which was not observed in the case of the iron-reducing bacterium Desmococcus radiodurans (Fredrickson et al. 2000a). Additionally, Benz et al. (1998) demonstrated that Propionibacterium freudenreichii, a fermenting bacterium, was only capable of reducing HFO, maghemite and hematite in the presence of AQDS. Together, these results suggest that strain ES6 may not be able to reduce Fe(III) to the same extent or at the same rate as most iron respiring bacteria, but it may possess a greater ability to reduce iron than other fermenting bacteria. Therefore, Cellulomonas species may play a valuable role in Fe(III) reduction and subsequent contaminant transformations in subsurface environments.

The exact mechanism of how different compounds enhance solid phase Fe(III) reduction is not always obvious since some compounds are capable of both chelation of Fe(III) and electron shuttling. Humic substances for instance are commonly believed to both, chelate iron and shuttle electrons (Lovley 1996; Lovley and Blunt-Harris 1999; Royer et al. 2002b). AQDS, a frequently used model compound in laboratory experiments, has been widely recognized as an electron shuttle only (Lovley et al. 1998; Newman 2001; Royer et al. 2002b). While Shyu et al. (2002) reported that AQDS can diffuse into the membrane of Shewanella oneidensis MR1 cells, the
mechanism of electron transfer from bacterial cells to AQDS is not completely understood.

Several groups have reported that fermenting bacteria are able to shift their fermentation patterns to slightly more oxidized compounds in the presence of humic substances, electron shuttling compounds, or other potential electron acceptors (Emde and Schink 1990; Benz et al. 1998). With sucrose as the substrate, strain ES6 produced approximately 25% more lactate in the presence of HFO and approximately 40% more if 100 μM AQDS were present in addition to HFO. The increased oxidation of sucrose in the presence of AQDS and HFO and the increased Fe(II) concentrations in these treatments indicate that more electrons can be transferred from sucrose to HFO in the presence of an electron shuttling compound. However, despite increased electron transfer to HFO and more oxidized fermentation products, no statistically significant difference in bacterial growth was observed. Assuming that the more extensive oxidation of sucrose in the presence of HFO or HFO and AQDS allowed strain ES6 to gain additional energy via substrate level phosphorylation, this additional energy was apparently not used to produce additional biomass. It is however possible that this additional energy was used to produce carbon storage compounds which would agree with observations made by Sani et al. (2002) and Viamajala et al. (2008).

The presence of quinone-like compounds capable of shuttling electrons, such as AQDS or humic substances, drastically increased the ability of strain ES6 to transfer electrons to solid phase Fe(III). While it has been shown that electron shuttling compounds such as AQDS can enhance the ability of several bacterial strains to reduce solid phase ferric iron, enhanced electron transfer from the bacterial cell to a dissolved electron acceptor has rarely been described. Figure 6 shows a significant increase in Fe(II) production rates from Fe(III)-citrate in the presence of AQDS. Borch et al. (2005) showed that the addition of AQDS can considerably enhance the rate of TNT transformation by strain ES6 and we have shown in separate experiments that AQDS also drastically increases Cr(VI) reduction rates by strain ES6 (manuscript in preparation). The electron transfer from the reduced form of AQDS to dissolved Fe(III), TNT, or Cr(VI) is basically instantaneous. The rate of electron transfer to solid phase Fe(III) is likely limited by the number of available reducible surface sites.

The addition of synthetic compounds, such as AQDS, capable of increasing electron transfer from bacterial cells to oxidized contaminants or terminal electron acceptors, such as Fe(III)-minerals, could have economic potential. At this point in time however, the addition of synthetic electron shuttling compounds such as AQDS might lack regulatory approval because the effects of AQDS on the environment have not been studied extensively. AQDS has been shown to be toxic to a tdC mutant of S. oneidensis MR1 (Shyu et al. 2002) and methanogenic bacteria (Cervantes et al. 2000) in millimolar concentrations but no other toxicological data appear to be available. Concentrations used in this study were all below 500 μM, and other authors have reported that AQDS concentrations in the 50–100 μM range are sufficient for maximum Fe(III) reduction stimulation (Lovley et al. 1998; Royer et al. 2002b).

Humic substance-enhanced iron mineral reduction

The addition of natural organic matter such as humic and fulvic acids or humic substances should not encounter significant regulatory resistance. Thus, the addition of humic substances or fractions thereof have the potential to be successfully used in the field to enhance electron transfer from bacterial cells to oxidized contaminants or oxidized minerals. Humic substances obtained from the IHSS stimulated Fe(III) production from HFO although to a lesser extent than AQDS. A slight production of Fe(II) was observed in uninoculated controls containing these humic substances and is believed to be based on the abiotic reduction of Fe(III) by reduced humic substance moieties, such as reduced quinone structures (Kappler et al. 2004). The extent of stimulation by natural or synthetic organic matter is likely to depend on the number and reactivity of quinone-like structures, although compounds without quinone groups have also been reported to increase electron transfer from bacterial cells to Fe(III)-minerals (Hernandez et al. 2004). Additionally, the size and solubility of electron shuttling compounds are likely to play a role, such that insoluble fractions of natural organic matter for instance might not be as efficient in shuttling electrons as soluble fractions.
Conversely, in a flowing environment, such as a contaminated aquifer, soluble fractions will likely be transported away from the treatment area with the flowing groundwater faster than fractions with low solubility and higher sorption tendency.

Conclusions

Important reactions involving dissolved and solid phase iron can be significantly influenced by fermenting, sulfate-reducing, and methanogenic bacteria (Benz et al. 1998; Cervantes et al. 2002; Kappler et al. 2004). This study demonstrates that the type of organic electron donor and other organic matter can significantly change the ability of Cellulomonas sp. strain ES6, a fermenting bacterium isolated from the Hanford site, to reduce dissolved and solid phase Fe(III).

It appears from our results that, while strain ES6 possesses a limited ability for Fe(III) reduction compared to iron respiring bacteria, it might possess a greater ability to reduce iron than other fermenting bacteria. This makes strain ES6 a potentially important organism for in situ contaminant remediation. Results demonstrate that carbon sources can be selected to control growth at least partly independently from Fe(III) reduction. In the potential application of in situ biobarriers in the field, this could be an important method for controlling the bacterial density and activity in subsurface environments and hence provide a mechanism to avoid the plugging of injection wells by gaining some control over subsurface bacterial growth.

While the reduction of Fe(III) minerals by respiratory metal-reducing bacteria such as Shewanella and Geobacter has been described extensively over the past decade, the importance of other genera on the iron cycle and contaminant transformations have received much less attention. Although Lovley (1987) reported that a large number of bacteria can reduce, but not grow on Fe(III) as their sole terminal electron acceptor, it was only recently accepted that these bacteria can have a significant influence on oxidation-reduction reactions in the environment. Based on the results presented here and recent results by Benz et al. (1998), Cervantes et al. (2002), Kappler et al. (2004), and Luitjen et al. (2004) we suggest that the presence of bacteria other than respiratory iron reducers, can be sufficient to achieve significant reduction of subsurface iron minerals especially in the presence of even very low concentrations of natural or synthetic organic matter. Reduced iron minerals could then serve to reductively transform oxidized contaminants such as Cr(VI), U(VI), trinitrotoluene, or chlorinated aliphatic compounds.

Acknowledgments The authors thank Kristy Weaver and Laura Jennings for their assistance in the laboratory. This research was supported by the U.S. Department of Energy, Office of Science, Environmental Management Science Program, under Grant No. DE-FOO2-03ER63582 and DOE-NE Idaho Operations Office Contract DE-AC07-05ID14517. Partial financial support was provided by a grant from the Inland Northwest Research Alliance (INRA) under contract MSU 002.

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APPENDIX C

CR(VI) AND U(VI) MOBILITY IN COLUMN STUDIES
The results presented in this appendix are the Cr(VI), U(VI) and pH summaries for column studies in which the wood waste soil layer from the Cold Test Pit South site was mimicked. Bacterial isolates were obtained from column effluents and are discussed in more detail in Chapter 2.

Figure C.1. Summary of effluent pH in all four columns over time. In columns 1-3 pH stabilized at approximately 7.0 after 25 pore volumes of CSS media flowed through for the remainder of the study while Column 4 effluent pH increased after 480 pore volumes and remained slightly higher than the other columns for the remainder of the study. This increase could not be correlated to any other parameter measured.
Figure C2. Summary of effluent pH through the first 94 pore volumes collected. A more rapid decrease in pH was observed in column 1 containing irradiated soil compared to the other columns. All column effluents stabilized at approximately 25 pore volumes.

Figure C3. Cr(VI) concentration in the column effluents over the first 5 pore volumes. Cr(VI) concentrations were below the detection limit (< 0.1 mg/L) for the remainder of the study. Results indicate that significantly less Cr(VI) leached out of the column that was run in batch mode before the study began compared to the other three columns. This suggests that the influx of water into the site and pooling in the wood waste soil layer may influence Cr(VI) mobility out of the site.
Figure C4. U(VI) concentration in the column effluents over the first 5 pore volumes. U(VI) concentrations decreased to below the detection limit (< 0.1 mg/L) in all columns by pore volume 209 and remained below detection limits for the remainder of the study. Column 4, run in batch mode before the start of the experiment, had two pulses of U(VI) leaching out of the column while the other columns only had one suggesting the influx of water in the site may influence U(VI) leaching out of the site. Column 3 had a higher peak of U(VI) leaching at pore volume 1.5 than columns 1 and 2.

Table C1. Percentage of Total Cr(VI) and U(VI) Leached Out of Each Column Mimicking a Low-Level Waste Site at the Idaho National Laboratory After 910 Pore Volumes.

<table>
<thead>
<tr>
<th></th>
<th>Column 1 (Irradiated Soil)</th>
<th>Column 2</th>
<th>Column 3</th>
<th>Column 4 (Batch Mode Start)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Cr(VI) Leached</td>
<td>33.1</td>
<td>45.9</td>
<td>45.9</td>
<td>14.8</td>
</tr>
<tr>
<td>% U(VI) Leached</td>
<td>13.4</td>
<td>7.2</td>
<td>10.2</td>
<td>83.1</td>
</tr>
</tbody>
</table>

A greater percentage of Cr(VI) leached from the columns that were not run in batch mode before flow began. This saturated conditions may have allowed for the reduction of Cr(VI) or the sorption of Cr(VI) to soil constituents within the column. The opposite effect was observed for U(VI) in which a greater percentage of U(VI) leached out of the column run in batch mode before flow began. This suggests that U(VI) is more easily leached from the soil under saturated conditions. These results provide insight into the potential of these metals leaching from a simulated low-level waste site.
APPENDIX D

SUPPLEMENTAL MATERIAL FOR CHAPTER 2 ISOLATE CHARACTERIZATION STUDIES
The results presented in this appendix are supplemental information for Chapter 2 in which the isolates obtained from the Cold Test Pit South at the Idaho National Laboratory are characterized. This data shows that both *Paenibacillus* sp. and *Serratia* sp. are capable of growing in the presence of and reducing Cr(VI).

![Graph](image)

Figure D1. Aerobic growth of *Paenibacillus* sp. on glucose in the presence and absence of 50 µM Cr(VI). Results indicate that *Paenibacillus* sp. can grow in the presence of 50 µM Cr(VI), though, a decreased maximum OD is observed. Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.
Figure D2. Cr(VI) concentrations after 78 hours when *Paenibacillus* sp. when glucose was provided for aerobic growth in the presence of 50 µM Cr(VI). Results indicate that *Paenibacillus* sp. was capable of reducing Cr(VI) as approximately 70% decrease in Cr(VI) concentration was observed. Error bars represent ± one standard deviation (n = 3).

Figure D3. Anaerobic growth of *Paenibacillus* sp. when glucose was provided in the presence and absence of 50 µM Cr(VI). Results indicate that *Paenibacillus* sp. can grow in the presence of 50 µM Cr(VI), though, an increased lag time is observed. Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.
Figure D4. Cr(VI) concentrations after 78 hours when *Paenibacillus* sp. was provided for anaerobic growth in the presence of 50 µM Cr(VI). Results indicate that *Paenibacillus* sp. was capable of reducing Cr(VI) under anaerobic conditions as a significant decrease in Cr(VI) concentration was observed. Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.

Figure D5. Aerobic growth of *Serratia* sp. when glucose was the sole carbon source in the presence and absence of 50 µM Cr(VI). Results indicate that *Serratia* sp. can grow in the presence of 50 µM Cr(VI) while an increased lag time was observed. Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.
Figure D6. Comparison of *Serratia* sp. growth (dark filled symbols) and Cr(VI) concentrations (white filled symbols) over time when glucose was the sole carbon source for anaerobic growth of *Serratia* sp. in the presence of 50 µM Cr(VI). Results indicate that growth by *Serratia* sp. does not occur until Cr(VI) concentrations approach zero. Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.
APPENDIX E

SUPPLEMENTAL MATERIAL FOR FIELD ET AL. (2010)
Supplemental Material

Table S1. Family-Specific Primer Pairs for Quantitative PCR

<table>
<thead>
<tr>
<th>Family</th>
<th>Primer Specificity</th>
<th>Primer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Primer Sequence (5'-3')</th>
<th>Primer Pair Annealing Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidimicrobiaceae</td>
<td>Acidimicrobiaceae</td>
<td>795f</td>
<td>TGGATACTAGGTGTGGCGGT</td>
<td>60</td>
</tr>
<tr>
<td>Bacteria</td>
<td></td>
<td>907r</td>
<td>CCGTCAATTCCTTTRAGTTT</td>
<td></td>
</tr>
<tr>
<td>Streptomycesaceae</td>
<td>Streptomycesaceae</td>
<td>126r</td>
<td>GCTTGTCAGTGAGCACAGG</td>
<td>65</td>
</tr>
<tr>
<td>Bacteria</td>
<td></td>
<td>8f</td>
<td>AGAGTGTGATCCTGGCTCAG</td>
<td></td>
</tr>
<tr>
<td>Flexibacteriaceae</td>
<td>Flexibacteriaceae</td>
<td>88r</td>
<td>TGTTACGCAACCCGATCGT</td>
<td>60</td>
</tr>
<tr>
<td>Bacteria</td>
<td></td>
<td>8f</td>
<td>AGAGTTGTGATCCTGGCTCAG</td>
<td></td>
</tr>
<tr>
<td>KSA Unclassified</td>
<td>KSA Unclassified</td>
<td>652r</td>
<td>ACCACGAATTCCACCGAC</td>
<td>60</td>
</tr>
<tr>
<td>Bacteria</td>
<td></td>
<td>533f</td>
<td>GTGCCAGCMGCCGCGTAA</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>General Bacteria primers: Lane 1991 (2)

Table S2. Number of Taxa Detected by Both Clone Library and PhyloChip Methods

<table>
<thead>
<tr>
<th>Taxonomic Rank (% cutoff)</th>
<th>Clone Library Only (%)</th>
<th>Both (%)</th>
<th>PhyloChip Only (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylum (≥ 80)</td>
<td>0 (0.0)</td>
<td>25 (61.0)</td>
<td>24 (39.0)</td>
</tr>
<tr>
<td>Class (≥ 85)</td>
<td>1 (1.2)</td>
<td>43 (53.1)</td>
<td>37 (45.7)</td>
</tr>
<tr>
<td>Order (≥ 90)</td>
<td>4 (2.5)</td>
<td>81 (51.3)</td>
<td>73 (46.2)</td>
</tr>
<tr>
<td>Family (≥ 92)</td>
<td>11 (3.8)</td>
<td>156 (54.4)</td>
<td>120 (41.8)</td>
</tr>
<tr>
<td>Subfamily (≥ 94)</td>
<td>16 (4.4)</td>
<td>221 (60.5)</td>
<td>128 (35.1)</td>
</tr>
<tr>
<td>OTU (≥ 97)</td>
<td>203 (10.1)</td>
<td>200 (10.0)</td>
<td>1599 (79.9)</td>
</tr>
</tbody>
</table>

Total unique sequences detected in all four soil layers by each method were compared at six taxonomic ranks. Percentages represent the percent that the clone library only, PhyloChip only, or both methods identified of the total unique sequences detected by both methods at the designated taxonomic rank. A total of 2002 unique OTUs were detected by the entire study. Of these, only 10% were detected by both the clone library and PhyloChip. Another 10% were detected by the clone library only while the remaining 80% were detected by the PhyloChip only. These results demonstrate the amount of diversity detected by the PhyloChip that the clone libraries did not detect.
However, the 203 unique OTUs detected by the clone library only is most likely due to the number of novel organisms that were absent from the database at the time the PhyloChip probes were designed, further supporting the use of these two methods together.

Detection of seven bacterial isolates by cultivation (isolation), PhyloChip analysis and clone library analysis were compared at the family level in the Fill Waste interface, Wood Waste, and Waste Clay interface soil layers. The *Paenibacillaceae* isolate was isolated only from the clay layer of which no molecular analysis was performed. Results suggest that the PhyloChip detected all of the families in all three soil layers. Meanwhile, the clone library detected some families, including *Enterobacteriaceae* and *Sphingobacteriaceae*, in soil layers from which they were not isolated suggesting either
we were unable to culture them even though they were present or that another member of
the family was present. In some soil layers a family was isolated but was not detected by
the clone library, such as the *Streptomycetaceae* isolate in the WW layer, which again
depicts the limited diversity that the clone library identified.

**Supplemental Isolation Methods.**

**Soil Sample Preparations.** 1.2g of soil obtained from either the Fill Waste Interface soil
layer, Wood Waste soil layer, Waste Clay Interface soil layer, or Clay soil layer
aseptically using a sterile spatula and added to 11mL sterile 0.85% NaCl and mixed.
This was not completed for the Fill layer soil as after molecular analyses there was not
enough soil to set up enrichments. 500 µL of soil slurry was added to aerobic
enrichments and 1 mL was added to anaerobic enrichments.

**Liquid Enrichments.** Aerobic and anaerobic enrichments contained a synthetic
groundwater medium (SGW) described by Borch et al. (2005) (1), without yeast extract,
and either Whatman No. 40 filter paper (Whatman, Inc., Florham Park, NJ) or methyl
cellulose (Sigma-Aldrich, St. Louis, MO) as the sole carbon source. The following
enrichments were set up using each of the four soil slurries prepared.

Aerobic enrichments were set up in 10 mL sterile screw cap test tubes and kept at
room temperature. Aerobic enrichments with filter paper contained 125 mg Whatman
No. 40 (Whatman, Inc., Florham Park, NJ) sterile filter paper, 500 µL of the soil slurry,
and SGW for a final volume of 5 mL. Aerobic enrichments with methyl cellulose
(Sigma-Aldrich, St. Louis, MO) contained a final concentration of 0.1% methyl cellulose,
500 µL of the soil slurry, and SGW for a final volume of 5 mL. Uninoculated sterile controls were also set up.

Anaerobic enrichments were set up in 40 mL sterile serum bottles that were sealed with butyl rubber stoppers and aluminum crimp seals. Enrichments were kept at room temperature. Anaerobic enrichments with filter paper contained 500 mg Whatman No. 40 sterile filter paper, 1 mL of the soil slurry, and SGW for a final volume of 10 mL. Anaerobic enrichments with methyl cellulose contained a final concentration of 0.1% methyl cellulose, 1 mL of the soil slurry, and SGW for a final volume of 10 mL. Uninoculated sterile controls were also set up.

Enrichments were monitored and 10% of the enrichment was transferred to new medium when they became visibly turbid. After two transfers to new media 100 µL of the final enrichment was spread plated onto direct isolation cellulose plates. Colonies that grew were re-streaked onto new cellulose plates until pure cultures were obtained.

**Direct Isolation Plates.** In order to try to isolate organisms on solid medium, 1 mL of each original soil slurry prepared was diluted to 10⁻¹, 10⁻², and 10⁻³ in sterile 0.85% NaCl. 100 µL of each dilution was spread plated onto plates containing 25g Gelrite Gellan Gum agar (Sigma-Aldrich, St. Louis, MO), 1.65g K₂HPO₄, 1.6g NH₄SO₄, 900mL DI Water, and 100mL 1% methyl cellulose sterile solution. Plates were stored at room temperature. This was repeated anaerobically in the glovebag after which plates were stored anaerobically in a GasPak Jar (BD Diagnostics, Franklin Lakes, NJ).

Colonies that appeared on any of the plates were re-streaked onto new cellulose plates until a pure culture was obtained.
**DNA Extraction, PCR Amplification, and Isolate Identification.** Seven bacterial isolates were obtained from the direct isolation plates and enrichments. DNA from the pure culture was extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH). Four primer sets were used to amplify different regions of the 16S rRNA gene: 8F primer (5’-AGAGTTTGATCCTGGCTCAG-3’), 533F primer (5’-GTGCCAGCMGCCGCGGTAA-3’), 907R primer (5’-CCGTCATTCTTTRAGTTT-3’), 1492R (5’-GGTTACCTTGTACGACTT-3’) (2). PCR amplification of 16S rRNA genes was performed using 20 µL reactions containing 1x PCR Master Mix (Promega, Madison, WI), 3.2 pmol/µL Forward primer, either 8F or 533F, 3.2 pmol/µL Reverse primer, 907R or 1492R. PCR reactions were heated to 94°C for 2 minutes, followed by 25 cycles of 94°C for 45 seconds, 55°C for 45 seconds, 72°C for 45 seconds with a final extension at 72°C for 7 minutes. Purified PCR products were sequenced at Idaho State University’s Molecular Core Research Facility. Reads were trimmed removing poor quality regions (Q<20) and contiguous sequences were assembled using Sequencher 4.5 software (Gene Codes Corporation, Ann Arbor, MI). The assembled sequence for each isolate was then analyzed and classified using the National Center for Biotechnology Information’s BLAST program (www.ncbi.nlm.nih.gov/BLAST).
Supplemental Figure 1. Soil core obtained from the Cold Test Pit South site from which soil samples were collected for analyses.
Supplemental Figure 2. Rarefaction curves for (A) the combined clone libraries ($n = 1719$) and (B) the combined PhyloChips ($n = 3282$ OTUs). Distance matrices were calculated in ARB using trimmed, aligned sequences (Clone Library) or NAST-aligned representative sequences (PhyloChip) and rarefaction curves were created using DOTUR with a phylotype cutoff of $\geq 93\%$ identity. The results of the rarefaction curve analyses suggest that neither the clone libraries nor the PhyloChip had reached diversity.
saturation, nor are very close to reaching saturation, in this study even with such large datasets. Still, the PhyloChip analyses are closer to reaching diversity saturation at 2002 OTUs (the number of unique OTUs detected in this study by PhyloChip analysis) than the clone libraries which is not surprising as the PhyloChip detected significantly more unique OTUs than did the clone libraries. It is estimated that if another 100 clones were sequenced approximately 100 additional phylotypes would be detected demonstrating that diversity saturation is far from being reached even with 1719 clones sequenced.
Supplemental Figure 3. (A) *Glycomycetaceae* and (B) *Micromonosporaceae* within the *Actinobacteria* phylum and (C) *Crenotrichaceae* and (D) *Sphingobacteriaceae* families within the *Bacteroidetes* phylum that had significant changes with depth as viewed by PhyloChip and clone library analyses. PhyloChip results are presented as a presence (black) absence (gray) heatmap for each OTU detected within the family. Each row, marked (*•*), represents a unique OTU. An OTU was determined present in a soil layer if the pf value was above or equal to 0.92 for both PhyloChips. Clone abundance of each family is reported as the percent of the total clones detected per soil layer.
Supplemental Figure 3 further demonstrates the differences between clone library and PhyloChip analyses at the family level. In the case of all four families, the trends observed by clone library analysis do not match those observed by PhyloChip analysis which is not surprising as clone library analyses detects changes in clone abundance and the PhyloChip analyses detects changes in diversity between soil layers. When used together, though, they complement each other and provide even more information about changes within these four families with depth at the CTPS. Interestingly, in regards to the *Glycomycetaceae* family, an increase in clone abundance was observed between the F and FW layers followed by a significant decrease between the FW and WW layers. Meanwhile, the PhyloChip detected only one *Glycomycetaceae* OTU (though probes for multiple *Glycomycetaceae* OTUs are on the PhyloChip). It is important to note that this OTU detected had a pf value of 0.92 on only one FW PhyloChip which was not enough to meet the criteria needed to be counted as present.

**Supplemental References**


APPENDIX F

SUPPLEMENTAL MATERIAL FOR ARTHROBACTER SP. ISOLATE EF01
CHROMATE TOXICITY STUDIES
The research presented in this appendix is supplemental material for Chapter 4 “The Influence of Carbon Source on Hexavalent Chromium Toxicity and Reduction by an Environmental *Arthrobacter* sp. Isolate”

Figure F1. Growth of *Arthrobacter* sp. isolate EF01 when ethanol (EtOH) was the sole carbon source in the presence of Cr(VI). Cr(VI) concentrations ranged from 0.5 to 5 µM. Results indicate that *Arthrobacter* sp. isolate EF01 only has significant growth if the Cr(VI) concentration is below 5 µM. Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.
Figure F2. Growth of *Arthrobacter* sp. isolate EF01 when butyrate was the sole carbon source in the presence of Cr(VI). Cr(VI) concentrations ranged from 0.5 to 5 µM. Results indicate that a significant decrease in growth was observed when 5 µM Cr(VI) was present. Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.

Figure F3. Chromium concentration over time when lactate was the sole carbon source provided for aerobic growth of *Arthrobacter* sp. isolate EF01 in the presence of 50 µM Cr(VI). Results compare total Cr (white filled symbols) as measured by Inductively Coupled Plasma Mass Spectrometry analysis with Cr(VI) measurements (dark filled symbols) as measured by the diphenylcarbazide assay. Results indicate that Cr(VI) was reduced in the system and not sorbed to the cells as total Cr concentrations do not change throughout the experiment.
Figure F4. Growth of *Arthrobacter* sp. isolate EF01 when xylose was the sole carbon source in the presence of 50 µM Cr(VI). Results indicate there is a significant lag in *Arthrobacter* sp. isolate EF01 growth in the presence of Cr(VI). Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.

Figure F5. Growth of *Arthrobacter* sp. isolate EF01 when sucrose was the sole carbon source in the presence of 50 µM Cr(VI). Results indicate that isolate EF01 can grow when sucrose is provided in the presence of Cr(VI). Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.
Figure F6. Growth of *Arthrobacter* sp. isolate EF01 when isopropanol was the sole carbon source in the presence of 5 µM Cr(VI) and 50 µM Cr(VI). Results indicate that no growth was observed in the presence of either Cr(VI) concentration. Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.

Figure F7. Growth of *Arthrobacter* sp. isolate EF01 when 1-butanol was the sole carbon source in the presence of 5 µM Cr(VI) and 50 µM Cr(VI). Results indicate that no growth was observed in the presence of either Cr(VI) concentration. Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.
Figure F8. Growth of *Arthrobacter* sp. isolate EF01 when methanol was the sole carbon source in the presence of 5 µM Cr(VI) and 50 µM Cr(VI). Results indicate that no significant growth was observed in the presence of either Cr(VI) concentration. Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.

Figure F9. Growth of *Arthrobacter* sp. isolate EF01 when succinate was the sole carbon source in the presence of 50 µM Cr(VI). Results indicate that no significant growth was observed in the presence of Cr(VI). Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.
Figure F10. Cr(VI) concentrations over time when xylose was the sole carbon source provided for *Arthrobacter* sp. isolate EF01 in the presence of 50 µM Cr(VI). Results indicate that a significant amount of Cr(VI) was reduced throughout the study. Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.

Figure F11. Cr(VI) concentrations after 73 hours when sucrose and succinate were the sole carbon sources provided for growth of *Arthrobacter* sp. isolate EF01 in the presence of 50 µM Cr(VI). Results indicate that Cr(VI) reduction occurred when sucrose was present but not succinate. Error bars represent ± one standard deviation (n = 3).
Figure F12. Cell viability of *Arthrobacter* sp. isolate EF01 when glucose was the sole carbon source in the presence and absence of 50 µM Cr(VI). Results indicate that there was an initial decrease in cell viability when isolate EF01 was in the presence of glucose and 50 µM Cr(VI) but that cell viability then increased over time. Plate count data representing cell viability are reported as log CFU/mL. Error bars represent ± one standard deviation (n = 5) and are smaller than the symbols where not visible.
Figure F13. Growth of *Arthrobacter* sp. isolate EF01 when a carbon source mix was provided (7.5mM carbon as glucose and 7.5mM carbon as ethanol) in the presence and absence of 50 µM Cr(VI). Results indicate that a mixture of carbon sources did influence growth of isolate EF01 in the presence and absence of Cr(VI). Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.
APPENDIX G

SUPPLEMENTAL MATERIAL FOR CHAPTER 6 CHROMATE TOXICITY MECHANISM STUDIES
Results presented in this appendix are supplemental material for Chapter 5 and are all demonstrating differences observed between *Arthrobacter aurescens* TC1 and *Arthrobacter* sp. isolate EF01 when utilizing various carbon sources for growth in the presence and absence of 50 µM Cr(VI). These results are discussed in more detail in the results section of Chapter 5.

Table G1. Growth Rate Comparisons for *Arthrobacter aurescens* TC1 and *Arthrobacter* sp. isolate EF01 on Various Carbon Sources

<table>
<thead>
<tr>
<th>Carbon Source</th>
<th>Growth Rate*</th>
<th>Growth Rate†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon Source + <em>A. aurescens</em> TC1 [hr⁻¹]</td>
<td>Carbon Source + <em>A. aurescens</em> TC1 + 50 µM Cr(VI) [hr⁻¹]</td>
<td>Carbon Source + <em>Arthrobacter</em> sp. isolate EF01 [hr⁻¹]</td>
</tr>
<tr>
<td>Glucose 0.089 ± 0.0046</td>
<td>0.105 ± 0.0054</td>
<td>0.140 ± 0.0060</td>
</tr>
<tr>
<td>Lactate 0.0069 ± 0.00016</td>
<td>0.007 ± 9.2E-05</td>
<td>0.013 ± 0.0005</td>
</tr>
<tr>
<td>Butyrate 0.0239 ± 0.0015</td>
<td>NG</td>
<td>0.044 ± 0.0021</td>
</tr>
<tr>
<td>Ethanol 0.007 ± 5.99E-05</td>
<td>NG</td>
<td>0.032 ± 0.0145</td>
</tr>
<tr>
<td>Xylose 0.034 ± 0.001</td>
<td>0.020 ± 0.0003</td>
<td>0.017 ± 0.0002</td>
</tr>
</tbody>
</table>

NG; No Growth Observed
*Errors are reported for three biological replicates
†Errors are reported for at least three replicate studies (each containing three biological replicates)

Table G2. Lag Time Comparisons for *Arthrobacter aurescens* TC1 and *Arthrobacter* sp. isolate EF01 on Various Carbon Sources

<table>
<thead>
<tr>
<th>Carbon Source</th>
<th>Lag Time*</th>
<th>Lag Time†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon Source + <em>A. aurescens</em> TC1 [hr]</td>
<td>Carbon Source + <em>A. aurescens</em> TC1 + 50 µM Cr(VI) [hr]</td>
<td>Carbon Source + <em>Arthrobacter</em> sp. isolate EF01 [hr]</td>
</tr>
<tr>
<td>Glucose 0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.09 ± 0.4</td>
</tr>
<tr>
<td>Lactate 0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>11.2 ± 7.3</td>
</tr>
<tr>
<td>Butyrate 0.0 ± 0.0</td>
<td>NG</td>
<td>16.3 ± 5.8</td>
</tr>
<tr>
<td>Ethanol 0.0 ± 0.0</td>
<td>NG</td>
<td>12.0 ± 0.02</td>
</tr>
<tr>
<td>Xylose 5.9 ± 0.6</td>
<td>0.0 ± 0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

NG; No Growth Observed
*Errors are reported for three biological replicates
†Errors are reported for at least three replicate studies (each containing three biological replicates)
Figure G1. Microbial growth comparison between *A. aurescens* TC1 and isolate EF01 when xylose was the sole carbon source in the presence and absence of 50 μM Cr(VI). Results demonstrate that in the presence of 50 μM Cr(VI) *A. aurescens* TC1 had a shorter lag time and greater maximum OD than isolate EF01. Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.

Figure G2. Cr(VI) concentration over time when *A. aurescens* TC1 and isolate EF01 were in the presence of ethanol. No reduction in Cr(VI) concentration over time was observed in the presence of either organism. Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible. EtOH; ethanol.
Figure G3. Cr(VI) concentration over time when *A. aurescens* TC1 and isolate EF01 were in the presence of butyrate. No reduction in Cr(VI) concentration over time was observed in the presence of either organism. Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.

Figure G4. Cr(VI) concentrations over time when *A. aurescens* TC1 and isolate EF01 were provided xylose as the sole carbon source. A significant decrease in Cr(VI) concentrations was observed in the presence of both *Arthrobacter* spp. suggesting they were both capable of reducing Cr(VI) when xylose was available. Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.
Figure G5. Fatty acid metabolism annotated for the *A. aurescens* TC1 genome. Enzymes are presented as EC classifications and crossed out enzymes are not found or have not been predicted to be in the *A. aurescens* TC1 genome. (www.microbesonline.org)
Figure G6. Pentose and glucuronate interconversions pathway through which xylose is metabolized annotated for the *A. aurescens* TC1 genome. Enzymes are presented as EC classifications and crossed out enzymes are not found or have not been predicted to be in the *A. aurescens* TC1 genome. (www.microbesonline.org)
Figure G7. Propionate metabolism pathway annotated for the *A. aurescens* TC1 genome. Enzymes are presented as EC classifications and crossed out enzymes are not found or have not been predicted to be in the *A. aurescens* TC1 genome. (www.microbesonline.org)
Figure G8. Starch and sucrose metabolism pathway annotated for the *A. aurescens* TC1 genome. Enzymes are presented as EC classifications and crossed out enzymes are not found or have not been predicted to be in the *A. aurescens* TC1 genome. (www.microbesonline.org)
Figure G9. Pyruvate metabolism pathway annotated for the *A. aurescens* TC1 genome. Enzymes are presented as EC classifications and crossed out enzymes are not found or have not been predicted to be in the *A. aurescens* TC1 genome. (www.microbesonline.org)
APPENDIX H

SUPPLEMENTAL MATERIAL FOR FIELD ET AL. (SUBMITTED 2011)
Supplemental Table H1. Summary of Normalized First Order Rate Coefficients

<table>
<thead>
<tr>
<th>Study</th>
<th>Treatment</th>
<th>Averaged normalized first order rate coefficient [mL/(CFU*hr)]</th>
<th>Stdev normalized first order rate coefficient [mL/(CFU*hr)]</th>
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<td>Carbon Sources</td>
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<td>Sucrose + AQDS</td>
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<td></td>
<td>Molasses</td>
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<td></td>
<td>Molasses + AQDS</td>
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<td>No C-source +</td>
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<td></td>
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<td>Microbially Produced Fe(II)</td>
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<td>Heat killed ES6 + reduced HFO + AQDS</td>
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<td>Active ES6 + reduced HFO</td>
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<td>Reduced HFO + AQDS</td>
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<td>AHDS</td>
<td>Abiotically reduced AQDS (500µM)</td>
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</table>

NC; Not able to calculate. * Apparent first order rate constant ≥263 hr⁻¹. † Apparent first order rate constant > 400h⁻¹. Unless indicated otherwise: all treatments contained strain ES6, 5 mg/L Cr(VI) as CrO₄²⁻, electron shuttle concentrations were 100 µM, carbon source concentrations were 10 mM sucrose and Fe(III) concentrations were 6 mM.
Supplemental Figure H1. Cr(VI) reduction by strain ES6 in the presence of different iron minerals without AQDS. All treatments contained strain ES6. Calculated first order rate coefficients determined that the presence of different iron mineral phases in the absence of AQDS did not influence Cr(VI) reduction rates. HFO; hydrous ferric oxide, HEM; hematite, GOE; goethite, MHM; maghemite, MGN; magnetite, AQDS; anthraquinone-2,6-disulfonate. Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.
Supplemental Figure H2. Fe(II) production by strain ES6 over time in the presence of 5mg/L Cr(VI) and increasing Fe(III) concentrations in the form of HFO. Results indicate that Fe(II) was not produced in significant quantities until Cr(VI) was completely reduced. Symbols denote first time point at which Cr(VI) was no longer detected in samples. (★), 12 hrs; (●), 72 hrs; (+) 49 hrs. Error bars represent ± one standard deviation (n = 3) and are smaller than the symbols where not visible.
Supplemental Figure H3. X-ray absorption near-edge structure (XANES) spectra of precipitates from various treatments containing strain ES6, 6 mM Fe(III) in the form of HFO and 100 μM AQDS.
APPENDIX I

SIMULTANEOUS DNA AND RNA EXTRACTION PROCEDURE FOR SOIL SAMPLES
TRIZOL® Simultaneous Extraction of RNA and DNA

Total Nucleic Acid Extraction

1. **Cell Lysis** – Weigh out 5g of soil and 2g sterile sand into an RNase-free mortar and pestle. Add 2 mL TRIZOL® Reagent (Invitrogen) to the soil. Perform freeze grind using liquid nitrogen three times. *Do not let the soil slurry thaw completely.* Transfer the soil slurry into a 50-mL falcon tube (*use liquid nitrogen to freeze remaining soil stuck in the mortar to scrape out easier*). Add 3 mL of TRIZOL® Reagent to the soil slurry in the 50-mL falcon tube. Mix soil slurry well and leave at room temperature for 20 min. (*Note- Added 1 mL TRIZOL® for every 1 x 10⁷ bacterial cells in sample, if more soil is to be extracted from adjust accordingly*).

2. **Phase Separation** – Centrifuge soil slurry at 6000 rpm for 10 min at 10°C. Pipette supernatant in 1 mL aliquots into 1.5 mL centrifuge tubes (can get about 3 mL out). Add 0.2 mL chloroform to each 1.5mL centrifuge tube (*if less then 1 mL in the centrifuge adjust volume of chloroform accordingly*). Shake tubes vigorously by hand for 15 seconds and incubate at RT for 2-3 min. Centrifuge samples at no more than 12,000 x g for 15 min. An upper aqueous phase (containing RNA), an inter-phase, and a lower phenol-chloroform phase (containing DNA) will form. Transfer the aqueous phase (~504 µL) to a fresh 1.5-mL centrifuge tube. The remaining two phases can be frozen at -80°C for future DNA extraction.

RNA Extraction

*Use the Qiagen RNeasy Mini Kit for procedure*

1. Add 280 µL 100% EtOH to each 1.5-mL centrifuge tube containing the RNA aqueous phase and mix thoroughly (*this is based on ~504 µL aqueous phase adjust volume accordingly*).

2. For each original soil sample extracted only one column will be used. Therefore, apply the 784 µL solution from one tube per each original sample to an RNeasy mini column placed in a 2-mL collection tube (supplied). Close tube gently and centrifuge for 15 s at 12,000 rpm. Discard flow-through. Repeat step as many times as necessary to add all the RNA from one original sample to the column (*ex. 3 mL extracted, will have to repeat procedure 3 times*).

3. Pipette 350 µL buffer RW1 into the RNeasy mini column and centrifuge for 15 s at >10,000 rpm to wash. Discard flow through.

4. Mix DNase I with RDD Buffer (10 µL: 70µL ratio per sample). *Can make a master mix for all samples.* Add 80 µL DNase I master mix solution directly onto the membrane of each column. Incubate at RT for 15 min. *This will remove any DNA contamination of the RNA.*
5. Pipette 350 µL buffer RW1 onto the column and centrifuge for 15 s at >10,000 rpm to wash. Discard flow-through.

6. Transfer the column to a new 2-mL collection tube (supplied). Pipette 500 µL Buffer RPE onto the column. Centrifuge for 15 s at 12,000 rpm to wash. Discard flow-through.

7. Add another 500 µL Buffer RPE to the column and centrifuge at 12,000 rpm for 2 min. Discard flow-through.

8. Centrifuge column for 2 min at 12,000 rpm to remove any residual Buffer RPE.

9. Transfer the column to a new 1.5-mL centrifuge tube. Pipette 60 µL RNase-free H₂O directly onto the membrane. Incubate at RT for 3-5 min. Centrifuge for 1 min at 12,000 rpm to elute. *Eluted RNA may be a slightly orange color. This will be removed after cleanup.* Quantify RNA using the nanodrop knowing the 280/260 ratio may be high if humics or other contaminants are still present. If so, continue on to purification. A 260/280 ratio ≥ 1.7 and a 260/230 ratio ≥ 1.5 is desired.

**RNA Purification**

*Use the QIAQuick PCR Purification Kit for purification*

1. Add 500 µL PBI solution (binding solution) to the 60 µL RNA. Transfer mixture to a spin column (supplied). Centrifuge for 45 s at 12,500 x g. Discard flow-through.

2. Add 500 µL PE solution (wash buffer) to the spin column. Centrifuge for 45 s at 12,500 x g. Discard flow-through.

3. Add another 500 µL PE solution (wash buffer) to the spin column. Centrifuge for 1 min at 12,500 x g. Discard flow-through.

4. Centrifuge spin column to remove any residual PE solution for 2 min at 12,500 x g.

5. Put spin column in a new 1.5-mL centrifuge tube. Elute RNA w/ 40 µL RNase-free H₂O. Let sit for 2-3 min and centrifuge for 1 min at 12,500 x g.

6. Quantify using the nanodrop. Separate out into two 20µL aliquots and store in the -80ºC freezer.
**DNA Extraction**

*Procedure obtained from TRIZOL® reagent information sheet*

1. **Precipitation** – Remove the remaining aqueous phase (from total nucleic acid extraction procedure) overlying the interphase and precipitate the DNA from the interphase and organic phase with ethanol. *Careful removal of the aqueous phase is critical for the quality of the isolated DNA.* Add 0.3 mL of 100% EtOH to each 1.5-mL centrifuge tube (if less then 1mL was added to the tube in step 2 of total nucleic acid extraction procedure adjust volume accordingly). Mix samples by inversion. Let samples sit at 15 to 30°C for 2-3 min. Sediment DNA by centrifugation for 5 min at no more then 2,000 x g at 2 to 8°C.

2. **Wash** – Remove the phenol-ethanol supernatant (*save for protein isolation if desired*). Wash the DNA pellet twice with a 0.1M Sodium Citrate in 10% EtOH solution. Add 1 mL per 1.5-mL centrifuge tube (if less ten 1mL was added to the tube in step 2 of total nucleic acid extraction procedure adjust volume accordingly). Resuspend pellet in solution and let sit for 30 min at 15 to 30°C (with periodic mixing). Centrifuge for 5 min at 2,000 x g at 2 to 8°C. Following these two washings suspend the DNA pellet in 1.5 mL 75% EtOH. Let sit for 10-20 min at 15 to 30°C (with periodic mixing). Centrifuge for 5 min at 2,000 x g at 2 to 8°C. A third wash in 0.1M sodium citrate in 10% EtOH can be performed if >200 ug DNA is expected or large amounts of non-DNA material is present.

3. **Redissolving DNA** – Air dry the DNA pellet 5 to 15 min (*Do not dry by centrifugation as it will be more difficult to dissolve the DNA*). Dissolve DNA in up to 600 µL of 8mM NaOH or TE Buffer pH 8.0. Put solution in 37°C water bath for ~5 min to help dissolve. Centrifuge resuspended DNA 12,000 x g for 5 min in order to pellet out any insoluble material such as membrane particles. Remove the supernatants and pool DNA that was obtained from the same original soil sample.

4. Quantify DNA using the nanodrop. *Ideally both the 260/280 and 260/230 ratios are >1.7. If the ratios are too low continue on with purification. If DNA concentrations are high gel purification can be performed. If DNA concentrations are low perform purification using the Wizard DNA Clean-Up System (with modifications, see Joy’s protocol). If 260/230 ratios are low after using the Wizard DNA Clean-Up System continue on with the desalting protocol (Joy’s protocol).*
DNA Purification using Wizard® DNA Clean-Up System (Promega)

*Procedure Modifications obtained from Joy Van Nostrand University of Oklahoma

** Purify only half the sample if DNA concentrations are low so as to not lose all the DNA

1. Bring volume of sample up to 50-100 µL with nuclease-free H2O

2. Add 100 µL Direct Purification Buffer (50mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl2, 0.1% Triton® X-100).

   100mL Direct Purification Buffer
   2.5 mL 2M KCl
   1 mL 1M Tris-HCL pH 8.8
   150 µL 1M MgCl2
   100 µL Triton® X-100

3. Add 1 mL resin (supplied) with DNA by inversion. Incubate at RT for 30 min, mixing every 10 min. Make sure the resin is well-mixed prior to use.

4. Attach the minicolumn to the syringe barrel (supplied). Add DNA/resin mix to the barrel. Apply a vacuum to draw the sample through the minicolumn. Once all the samples have passed through the column, release the vacuum.

5. Transfer 2 mL 80% isopropanol to the column and apply a vacuum. Continue to apply a vacuum for 30 s after the wash solution has been pulled through the column (no more than 30 s). Remove the syringe barrel and place minicolumn into a 1.5-mL microcentrifuge tube.

6. Centrifuge the minicolumn for 2 min at 10,000 x g to remove excess isopropanol.

7. Transfer the minicolumn to a new microcentrifuge tube and place 100 µL prewarmed (80°C) nuclease-free water to the minicolumn. Incubate at 80°C for 10 min (you can keep the microcentrifuge in a heat block set at 80°C or a water bath).

8. Centrifuge the minicolumn for 20 s at 10,000 x g to elute the DNA.

9. Repeat steps 7 and 8. You do not need to put minicolumn in a new 1.5-mL centrifuge tube to elute the next 100 µL.

10. Quantify DNA with the nanodrop. If the 260/280 ratio is too low (<1.8) repeat purification process. If the 260/230 ratio is low (<1.7) proceed with desalting protocol (essentially an EtOH precipitation procedure). Know that there may be a loss of DNA with each of these procedures so if concentrations are very low it
may not be worth performing, but rather dilute DNA before amplification. Regardless, only use half of the DNA to perform each procedure as to not lose it all.

**Desalting Protocol (Ethanol Precipitation)**

*Procedure Modifications obtained from Joy Van Nostrand University of Oklahoma

** Purify only half the sample if DNA concentrations are low so as to not lose all the DNA

If the 260/230 ratio is low after purification (<1.7) the desalting protocol can be used to remove contaminants (most likely guanidine from the resin in the purification kit).

1. Precipitate the Wizard purified product with 2.5 vol of ice-cold 100% EtOH and 1:10 vol 3M NaOAc (pH 5.2). Incubate samples overnight at -20°C (can ppt longer if desired).

For 100 µL purification product add 250 µL EtOH and 10 µL NaOAc

2. Centrifuge 30 min at 13,000 x g to pellet the DNA.

3. Decant the supernatant and wash the pellet with 1 mL ice-cold 70% EtOH. *Keep the supernatant until you are sure the DNA has been recovered.*

4. Centrifuge for 10 min at 13,000 x g.

5. Decant the supernatant and air dry the pellet. *Do not over dry the sample as it will be harder to resuspend the pellet. Keep the supernatant until you are sure the DNA has been recovered.*

6. Resuspend the pellet in nuclease free water. Put resuspended DNA in 37°C water bath for ~5 min. Centrifuge for 5 min at 13,000 x g to remove insoluble material such as membrane particles. Transfer supernatant into a clean 1.5-mL centrifuge tube and quantify using the nanodrop.
Preliminary Results of GeoChip Analysis of RNA Extracted from the Wood Waste Soil Layer

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<tr>
<th>No.</th>
<th>Gene ID</th>
<th>Gene Info</th>
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<th>SNR</th>
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