BIOCHEMISTRY OF CHROMIUM(VI) REDUCTION: FORMATION, FATE, AND IMPLICATIONS OF SOLUBLE ORGANO-CHROMIUM(III) COMPLEXES ON THE BIOGEOCYCLE OF CHROMIUM.

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation/thesis of GEOFFREY J. PUZON find it satisfactory and recommend that it be accepted.

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Abstract

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Chromium is widely distributed in the environment, existing in either the +3 or +6 state under environmental conditions. Trivalent chromium [Cr(III)] is the predominant oxidation state and considered relatively insoluble and non-toxic. In contrast, hexavalent chromium [Cr(VI)] forms oxyanions, i.e. CrO_4^-, at neutral pH, which are highly soluble and mobile in groundwater. Chromium is an important metal for use in industry based on its corrosion resistant properties. Large amounts of Cr(VI) have been introduced into the environment as industrial waste, posing a significant contamination problem requiring remediation.

Bioremediation has been proposed as a method for treating Cr(VI) contamination. Many bacteria reduce Cr(VI), but the reduction mechanism(s) and end-products are poorly understood. Research presented here focuses on understanding the biochemistry of Cr(VI) reduction and the Cr(III) end-products formed. An *Escherichia coli*

NAD(P)H:flavin oxidoreductase was found to reduce Cr(VI) via free reduced flavins. This system rapidly reduced chromate, whereas chemical reduction by NADH and glutathione was very slow. The reduced end-product was identified as a soluble and stable complex composed of multiple Cr(III) ions bound to NAD^+, NAD^+-Cr(III), instead of Cr(III) precipitate as expected for microbial reduction of Cr(VI). We further demonstrated that Cr(VI) reduction in the presence of several individual intracellular organic compounds formed soluble organo-Cr(III) end-products, which remained soluble and stable upon dialysis against H_2O and over a broad pH range. The fate and recalcitrance of these soluble organo-Cr(III) complexes in the environment were unknown. Microbial transformation of soluble organo-Cr(III) end-products was discovered. Two bacteria, PTX1 and PTX2, utilizing an organo-Cr(III) complex, NAD^+-Cr(III), were isolated. Phylogenetic classification designated PTX1 as a *Leifsonia* species and PTX2 as a *Rhodococcus* species, both common soil bacteria. The bacteria utilize the NAD^+ in the NAD^+-Cr(III) complex as a carbon and energy source. Mineralization of the NAD^+-Cr(III) resulted in precipitation of Cr(III) on the bacterial surface, signaling a probable long term insoluble Cr(III) form.

This work furthers the knowledge of mechanisms proposed to play a role in Cr(VI) reduction and expands the biogeocycle of Cr to include the formation of soluble organo-Cr(III) species and microbial mineralization of these species to produce an insoluble Cr(III) form.

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DEDICATION

This work is dedicated to my wife, Adeline Tan, and sons, Ian and Aidan Puzon. Thank you for your love and support for the last 14 years, especially the last 6 years. To my father Leon M. Puzon, the first scientist I knew, to my mother, Patricia A. Puzon, and my siblings Matt and Susan. I would also like to dedicate this dissertation to my Singapore family members and my friends throughout the globe for their encouragement and especially to Jose Reyes, Paul Hewson, and Peter Garrett for their perspective of the greater picture. Ian, I will remember the wisdom of the Lorax and Horton.

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CHAPTER ONE

INTRODUCTION AND BACKGROUND

Chromium

Chromium, Cr, was first discovered by a French chemist, Vauquelin, in 1798. It is a block VI transition metal on the periodic table and is the 21st most abundant element in the earth's crust at 100 parts per million (ppm) (5). It is also widely distributed in the environment being found in air, water, and biota. Cr metal is lustrous, steel gray, hard, and brittle (2). Based on its chemical properties, e.g. corrosion resistance (2), it is used in many industrial processes such as, stainless steel manufacturing, chrome plating, leather tanning, and wood treating (74). Cr is also used in the production of dyes and pigments (74). As a result, it is one of the United States' most important and strategic metals. The United States consumed 14% of the world's Cr production in 2001, approximately one half million metric tons, mainly used in the production of important metals (70). The primary global suppliers of Cr are South Africa, Zimbabwe, Russia, Kazakhstan, and Turkey (70).

Chromium has several oxidation states from -2 to +6, but it primarily exists in either the +3 or +6 state under environmental conditions. Trivalent chromium [Cr(III)] is the predominant oxidation state in the natural environment (54), typically found as chromite ore ($FeO*Cr_2O_3$) (74). It is relatively insoluble (12) and generally non-toxic. In mammals, Cr(III) is a necessary trace element, functioning in the glucose tolerance factor for proper carbohydrate metabolism (46, 76). In contrast, hexavalent chromium [Cr(VI)], derived from the oxidation of Cr(III), forms oxyanions, CrO_4^- and $Cr_2O_7^-$, at neutral pH.

These forms are highly soluble, up to 629 g/L of CrO_4^- and 49 g/L of $Cr_2O_7^-$ (14), and mobile in groundwater (19). Introduction of Cr(VI) into the environment is a result of waste from industrial processes (36, 54). Studies on total Cr, both Cr(III) and Cr(VI), released into the environment over a 7 year period, 1987-1993, showed nearly 200 million pounds released into land and water with the primary pollution from industrial organic chemical production (73). The introduction of large amounts of Cr(VI) through industrial processes has caused a serious contamination problem. Cr(VI) is a noted pollutant at 1,036 of the 1,591 National Priority List sites (72).

Cr(VI) toxicity

In humans, exposure to Cr(VI) is typically through ingestion, inhalation, or direct skin contact. Once humans are exposed, Cr(VI) crosses eukaryotic membranes via the sulfate uptake system (4, 53). The Environmental Protection Agency lists Cr(VI) with a maximum contaminant level (MCL) of 0.1 ppm in drinking water (73). Cr(VI) compounds are known to be highly toxic (81) causing perforations and ulcerations of the nasal septum, skin ulcerations, kidney damage, and cancer (39). After Cr(VI) is transported into eukaryotic cells, it is reduced to Cr(III) by glutathione [GSH], ascorbate, and citrate (20, 41, 64, 77, 82). The reduction process results in the production of reactive oxygen species (ROS) and can result in the formation of various Cr(III) cross-links between DNA-DNA, DNA-peptide, and DNA-amino acid (20, 41, 64, 77, 82). These all contribute to the carcinogenic effects of Cr(VI).

In prokaryotes, Cr(VI) is transported into the cytoplasm via the sulfate uptake system (32, 34, 55). Similar to eukaryotes, alterations of the genetic material also occur in prokaryotes, resulting in mutagenic effects (16). Cr(VI) levels between 10-12 ppm are

noted to be inhibitory to most soil bacteria while similar concentrations of Cr(III) have no effect (61).

Microbial Cr(VI) resistance

Microorganisms have evolved various mechanisms to enhance their survival in the presence of toxic heavy metals found in the environment (50, 51). Microbial reduction of Cr(VI) is one mechanism of resistance. A second resistance mechanism was first described by Ohtake *et al.* (55). The mechanism was originally thought to reduce the uptake of Cr(VI) (55), but has since been demonstrated to be a chromate efflux pump, ChrA (3, 9, 13, 57). ChrA efflux pumps are made of about 400 amino acid residues forming 10 transmembrane α-helical segments (52). The efflux pumps allow the energy dependent translocation of Cr(VI) oxyanions out of the cytoplasm (52). The ChrA proteins are known to be both plasmid and genome coded (57) and are present in both gram positive and gram negative bacteria (52).

Physical-chemical remediation of chromium

The biogeochemical cycle of Cr is proposed as being a transition between the +6 and +3 forms. Cr(VI) is reduced to Cr(III) in soil by inorganic and organic electron donors with the final forms identified as being inert hydroxyl Cr(III) precipitates (7). Due to the large input of Cr(VI) into the environment as industrial waste, the natural Cr cycle has been adversely augmented. In order to deal with the increased Cr(VI) waste and the danger it poses, different methods have been investigated for Cr(VI) remediation. Chemical treatment of contaminated waste first requires the physical excavation or pumping of waste followed by reductant addition, precipitate formation, and Cr

separation (25). Theses methods have high costs associated with production of secondary wastes requiring additional treatment, disposal of treated soil, and the energy and chemicals required for the process. Other methods such as, in situ redox manipulation (ISRM) by directly injecting reductant into the subsurface (71), electrodialytic removal (59), and sorption of Cr(VI) onto constructed materials (37) or biomass (83) are also actively being investigated as possible methods for remediation.

Biological remediation of chromium

Biological remediation of Cr is proposed to reduce soluble Cr(VI) to insoluble Cr(III) using microorganisms in either an *in situ* soil or batch reactor system. Biological methods for Cr(VI) remediation were first described by Romaneko and Koren'kov in 1977 (60). They demonstrated that *Pseudomonas* was able to reduce Cr(VI) from crocoite ($PbCrO_4$) to Cr(III) (60). Since then bioremediation has been actively studied as an affordable alternative for cleaning up Cr(VI) contaminated sites. Thus far, several different bacteria have been investigated for their ability to reduce Cr(VI). Researchers have primarily focused on the isolation of individual strains and identification of the ideal reducing conditions. Bacterial Cr(VI) reduction has been demonstrated in gram positive (11, 24, 45, 66) and gram negative eubacteria (48, 55, 56, 62, 65, 80) as well as Archaea (22). Reduction has been investigated as being condition specific (e.g. anaerobic-denitrifying), but has since been demonstrated to occur under several different conditions, both aerobically and anaerobically (24, 47, 56, 62, 65, 66). This indicated that Cr(VI) reduction occurs over a broad range of reducing conditions.

Overall, investigation into the mechanisms of Cr(VI) reduction has been more limited than the pursuit of reducing organisms. Initially, microorganisms able to respire

on Cr(VI) as the terminal electron acceptor were actively pursed. However, only two bacteria have been described (22, 66). The identification of the broad range of reducing conditions lead to the hypothesis that Cr(VI) reduction mechanisms are probably a secondary function of enzymes already present. Cr(VI) reduction mechanisms based on specific growth conditions were the next area investigated by researchers. Denitrifying and sulfate reducing enzymes have been actively studied based on the similarity of the ion size and charge (CrO_4^-, NO_3^-, SO_4^-) (15, 38, 75). Extracellular mechanisms of Cr(VI) reduction using both organic electron shuttles, i.e. humic acids (AQDS) (23), inorganic electron carriers (Fe^{2+}) (80), and using microbially produced reductants (H_2S) (63) have also been identified. Direct microbial reduction has been shown to take place on both the cell surface and intracellularly. Membrane associated reduction has been identified in *Enterobacter cloacae* (79), *Pseudomonas fluorescens* (10), and *Desulfovibrio vulgaris* (40). Intracellular locations have been proposed to be the main sites of Cr(VI) reduction based the known transport of the soluble Cr(VI) anion via the sulfate uptake system (32). Several groups have cited soluble Cr(VI) reduction mechanisms associated with the cytoplasmic fraction (11, 24, 28, 43, 45, 48, 62). Thus far, identification of specific intracellular mechanisms has been limited to the following, hydrogenases (47), cytochromes *c* (40, 47), cellular reducing agents (glutathione and cysteine) (17), and NADH-dependent reductases (56, 65). Our work has demonstrated and characterized the reduction of Cr(VI) by an NAD(P)H:flavin oxidoreductase (Fre) from *Escherichia coli* with free flavins (58). The reduction mechanism works as a system where Fre oxidizes NADH and in turn reduces the transiently bound flavin. The reduced flavin is released and in turn reduces Cr(VI). The system consumes 1.5 NADH for every Cr(VI) reduced,

stoichiometrically confirming Cr(III) as the reduced end-product. The Fre-flavin system rapidly reduced Cr(VI) under both aerobic and anaerobic conditions, whereas chemical reduction by NADH and glutathione was very slow. This signaled that enzymatic chromate reduction is likely the dominant mechanism in bacterial cells. Additional flavoproteins have since been identified as using a similar mechanism of Cr(VI) reduction via reduces flavins (1, 35).

Cr(VI) bioremediation end-products

The advantages of Cr(VI) bioremediation are based on the lower costs and that the Cr(III) end-product is expected to form precipitates, i.e. $Cr(OH)_3$. The foundation for this hypothesis is based on the properties of inorganic Cr(III) at neutral pH in organic poor environments (5). Identification of the actual reduced end-product however remains unclear. Some groups have reported the observation of a Cr(III) precipitate post reduction (13, 22, 24, 33, 40, 60, 66). However, other researchers have not identified the reduced Cr(III) end-product (10, 15, 26, 28, 47, 49, 56, 65, 69, 78). A few researchers have analyzed the mass balance during Cr(VI) reduction and quantified that significant amounts of reduced Cr(III) remained in the supernatant and did not precipitate after reduction (11, 43, 45, 48, 62). Field studies have also reported elevated amounts of soluble Cr(III) in the environment (27, 42) greater than thermodynamically predicted for $Cr(OH)_3$. These reduced end-products are thought to be in a complexed form with organics (27). Soluble organically bound Cr(III) complexes have been demonstrated to form between Cr(III) and natural chelators, i.e. citrate, in soil (30). In contrast to prokaryotes, biologically generated Cr(III) in eukaryotes is known to form organo-Cr(III) complexes, Cr(III)-DNA/DNA and Cr(III)-protein/DNA (4, 20, 41, 64, 77, 82). Work in

our lab has demonstrated that enzymatic Cr(VI) reduction produces soluble organo-Cr(III) complexes, NAD^+-Cr(III), at neutral pH and physiological ion concentrations (58). Similar enzymatically produced soluble NAD^+-Cr(III) end-products have since been reported by others (35). In addition, we have demonstrated that Cr(VI) reduction in the presence of cellular organics result in the formation of organo-Cr(III) products, which remain soluble at neutral pH. These soluble end-products remained stable upon dialysis in water and across a broad pH range. The results suggest that microbial reduction of Cr(VI) does not directly produce insoluble Cr(III) precipitates, but rather soluble organo-Cr(III) products. These soluble, stable organo-Cr(III) complexes are the likely forms of mobile trivalent chromium detected in the environment and are probably an integral part of the biogeocycling of Cr.

Cr(III) end-product recalcitrance and environmental fate

The fate of the Cr(III) end-products remains largely unknown. Because Cr(III) is noted to be substitution-inert (8) with very slow ligand exchange rates (18), the organo-Cr(III) complexes may remain stable and soluble for extended periods of time. In the environment, Cr(III) is known to be re-oxidized to Cr(VI) by MnO_2 (6) in Mn rich soil, thereby resulting in secondary Cr(VI) contamination. The long-term environmental implications of many toxic and radioactive metals have been the subject of more intense research. Investigations have focused on understanding the recalcitrance and movement of these metals in the environment. Metal-organic complexes have been identified to form between natural chelators and various metal ions: Fe, Zn, Ca, Cu, Co, Ni, Cd, Pb, U, and Cr (21, 29, 31, 67). Industrial chelators, such as EDTA, can also form complexes with Fe, Zn, Ca, Cu, Co, Ni, Cd, Pb, U, and Cr (44, 68). The metal-EDTA complexes

formed are both soluble and mobile, resulting in the movement of toxic metals through the subsurface environment and away from contaminated sites, i.e. ^{60}Co-EDTA, (44). Similar results have been reported for metal-citrate complexes (21, 30). This demonstrates the ability of both natural and industrial chelators to mobilize and spread metals, including Cr, through the environment. Degradation of these chelator-metal complexes by bacteria has been investigated as a means of preventing their movement. Bacteria able to biodegrade some of the metal citrate complexes (21, 22, 31, 67) and metal-EDTA complexes (68) have been identified. The degradation of the organic chelator results in further immobilization of the metal through precipitation. In the course of our research, two bacteria, PTX1 and PTX2, capable of mineralizing an enzymatically produced organo-Cr(III) complex, NAD^+-Cr(III), were discovered, isolated, and characterized. The bacteria utilized NAD^+, in both the free, NAD^+, and complexed, NAD^+-Cr(III), forms as a carbon and energy source. Bacterial mineralization of the NAD^+-Cr(III) resulted in the precipitation of Cr(III) on the bacterial surface as detected by transmission electron microscopy. Phylogenetic classification based on 16s rDNA analysis placed PTX1 in the *Leifsonia* genus and PTX2 in the *Rhodococcus* genus. The new *Leifsonia* and *Rhodococcus* isolates highlight a novel aspect of the biogeocycle of chromium, where soil microbes mineralize soluble organo-Cr(III) complexes. After mineralization, the Cr(III) then precipitates in an insoluble form, thus limiting further contamination problems.

Significance of our research

Our studies presented here contribute significantly to the understanding of the role of microorganisms in the bioremediation of Cr(VI) and the biogeocycle of chromium. We identify a general Cr(VI) reductase mechanism present in all bacteria that is capable of fast and efficient Cr(VI) reduction. Through this enzymatic reduction mechanism, we identified the formation of a soluble organo-Cr(III) complex, NAD^+-Cr(III). Furthermore, we demonstrated that other soluble organo-Cr(III) complexes readily form between cellular organics and Cr upon reduction. We also demonstrated that microorganism can mineralize the organo-Cr (III) end-products, resulting in the precipitation of Cr(III) on the cell wall. Identification of the production of soluble Cr(III) end-products and their mineralization are important in understanding the behavior and fate of Cr in *in situ* bioremediation efforts.

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CHAPTER TWO

A BACTERIAL FLAVIN REDUCTASE SYSTEM REDUCES CHROMATE TO A SOLUBLE CHROMIUM(III)-NAD^+ COMPLEX

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ABSTRACT

Biological reduction of carcinogenic chromate has been extensively studied in eukaryotic cells partly because the reduction produces stable chromium(III)-DNA adducts, which are mutagenic. Microbial reduction of chromate has been studied for bioremediation purposes, but little is known about the reduction mechanism. In eukaryotic cells chromate is mainly reduced nonenzymatically by ascorbate, which is usually absent in bacterial cells. We have characterized the reduction of chromate by a flavin reductase (Fre) from *Escherichia coli* with flavins. The Fre-flavin system rapidly reduced chromate, whereas chemical reduction by NADH and glutathione was very slow. Thus, enzymatic chromate reduction is likely the dominant mechanism in bacterial cells. Furthermore, the end product was a soluble and stable Cr(III)-NAD^+ complex, instead of Cr(III) precipitate. Since intracellularly generated Cr(III) forms adducts with DNA, protein, glutathione, and ascorbate in eukaryotic cells, we suggest that the produced Cr(III) is primarily complexed to NAD^+, DNA, and other cellular components inside bacteria.

INTRODUCTION

Chromium(VI) [Cr(VI)] has been released into the environment in large quantities from a broad range of industrial applications, posing a severe environmental problem (13). Cr(VI) exposure leads to cancer and hereditary disease (32). Under typical environmental conditions, Cr(VI) forms oxyanions, primarily as chromate, which is highly soluble (6).

Chromate reduction in eukaryotic cells has been extensively studied because the reduction process generates reactive intermediates, Cr(V) and Cr(IV), and leads to the incorporation of Cr(III) into DNA molecules. Recent studies have demonstrated the role of Cr(V) in chromate-induced DNA damage, cancer, and apoptosis (26, 30). The biologically generated Cr(III) has also been shown to be incorporated into DNA, causing DNA-DNA, DNA-peptide, and DNA-amino acid cross-links that are mutagenic (7, 14, 24, 31, 33). Chromate can readily cross the membranes of both prokaryotic and eukaryotic cells (16) and can be reduced to Cr(III) inside eukaryotic cells primarily by cellular reducing agents, such as ascorbate, glutathione, and cysteine (4), of which ascorbate is shown to be the principal reductant of chromate (21).

Bacterial reduction of chromate to Cr(III) has been studied mainly for the purpose of bioremediation, which is based on the concept that Cr(III) has negligible toxicity and minimal solubility compared with Cr(VI) (12). Many bacteria can reduce chromate under both aerobic and anaerobic conditions, including *Escherichia coli* (20). Anaerobic bacteria may use chromate as a terminal electron acceptor (28) or reduce chromate in the periplasmic space by hydrogenase (15) or reduced cytochrome *c* (15). Aerobic bacteria can reduce chromate by cellular reducing agents and NADH-dependant chromate reductases (10, 17, 20, 27). Since ascorbate is usually absent in bacterial cells, the

primary reductant is glutathione that reduces chromate slowly (22). Thus, it is unknown whether enzymatic or nonenzymatic reduction of chromate is dominant in bacterial cells under aerobic conditions. Furthermore, a significant issue that has not been vigorously demonstrated is the species and solubility of Cr(III) produced by microbial reduction of chromate. It also remains unsolved whether the NADH-dependant reductases are specific chromate reductases. In this manuscript, we address these three important questions. NAD(P)H:flavin oxidoreductases (flavin reductases) catalyze the reduction of flavin by NAD(P)H. One of the functions of flavin reductases is to reduce ferrisiderophores (5) and Cob(III)alamin (8). Despite this known function of flavin reductases for metal reduction, the role of flavin reductases in chromate reduction has not yet been documented. Here, we report that a previously characterized *E. coli* flavin reductase (9) with flavins rapidly reduced chromate to a soluble and stable Cr(III)-NAD^+ complex, which is especially surprising as it is generally assumed that the produced Cr(III) forms precipitate, usually $Cr(OH)_3$ or Cr_2O_3 (1).

MATERIALS AND METHODS

Chromate reduction by the Fre system. The production and purification of Fre were done as previously reported (34). One unit of Fre was defined as the amount of Fre required to catalyze the consumption of one nmol of NADH per min with 10 μM FMN. The specific activity of the purified Fre was 49,600 U per mg of protein. Chromate reduction was assayed under both aerobic and anaerobic conditions. Reaction mixtures contained 38 U Fre, 400 μM NADH, and 250 μM chromate in 1 ml of 40 mM KPi buffer (pH 7.0) with 10 μM FMN, FAD, or riboflavin. Reaction was started by the addition of Fre. The reaction was stopped by mixing 100 μl sample with 5 μl of 2 M Na_2CO_3, and chromate was measured. Anaerobic chromate reduction was done identically, but inside an anaerobic chamber (Coy Laboratory Product INC., Grass Lake, Mich.) under a N_2 atmosphere with 2-3% H_2.

Chromium detection. Chromate was measured by a reported method (19) using K_2CrO_4 (Sigma, St. Louis, Mo.) as the standard and using ChromaVer powder pillows (HACH, Loveland, CO) containing diphenylcarbazide. Soluble Cr(III) species were detected by absorption spectra (Ultrospec 4000, Amersham Pharmacia, Piscataway, NJ). Because Cr(III) precipitates out in aqueous solutions at neutral pH, the spectrum of $Cr(NO_3)_3$ in distilled water (pH <3) was used as a control. Total soluble chromium was determined using an inductively coupled plasma/mass spectrophotometer (ICP/MS) (HP 4500 Plus Series, Agilent Technologies Inc., Palo Alto, CA). The size of the Cr(III)-NAD^+ complex was estimated by high performance liquid chromatography (HPLC) equipped with a size-exclusion BioSep SEC 3000 column (300 by 7.8 mm, Phenomenex,

Torrance, Calif.) and a photodiode array detector (Waters, Milford, Mass.). The running condition was 0.5 ml min^{-1} of 50 mM KPi (pH 7.0).

NADH consumption. NADH consumption was monitored by the absorbance at 340 nm (A_{340}, ε = 6,220 M^{-1} cm^{-1}). The reaction contained 400 μM NADH, 10 μM flavin, and 38 U Fre in 1 ml of 40 mM KPi buffer (pH 7.0) with or without 250 μM Cr(VI). Anaerobic experiments were assembled with identical reagents but in rubber-stopper sealed cuvettes in the anaerobic chamber. Reactions were initiated by adding Fre. Michaelis-Menten kinetic parameters were determined with 1, 2, 3, 4, or 5 μM FMN in triplicate, and averages were used to calculate the kinetic parameters.

Electron paramagnetic resonance spectroscopy (EPR). The conversion of Cr(VI) to Cr(III) by the Fre system was further analyzed by EPR. EPR spectra were obtained using a Bruker 200tt EPR spectrometer (Bruker instruments, Billerica, MA) in a GFS-300 transfer tube. All experiments were carried out at 70K with the following EPR settings: microwave frequency = 9.428 GHz, microwave power = 6.32 mW, gain = 6.3 x 10^5, time constant = 500 ms, modulation amplitude = 2 mT, center field = 330 mT, sweep width = 200 mT, and sweep time = 2 min.

Dialysis. A sample (0.5 ml) of enzymatically reduced Cr(III) mixture with 23.7 mM chromium was transferred into dialysis tubing having a molecular weight cutoff of 3,500 (Spectrum, Houston, Texas) and dialyzed against 500 ml of 40 mM KPi buffer (pH 7.0) for 12 h. The dialysis was then continued in 500 ml of fresh 40 mM KPi buffer for another 12 h. The first and second dialysis solutions as well as the dialyzed sample inside the tubing were analyzed for chromium by ICP/MS. NAD^+ was estimated by absorbance at 260 nm (ε = 18,000 M^{-1} cm^{-1}).

RESULTS

Reduction of chromate by a Fre-flavin system. The ability of Fre, an *E. coli* general flavin reductase (9), to reduce chromate was tested. The recombinant Fre overproduced in *E. coli* was purified as previously reported (34). Fre, together with riboflavin, FMN, or FAD rapidly reduced chromate under both aerobic and anaerobic conditions (Fig. 1). Chromate reduction was faster under anaerobic conditions than under aerobic conditions. Chromate reduction by NADH without the enzyme or without flavins was not apparent (Fig. 1). When NADH was replaced by glutathione, chromate reduction was observed but was much slower than that catalyzed by Fre (Fig. 1). Oxygen did not affect chromate reduction by either glutathione or NADH. The initial rates of chromate reduction under aerobic conditions were about 1/3 of those observed under anaerobic conditions (Table 1).

NADH consumption and kinetic analysis. The effects of chromate on NADH consumption by Fre were studied. Under aerobic conditions, the NADH consumption rates increased in the presence of chromate (Table 2). Under anaerobic conditions, NADH was oxidized only in the presence of chromate, and the rates were faster than those observed under aerobic conditions (Table 2). The apparent K_M and k_{cat} values of Fre (molecular weight of 26,110) for FMN were 1.2 $\pm$ 0.18 μM and 27.6 $\pm$ 1.4 s^{-1} when oxygen was the sole electron acceptor. When 250 μM chromate was the sole electron acceptor, the apparent K_M and k_{cat} values were 1.0 $\pm$ 0.16 μM and 41.3 $\pm$ 1.5 s^{-1} respectively.

Stoichiometry. The amount of NADH consumed for chromate reduction was measured under anaerobic conditions. NADH was analyzed before reaction initiation and

after reaction completion. Corrections to account for changes in NADH due to residual oxygen were accomplished by using samples without chromate as controls. The average amount of NADH consumed for the complete reduction of 1000 nmol of chromate in the presence of a specific flavin was as follows: FMN, 1633 $\pm$ 126 nmol; FAD, 1475 $\pm$ 81 nmol; and Riboflavin, 1650 $\pm$ 79 nmol. Thus, approximately 1.5 NADH was consumed for each chromate reduced, indicating that Cr(III) was the end product.

Conversion of chromate to Cr(III). Cr(III) precipitate was expected to be the end product of chromate reduction. However, no Cr(III) precipitate was observed in any reactions, but addition of similar concentrations of $Cr(NO_3)_3$ to the same reaction mixture resulted in immediate precipitation. The nature of the chromium end product was further tested. Anaerobic reactions containing 5 mM chromate, 10 mM NADH, and 76 U Fre in 1 ml of 40 mM KPi buffer (pH 7.0) were assembled in sealed cuvettes. Chromate alone in KPi buffer gave an absorption peak at 370 nm (ε = 4,700 M^{-1} cm^{-1}). After reduction by Fre for 90 min, Cr(VI) was completely reduced as confirmed by diphenylcarbazide analysis and by the lack of an absorption peak at 370 nm. The Cr(III) end product was soluble with a greenish color. The absorption spectra of the end product and 5 mM Cr(III) in distilled water gave peaks at a similar wavelength (Fig. 2) with a red shifting of the end product peak, as reported for a Cr(III) tetramer (25). The product was stable after one year at room temperature in a non-sterile container.

EPR was used to analyze the reaction intermediate and end product. Samples frozen during the reduction of 5 mM chromate (3 min after reaction initiation) showed a distinctive, sharp EPR spectrum attributable to Cr(V), centered at g = 1.97 (342.5 mT) and line width of ca. 2 mT, and essentially identical to those observed previously (27).

Samples taken after completion of the reaction (3 hours incubation) had no Cr(V) signal, but showed a broadened EPR spectrum (Fig. 3a) that was centered at g = 1.94 (347.3 mT) with a line width of approximately 50 mT, very similar to EPR spectra obtained for Cr(III) polymers (29). Given the reaction stoichiometry and the spectrum of the end product, we deduced that the EPR spectrum represents a paramagnetic Cr(III) species. In contrast, a standard solution of 5 mM $Cr(NO_3)_3$ in H_2O gave a considerably narrower EPR spectrum, centered at g =1.94 (347.3 mT) and line width of ca. 5 mT (Fig. 3b). These two signals represent two different Cr(III) states, where both peaks were centered at the same magnetic field. The broadening observed in the reaction product is likely due to the interaction of the unpaired electrons of Cr(III) in a complex. Double integrations of EPR spectra of the standard Cr(III) and end product were within 3% of each other. This information indicates that the EPR relaxation properties of the two signals are reasonably similar, further supporting our assignment of the spectrum to Cr(III).

Characterization of the soluble Cr(III) product. The end product was further analyzed by HPLC size-exclusion chromatography. The reaction product gave a retention time of 20.9 min with absorbance peaks at 260 and 590 nm, indicating the presence of NAD^+ and Cr(III). When compared to the protein standards run under the same conditions, the estimated size of the Cr(III)-NAD^+ complex was 12 kDa. To test the enzymatic reduction of chromate at high chromate concentrations, an anaerobic reaction containing 25 mM chromate, 50 mM NADH, and 78 U Fre per ml was done in 40 mM KPi. The reaction produced a green solution with neither detectable Cr(VI) nor $Cr(OH)_3$ precipitate. When the reaction product was dialyzed against 40 mM KPi, the distribution of chromium was 9,406 nmol inside the tubing and 644 nmol in the dialysis solution.

After continued dialysis in a fresh 40 mM KPi buffer, the chromium was 8,898 nmol inside the tubing and 508 nmol in the buffer. The total loss of chromium through dialysis was only 11%. The NAD^+ content inside the tubing as estimated to be 16372 nmol by absorption at 260 nm. The molar ratio of NAD^+ to Cr(III) was approximately 2 to 1 after dialysis.

DISCUSSION

Chromate reduction in eukaryotic cells is primarily through nonenzymatic reduction by ascorbate (21). Since bacterial cells normally do not contain ascorbate, glutathione becomes the major reductant (4). However, chromate reduction by glutathione is much slower than by the Fre-flavin system (Fig. 1) (22), suggesting that chromate reduction is primarily enzymatic inside cells. Fre, a general flavin reductase in *E. coli,* does not contain bound flavins (9). As shown here, the Fre system is highly effective in chromate reduction with the highest activity in the presence of riboflavin at 33.3 or 96.4 μmol mg^{-1} min^{-1} at 24°C under aerobic or anaerobic conditions (Table 1). The activities are significantly faster than those of two purified NAD(P)H dependent chromate reductases previously reported. The soluble *Pseudomonas putida* chromate reductase has a specific activity of 0.533 μmol mg^{-1} min^{-1} at 30°C (17), and the soluble *Pseudomonas ambigua* G-1 chromate reductase (ChrR) has a specific activity of 0.025 μmol mg^{-1} min^{-1} at 50°C (27). It has previously been suggested that the primary functions of these enzymes may not be for chromate reduction (17). This is likely true as the gene sequences available from GenBank indicate that the *P. putida* chromate reductase is a quinone reductase (AF375641) that has a bound flavin and *P. ambigua* ChrR is a flavin reductase (D83142). The high rate of chromate reduction by the Fre-flavin system and the ubiquity of flavins and flavin reductases in cells suggest that flavin reductase systems may play a significant role in chromate reduction. Flavin reductases may also be used in bioremediation of Cr(VI) contamination.

Chromate reduction by the Fre-flavin system is likely via reduced flavins, in a similar manner as the reduction of ferrisiderophore (5) and Cob(III)alamin (8) by Fre in

E. coli and *Salmonella enterica*. Reduced flavins are highly reactive and can be reoxidized by either O_2 (34) or chromate. Since the oxidized forms of flavins are continuously regenerated, flavin concentrations less than 10 μM (ca. 10 K_M) are required to support maximal activity for Fre as measured by NADH consumption (34). However, the rates of NADH consumption were increased by replacing O_2 with chromate. Kinetic analysis suggests that the increased rate of NADH consumption with chromate as the oxidant is due to the increased V_{max}. The increased NADH consumption could be a result of the direct reaction of NADH with reactive intermediates Cr(IV) and Cr(V). Thus, the K_M for FMN remains the same, while V_{max} is the sum of enzymatic and chemical oxidation of NADH. The hypothesis is also in agreement with the moderate increase in NADH consumption rates by chromate under aerobic conditions due to an intermediate level of chromate reduction (Table 2).

Because most enzymatic chromate reduction has been studied at micromolar concentrations, the form of Cr(III) produced has not been carefully studied. Since Cr(III) precipitates in neutral solutions, the formation of Cr(III) precipitate is expected. To our surprise, no Cr(III) precipitate was formed even when 25 mM chromate was reduced. Instead, a soluble Cr(III)-NAD^+ complex was produced. The reaction stoichiometry and absorption spectrum (Fig. 2) indicate that the end product is Cr(III). The EPR spectrum further demonstrated that Cr(III) is the end product but in a complexed form (Fig. 3). Similar EPR spectra have been observed for Cr(III) species in ruby (18, 23), where exchange-coupling of the two Cr(III) spin 3/2 systems, resulting in a total spin 3 system. Similar conclusions can also be inferred from the spectra of Cr dimer, $Cr(III)_2O_6H_4$ (29). The composition of the Cr(III) complex was composed of Cr(III) and NAD^+, at a molar

ratio of 1 to 2. The complex may also contain some NADH. The soluble Cr(III) complex does not result from an association of the Cr(III) with the phosphate buffer because the soluble end product was also formed in 40 mM Tris (pH 8.0), HEPES (ph 7.8), or MOPS (pH 7.2) buffer (Data not shown). The Cr(III) complex was only produced by chromate reduction and not by mixing $Cr(NO_3)_3$ with NAD^+ in a KPi buffer. A Cr(III)-NAD^+ complex has not been reported to date.

The conditions used for the production of soluble Cr(III) complex are similar to physiological conditions inside cells, where sufficiently high concentrations of FMN, FAD, NADH, and NAD^+ are available. There are 88 μM FMN, 51 μM FAD, 16 μM NADH, and 790 μM NAD^+ in *Salmonella typhimurium*, a close relative of *E. coli* (3). Thus, chromate reduction by soluble chromate reductases may result in soluble Cr(III) complexes inside cells. The complex could be leaked out of the cells or released after cell lysis. The stability of the complex in the environment is unknown, but the complex in the reaction solution was stable for over a year. Cr(III) is substitution-inert (2), which explains why the complex is not slowly converted to insoluble $Cr(OH)_3$. In addition, intracellularly produced Cr(III) is able to form adducts with DNA, protein, glutathione, and ascorbate as demonstrated in eukaryotic cells (7, 14, 24, 31, 33, 35). Thus, the Cr(III) produced intracellularly from chromate reduction is primarily bound to organic compounds including NAD^+, nucleic acids, and other cellular components. However, our results do not rule out the formation of Cr(III) precipitate outside the cytoplasm, but the production of soluble Cr(III), being mobile in groundwater, should be considered for bioremediation. Organically complexed Cr(III) species in wastewater have also been

reported (11), but the exact structures and bioavailability of these complexes are unknown.

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Table 1. Rates of chromate reduction by the Fre-flavin system*.

Flavin	Aerobic Cr(VI) Reduction (μmol mg^{-1} min^{-1})	Anaerobic Cr(VI) Reduction (μmol mg^{-1} min^{-1})
FAD	26.0 $\pm$ 1.7	76.7 $\pm$ 0.6
FMN	27.1 $\pm$ 1.9	71.3 $\pm$ 1.1
Riboflavin	33.3 $\pm$ 0.5	96.5 $\pm$ 6.4

* The rates were determined after incubating for 1 min. Results were averages of triplicate measurements with standard deviations.

Table 2. NADH oxidation rates by the Fre-flavin system*.

Flavin	O_2 only (μmol mg^{-1} min^{-1})	O_2+Cr(VI) (μmol mg^{-1} min^{-1})	Cr(VI) – O_2 (μmol mg^{-1} min^{-1})
FAD	43.8 $\pm$ 0.5	58.9 $\pm$ 2.8	84.3 $\pm$ 1.0
FMN	49.6 $\pm$ 0.8	63.4 $\pm$ 2.7	85.9 $\pm$ 2.4
Riboflavin	78.0 $\pm$ 0.4	98.8 $\pm$ 4.4	124.6 $\pm$ 3.3

* The initial rates were determined by monitoring NADH oxidation at 340 nm.

Results were averages of triplicate experiments with standard deviations.

Figure 1. Time course of chromate reduction. Controls contained only NADH (1) or glutathione (2). Chromate reduced by Fre with FAD (3), FMN (4), or riboflavin (5) under aerobic conditions. Chromate reduced by Fre with FAD (6), FMN (7), or riboflavin (8) under anaerobic conditions.

Figure 1.

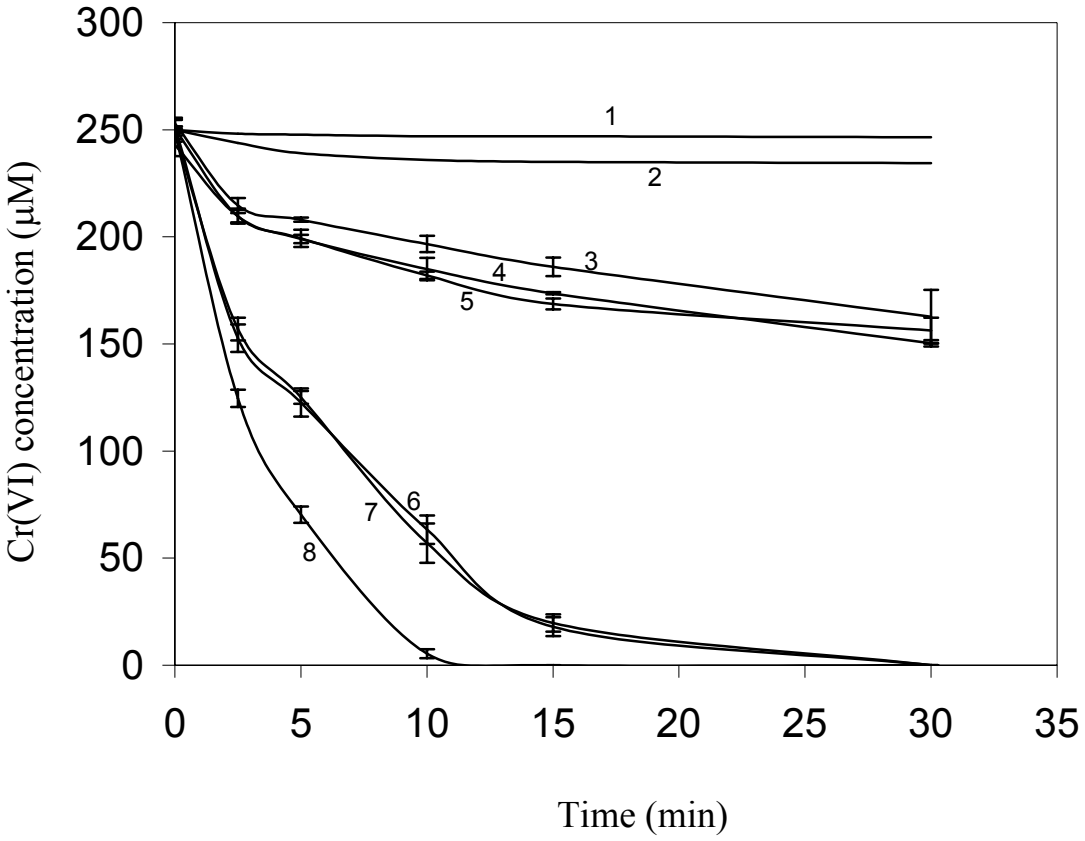

300
250
200
150
100
50
0
0
5
10
15
20
25
30
35
Cr(VI) concentration (μM)
Time (min)
1
2
3
4
5
6
7
8

Figure 2. Absorbance spectra of 5 mM Cr(III) samples. (A) Cr(III) produced by the Fre-FMN reduction of chromate with an absorption peak at 593 nm; and (B) Cr(III) monomer obtained by dissolving $Cr(NO_3)_3$ in distilled H_2O with an absorption peak at 579 nm.

Figure 2.

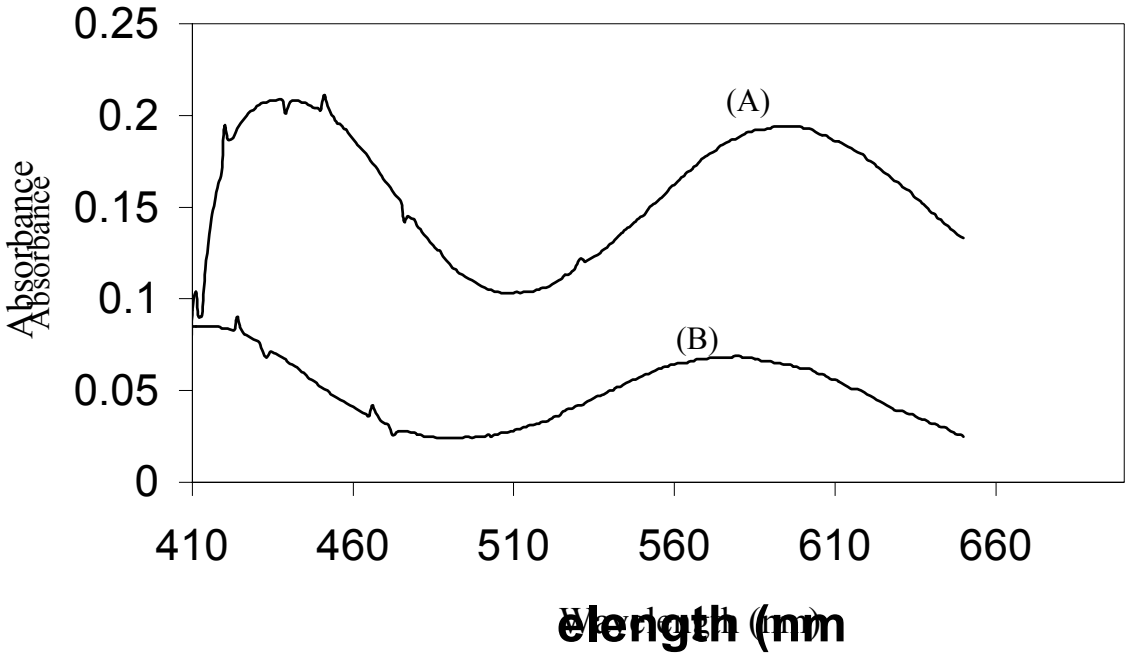
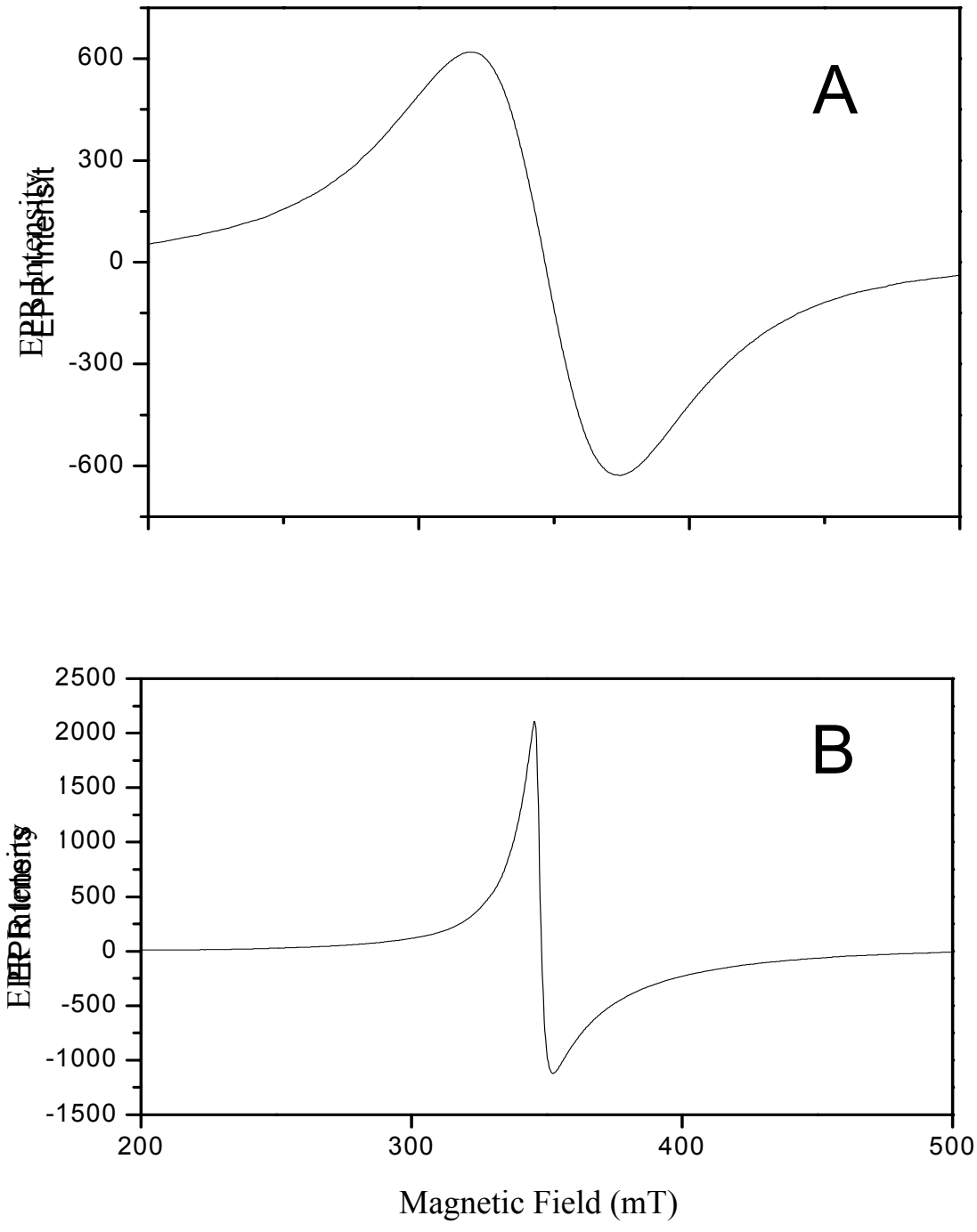

0.25
0.2
0.15
0.1
0.05
0
Absorbance
(A)
(B)
410
460
510
560
610
660
Wavelength (nm)

Figure 3. EPR analysis of the reaction end product and standard Cr(III). (a) Enzymatic reaction end product produced from 5 mM chromate in 40 mM KPi; and (b) Standard 5 mM $Cr(NO_3)_3$ in distilled H_2O.

Figure 3.


A
600
300
0
-300
-600
B
2500
2000
1500
1000
500
0
-500
-1000
-1500
200
300
400
500
Magnetic Field (mT)

CHAPTER THREE

FORMATION OF SOLUBLE ORGANO-CHROMIUM(III) COMPLEXES AFTER CHROMATE REDUCTION IN THE PRESENCE OF CELLULAR ORGANICS

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ABSTRACT

Microbial reduction of hexavalent chromium [Cr(VI)] to trivalent [Cr(III)] has been investigated as a method for bioremediation of Cr(VI) contaminated environments. The reduced Cr(III) end-product is thought to be insoluble $Cr(OH)_3$; however, recent reports suggested a more complicated fate of Cr(III). A bacterial enzyme system, using NADH as the reductant, converts Cr(VI) to a soluble NAD^+-Cr(III) complex, and cytochrome *c* mediated Cr(VI) reduction produces cytochrome *c*-Cr(III) adducts. In this study, Cr(VI) reduction in the presence of individual intracellular organic compounds formed both soluble and insoluble organo-Cr(III) end-products. The soluble end-products were characterized by absorbance spectroscopy and electron paramagnetic resonance spectrometry as organo-Cr(III) complexes. The complexes remained soluble and stable upon dialysis against distilled H_2O and over a broad pH range. The readily formation of soluble, stable organo-Cr(III) complexes from Cr(VI) reduction in the presence of cellular organic compounds suggest that organo-Cr(III) complexes are an integral part of the natural cycling of chromium. Thus, organo-Cr(III) complexes are the likely forms of mobile trivalent chromium detected in the environment.

INTRODUCTION

Chromium is one of the United States' most important and strategic metals (44). Hexavalent chromium [Cr(VI)] is widely used in industrial processes such as, stainless steel manufacturing, chrome plating, leather tanning, wood treatments, dyes, and pigments. Because of its wide usage, Cr(VI) has been released into the environment in large quantities, posing a severe environmental problem (24). Cr(VI) is a known contaminant at 1,036 of the 1,591 National Priority List sites (43). In the environment, Cr(VI) typically forms oxyanions, e.g. chromate, at neutral pH, which are highly soluble and mobile in groundwater (13).

Chromate can readily cross cell membranes (2) and human exposure is a considerable health issue. In the cytoplasm, chromate is converted to reactive Cr(V) and Cr(IV) species as well as stable Cr(III)-DNA adducts, causing mutation and cancer (47). In contrast, the inorganic trivalent chromium [Cr(III)] salts are typically insoluble (5), non-toxic, and used as food supplements (20). To minimize Cr(VI) contamination, various methods converting Cr(VI) to less toxic Cr(III) have been investigated, including bioremediation.

Many different bacterial species are capable of reducing Cr(VI) to Cr(III) (16, 25, 30, 33, 36, 40, 41), and they are the agents in bioremediation of Cr(VI). However, identification and understanding of the Cr(III) end-product have largely been ignored. Microbial Cr(VI) reduction is thought to primarily form insoluble Cr(III) as $Cr(OH_3)_3$ or Cr_2O_3 (5) based on the chemical nature of Cr(III) in inorganic environments and not in organic rich environments, such as the intracellular environment of microbes. Most reports on bacterial reduction of Cr(VI) emphasize on the reduction of Cr(VI) and often

do not specify the forms of Cr(III) end-products. However, growing evidence indicates insoluble Cr(III) salts may not be the end-product after microbial chromate reduction. First, several research groups have reported that significant amounts of Cr(III) remain in the supernatant of bacterial cultures and do not precipitate after reduction (9, 28, 31, 36). Second, field studies have identified elevated amounts of soluble Cr(III) in the environment (17, 27) greater than that predicted if $Cr(OH)_3$ and Cr_2O_3 were the sole species. Third, enzymatic reductions of chromate have formed organo-Cr(III) complexes, where Cr(III) is bound to the microbial organic reductant, such as cytochrome c_7 (3) and NAD^+ (34). Similar results are noted in eukaryotes with reduced Cr(III) bound to organic components such as protein, DNA, and certain metabolites (14, 26, 38, 45, 48).

After identifying the formation of NAD^+-Cr(III) complex from chromate reduction by a bacterial enzyme system, we have further investigated the formation of organo-Cr(III) complexes from chromate reduction in the presence of cellular, small organic compounds that are not chelating agents. Here we report the identification and characterization of soluble organo-Cr(III) end-products upon the reduction of Cr(VI) to Cr(III) with various intracellular components.

MATERIALS AND METHODS

Chromium detection. Potassium chromate, K_2CrO_4 (Sigma, St. Louis, Mo.), was used as the Cr(VI) source in all experiments. Cr(VI) was measured by a reported method (35) using chromate as the standard and using ChromaVer powder pillows (HACH, Loveland, CO) containing diphenylcarbazide. Total soluble chromium, Cr(VI) + Cr(III), samples were prepared for analysis by first centrifugation at 16,500 x g for 5 min at 23^{o}C and then filtered through a 0.22 μm MILLIPORE MILLEX^{TM}GP syringe filter to remove any precipitates and large complexes. Samples were analyzed using an inductively coupled plasma/mass spectrophotometer (ICP/MS) (HP 4500 Plus Series, Agilent Technologies Inc., Palo Alto, CA). Soluble Cr(III) was calculated as the difference between the detected total soluble chromium and remaining Cr(VI). Monomeric chromium (III), $Cr(NO_3)_3$ (Aldrich, Milwaukee, WI), in distilled water (pH <3) was used as a control in UV/VIS spectrophotometry, EPR, and to generate a standard curve for ICP/MS and ICP/OES.

Organo-Cr(III) complex production and detection. Organo-Cr(III) complexes were produced by reduction of Cr(VI) in the presence of excess organic component. Reductions were buffered with 100 mM KPi, pH 7.0, to simulate the physiological intracellular conditions (15). Elevated concentrations of Cr(VI), 5 mM, were used in order to allow for detection by spectrophotometric methods. Samples were reduced under anaerobic conditions in a COY anaerobic chamber (COY Laboratory Product, Grass Lake, MI) under an N_2 atmosphere with 3-4% H_2 at 24^{o}C. Reactions mixtures contained 5 mM Cr(VI), in 100 mM KPi pH 7.0, with 20 mM of the corresponding organic component (Table 1). Reductions were started by the addition of 20 mM $Na_2S_2O_4$, except

in reactions with cysteine and glutathione [GSH] which completely reduced Cr(VI) after 24 h. Reductions with ethanol contained a final concentration of 19% ethanol. All samples were tested for complete reduction of Cr(VI) by the diphenylcarbazide assay and assayed for total soluble Cr(III) by ICP/MS. Soluble Cr(III) species were analyzed by absorption spectroscopy on a Ultrospec 4000 UV/VIS spectrophotometer (Amersham Pharmacia, Piscataway, NJ) by a characteristic peak around 580-600nm (34, 39).

Electron paramagnetic resonance spectroscopy [EPR]. The organo-Cr(III) products were further analyzed by EPR. Spectra were obtained using a Bruker 300e EPR spectrometer (Bruker instruments, Billerica, MA) in a GFS-300 transfer tube. All experiments were carried out at 70K with the following EPR settings: microwave frequency = 9.256 GHz, microwave power = 6.32 mW, gain = 6.3 x 10^5, time constant = 500 ms, modulation amplitude = 2 mT, center field = 330 mT, sweep width = 200 mT, and sweep time = 2 min.

Total organic carbon analysis and stability of organo-Cr(III) complexes. Samples (1.5 ml) of the organo-Cr(III) complexes were transferred into dialysis tubing having a molecular weight cutoff of 6,000-8,000 (Spectrum, Houston, TX) and dialyzed against 500 ml of ddH_2O for 2 h at 23^oC. After dialysis samples were analyzed for total organic carbon (TOC) using a total organic carbon analyzer, TOC-5000 (Shimadzu, Kyoto, Japan), equipped with a nondispersive infrared detector. Samples were first measured for total carbon (TC). Samples were then treated with phosphoric acid and measured for inorganic carbon (IC). IC was deducted from TC to calculate TOC. Total organic was then calculated from the TOC based the number of carbons per organic compound. Standards curves were generated by analysis of each organic component

without Cr(III) present. Organo-Cr(III) complexes were analyzed and compared to the corresponding standard curve to calculate total organic component. The dialyzed samples inside the tubing and the dialysis buffer were analyzed for Cr(III) by ICP/MS. The concentrations of organic ligands and corresponding Cr(III) inside the dialysis tubing were compared to calculate the ratio of the organo:Cr(III) in the complexes.

The ability of the organo-Cr(III) complexes to resist dissociation upon dialysis in water, i.e. stability in groundwater, was calculated as the difference in Cr(III) concentrations in the dialysis tubing before dialysis and after dialysis. Changes in the solubility of the organo-Cr(III) complexes due to changes in the pH of the environment were measured over a broad pH range. One ml samples were divided into 2 vials of 0.5 ml each and titrated with either acid, 12.1 N HCl, or base, 10 N KOH, until precipitation of the complex occurred. The solution pH was measured before and after addition of acid or base using colorpHast^{TM} pH indicator strips (EM Science, Gibbstown, NJ.). The amount of Cr(III) lost by precipitation due to changes in pH was calculated as the difference between the initial amount of soluble Cr(III) before acidification and the amount remaining soluble after acidification. Samples were centrifuged at 16,500 x g for 5 min at 23^{o}C before analysis by ICP/MS for total soluble Cr(III).

RESULTS

Organo-Cr(III) production. Formation of soluble Cr(III) end-products was investigated using individual organic components present in the cytoplasm (Table 1). All samples were reduced with excess $Na_2S_2O_4$, except cysteine and GSH that reduced Cr(VI) at a slower rate but reduction was completed within 24 h, and analyzed to confirm the complete reduction of Cr(VI). Reductions in the presence of organic components immediately produced clear greenish color solutions, except cysteine-Cr(III) and histidine-Cr(III) which were pale blue. Precipitates formed in the following reaction tubes within 24 h after reduction, glycine-Cr(III), leucine-Cr(III), lactate-Cr(III), succinate-Cr(III), acetate-Cr(III), ethanol-Cr(III), fumarate-Cr(III), tyrosine-Cr(III), oxaloacetate-Cr(III), and serine-Cr(III). Control experiments composed of inorganic Cr(III) mixed with individual organic components in 100 mM KPi buffer, pH 7.0, as well as reductions reactions composed of 5 mM Cr(VI) with $Na_2S_2O_4$ in the buffer, immediately turned cloudy and formed an insoluble Cr(III) precipitate. Solubility of Cr(III) in the reductions containing organics was investigated 21 days after reduction by ICP/MS and compared to the controls containing no organic component. Control reductions contained < 0.01 mM soluble Cr(III) (Table 1). Reductions containing organics resulted in both soluble Cr(III), from 5.0 mM to 0.68 mM, and insoluble Cr(III) end-products, < 0.1 mM (Table 1). The precipitated Cr(III) end-products were composed of organic bound to Cr(III), organo-Cr(III) precipitate, as detected by the loss of both organic ligand and Cr(III) from solution, e.g. fumarate lost 5.76 mM organic and 5 mM Cr(III) from solution. Differences between the total chromium (5 mM) and the total soluble Cr(III) detected were attributed

to loss through precipitation. The soluble Cr(III) end-products formed were likely organo-Cr(III) complexes.

Identification of the Organo-Cr(III) complexes. The soluble organo-Cr(III) end-products formed with cysteine, serine, malate, oxaloacetate, and GSH were selected for further characterization. Absorbance spectrometry and EPR were used to further characterize these organo-Cr(III) end-products. Samples used in both the absorbance spectra and EPR analyses were at concentrations as reported in Table 1. Absorbance spectra of the individual organo-Cr(III) samples were compared to that of 5 mM $Cr(NO_3)_3$, a monomeric form of Cr(III) (Figure 1). Reduction samples with cysteine, serine, malate, oxaloacetate, or GSH showed comparable absorption curves but with a red shifting of their peaks in contrast to the monomeric $Cr(NO_3)_3$. Similar results were found with other complexes composed of multiple Cr(III) ions, NAD^+-Cr(III) (34), and Cr(III) trimer (39).

EPR analysis of the soluble organo-Cr(III) samples were done, and results were compared to a 5 mM Cr(III) control in H_2O. The $Cr(NO_3)_3$ control gave a sharp peak centered at g = 1.94 with a line width of ca. 5 mT (Figure 2A). The organo-Cr(III) samples were also centered at g = 1.94, but broader peaks of ca. 50 mT line width, except malate, with different shapes and intensities (Figure 2B-F). The malate-Cr(III) samples (Figure 2D) gave at least 2 paramagnetic species focused at g = 1.94. One of the paramagnetic species had an isotropic EPR spectrum with a line width of ~ 3 mT, considerably narrower than the $Cr(NO_3)_3$ control. The other paramagnetic species showed a rhombic EPR spectrum with a line width of approximately 40 mT and peaks at gz = 2.45, gy = 1.94, and gx = 1.56. These distinct EPR line shapes may represent different

binding states of Cr(III) to malate. The focusing of the EPR spectra for both the control and samples at a g = 1.94 further confirms the assignment of the end products as being composed of Cr(III) and not a Cr(IV) or Cr(V) intermediate. The broadness of the spectra is similar to that seen in other complexes containing multiple paramagnetic Cr(III) ions (34, 42) attributed to the interaction of the unpaired electrons. Hence, the organo-Cr(III) end-products are highly likely to be composed of multiple Cr(III) ions bound to the organics in a complex form, organo-Cr(III) complexes.

Characterization of Organo-Cr(III) complexes. Samples were analyzed for the molar ratio of organic bound to a single Cr(III) to understand the composition of the organo-Cr(III) complexes. The complexes were dialyzed against 500 ml ddH_2O for 2 h at 23^oC to remove any unbound organic components. The total concentration of organic components, determined by TOC analysis, was compared to the total Cr(III) remaining in the dialysis tubing. The molar ratios of the organo-Cr(III) complexes ratios varied from approximately 0.5 to 1.5 organic bound to 1 Cr(III) after dialysis (Table 2), indicating that intracellular organic components do not bind to Cr(III) at a fixed ratio. Complexes were analyzed for possible dissociation by dialysis. The total amount of Cr(III) remaining in the dialysis tubing was compared to that in the dialysis buffer. All the complexes, except malate-Cr(III), appear to be resistant to dissociation with no Cr(III) detected in the dialysis buffer. The dialysis of the malate-Cr(III) complex resulted in 5,988 nmol Cr(III) remaining in the dialysis tubing and 1214 nmol in the dialysis buffer, indicating that 20.3 % of the total Cr(III) was able to move through the dialysis tubing, either due to dissociation of the malate-Cr(III) complex or the small size of the complex.

In addition, complexes were investigated for changes in solubility due to pH alteration. Acidification of the complexes using 12.1 N HCl resulted in significant precipitation of the serine-Cr(III) below pH 4.5 and GSH-Cr(III) below pH 3 with only 5% of the Cr(III) remaining soluble (Table 3). Oxaloacetate-Cr(III) precipitated below pH 3 with approximately 30% Cr(III) remaining soluble (Table 3). Both malate-Cr(III) and cysteine-Cr(III) complexes remained very soluble at extremely low pH 1.0 with 92% and 79% Cr(III) remaining soluble, respectively (Table 3). Addition of 10 N NaOH up to pH 14 did not result in any precipitation nor re-oxidation of the Cr(III) to Cr(VI). This indicated that the complexes remain soluble over a broad pH range. A similar soluble range, pH 4.5-14, was obtained with the previously identified Cr(III)-NAD^+ complex. In contrast, inorganic $Cr(NO_3)_3$ was soluble only below pH 5.

DISCUSSION

Formation of organo-Cr(III) end-products with cellular components. Chromate reduction by bacteria has primarily focused on the strain isolation and reduction mechanisms. In bacteria, chromate is known to be transported into the cytoplasm of bacteria via the sulfate uptake system (21, 22, 32) and then is reduced in the cytoplasm (11, 36). The reduced end-products are proposed to be insoluble inorganic Cr(III) (5), on the basis of the inorganic chemistry of Cr(III) which precipitates at pH > 5 forming chromium oxides and hydroxides (10). However, these precipitates have not been clearly identified. Mass balance analysis of chromate reduction by an *E. coli* strain (ATCC 33456) (36) as well as bacteria isolated from Cr(VI) contaminated soils, including *Bacillus* strain QC1-2 (9), *Bacillus* sp., and *Arthrobacter* sp., (29), and a pseudomonad (CRB5) (28), indicates that reduction of Cr(VI) results in soluble Cr(III) end-products and not $Cr(OH)_3$ precipitates. *E. coli* reduction of Cr(VI) to soluble Cr(III) end-products has also been observed in our lab (data not shown). The soluble Cr(III) is likely derived from Cr(VI) reduction, which occurs mainly in the cytoplasm (9, 16, 18, 23, 28, 29, 31, 33, 34, 36, 40). We have reported that an *E. coli* enzyme system converted Cr(VI) to a soluble and stable NAD^+-Cr(III) complex (34). In this report, a range of possible organo-Cr(III) end-products was identified by reducing Cr(VI) in the presence of different organic components readily available in the cytoplasm. Reduction of Cr(VI) in the presence of different organic components formed organo-Cr(III) end-products with varying solubilities, 0.68 mM to 5.0 mM (Table 1). The amount of soluble Cr(III) represents a significant portion of the total 5 mM chromium reduced, from 13.6% to

100%. Soluble end-products were formed by the reduction of Cr(VI) to Cr(III) in the presence of organic compounds and not by mixing Cr(III) and organics.

Characterization of the organo-Cr(III) end-products by both absorbance spectrometry and EPR analysis (Figures 1 & 2) produced spectra similar to those identified for the NAD^+-Cr(III) complex (34) and other Cr(III) polymers (39, 42) confirming the organo-Cr end-products being composed of a multiple paramagnetic Cr(III) species in a complex form. In addition, the organo-Cr(III) complexes have different ratios of organic:Cr(III) (Table 2) which are variable and different from the NAD^+:Cr(III) ratio of 2:1 (34). In vivo formation of intracellular soluble organo-Cr(III) complexes is supported by the electron microscopy (EM) and electron energy loss spectroscopy (EELS) work with the environmentally significant *Shewanella oneidensis* MR-1. Combined EM/EELS analysis of reduced intracellular chromium in *S. oneidensis* MR-1 showed significant amounts of chromium dispersed throughout the bacterial cytoplasm as well as precipitated in larger intracellular globules (31). EELS analysis identified the intracellular chromium as being Cr(III), but in a different form from inorganic $Cr(NO_3)_3$ and $CrCl_3$ controls. These intracellular Cr(III) forms are likely organo-Cr(III) adducts, with DNA, protein, and metabolites, as previously identified in eukaryotic systems (14, 26, 38, 45, 48).

Mechanisms for release of the intracellular formed soluble organo-Cr(III) complexes are unknown but could be through either cell lysis or by methods similar to that for protein export (1), or other ATP-binding cassette (ABC) protein transporters (4), or by a yet unknown export method based on metal mimicry. Soluble complexes could also be formed outside the cell via interaction of chromium with intracellular components

released from cells. Insoluble Cr(III) precipitates may still be directly formed by extracellular microbial reduction systems utilizing reduced extracellular shuttling agents, Fe^{2+} (46), or bacterial excreted end-products, H_2S (37).

Environmental implications. The fate of the complexes in the environment remains unknown. Because Cr(III) is noted to be substitution-inert (7) with very slow ligand exchange rates (12), the organo-Cr(III) complexes may remain stable and soluble for extended periods of time in the environment. All the complexes, except malate-Cr(III), appear to remain stable and soluble upon interaction with H_2O. Malate-Cr(III) complexes either partially dissociate or are simply smaller complexes as detected by the presence of Cr(III) in the dialysis solution. Dissociation could be a result of the formation of a less stable complex. This can be inferred from the EPR analysis showing two distinct spectra which indicate possible multiple binding of a single Cr(III) to malate. However, since no Cr(III) precipitate was visible in the dialysis buffer, it is plausible that the detected Cr(III) was composed of smaller complexes which remain soluble. The complexes appear stable and resist precipitation over the pH range 4-8 typically encountered in soils (10) with malate-Cr(III) and cysteine-Cr(III) remaining soluble even at extremely low pH (Table 3). Upon release into the environment, the soluble organo-Cr(III) complexes may represent further problems by remaining stable and mobile in groundwater for an extended time. Soluble Cr(III) complexes have been detected in the environment. Soluble chromium, which was not Cr(VI), has been identified in wetland pore water at neutral pH (17, 27). The concentrations were greater than expected if $Cr(OH)_3$ was formed and were primarily composed of anionic species (96%) (17). It is hypothesized that these soluble Cr(III) species are organo-Cr(III), possibly consisting of

microbially and chemically produced Cr(III) end-products, as presented here as well as those previously reported including, Cr(III)-diethylenetriaminepentaacetic acid (DPTA), Cr(III)-citrate, Cr(III)-oxalate, Cr(III)-bisoxalate, Cr(III)-salicylate, and Cr(III)-bisalicylate (8, 19). The toxicity of these organo-Cr(III) complexes remains unknown. However, the movement of soluble organo-Cr(III) complexes in the subsurface may permit their interaction with Mn rich soils resulting in the chemical re-oxidized of Cr(III) to Cr(VI) (6). The complexes may also eventually be converted to $Cr(OH)_3$ or Cr_2O_3 in the environment. Thus, our results suggest that the reduction of Cr(VI) is more complex than just the direct production of insoluble $Cr(OH)_3$ and the formation of organo-Cr(III) complexes is possibly an integral part of the biogeochemical cycle of chromium.

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Table 1. ICP/MS analysis of total soluble chromium in the organo-Cr(III) complexes.

Complex solubility [a]	Organic ligand	Soluble Cr(III) (mM) [b]	Percent soluble Cr(III)
Highly soluble organo-Cr(III) complexes	Histidine	5.01 ± 0.06	100%
	Glutathione	4.76 ± 0.15	95%
	α-ketoglutarate	4.65 ± 0.05	93%
	Citrate	4.30 ± 0.10	86%
	Malate	3.88 ± 0.04	78%
	Serine	3.62 ± 0.14	72%
	Cysteine	3.43 ± 0.10	69%
	Pyruvate	3.25 ± 0.17	65%
	Oxaloacetate	2.86 ± 0.05	57%
Slightly soluble organo-Cr(III) complexes	Leucine	0.71 ± 0.04	14%
	Glycine	0.68 ± 0.01	13%
Insoluble organo-Cr(III) complexes	Succinate	0.02 ± 0.01	0.4%
	Fumarate	< 0.01	0%
	Lactate	< 0.01	0%
	Tyrosine	< 0.01	0%
	Acetate	< 0.01	0%
	Ethanol	< 0.01	0%
KPi-Cr(III) Control	100 mM KPi pH 7.0	< 0.01	0%

[a] Complexes were grouped based on the amount of soluble Cr(III) present, highly soluble >1mM, slightly soluble < 1mM > 0.02 mM, and Insoluble < 0.02 mM.

[b] Total initial chromium in each reduction reaction was 5 mM. Reductions were performed under anaerobic conditions in the presence or absence of 20 mM organic component and buffered with 100 mM KPi pH 7.0 at 23°C. Results are averages of triplicate analysis with standard deviations.

Table 2. Compositional analysis of organo-Cr(III) complexes.

Organo-Cr(III) complexes [a]	Total Organic ligand (mM)[b]	Total Cr(III) (mM)	Ratio of Organo : Cr(III)
Cysteine-Cr(III)	4.63 ± 0.11	3.17 ± 0.02	1.46 : 1
Serine-Cr(III)	1.40 ± 0.08	3.18 ± 0.08	0.44 : 1
Malate-Cr(III)	3.08 ± 0.02	2.66 ± 0.07	1.16 : 1
Oxaloacetate-Cr(III)	2.39 ± 0.11	2.50 ± 0.07	0.96 : 1
GSH-Cr(III)	2.97 ± 0.06	3.33 ± 0.05	0.89 : 1

[a] All samples were dialyzed for against 500 ml ddH_2O for 2 h at 23^oC before analysis. Results are averages of triplicate analysis with standard deviations.

[b] Total organic concentrations were calculated by first analyzing for total organic carbon and then calculating based on the number of carbons per organic molecule.

Table 3. Changes in soluble Cr(III) concentrations due to changes in pH [a]

Organo-Cr(III) complex	Initial soluble Cr(III) (mM)	Soluble Cr(III) (mM) after acidification[b]	Percent soluble Cr(III) Remaining	Precipitation pH
Malate-Cr(III)	5.11 ± 0.07	4.70 ± 0.07	92%	< 1
Cysteine- Cr(III)	5.28 ± 0.06	4.17 ± 0.04	79%	< 1
Oxaloacetate-Cr(III)	4.27 ± 0.11	1.28 ± 0.07	30%	< 3
Serine-Cr(III)	4.97 ± 0.16	0.274 ± 0.01	5.5%	< 4.5
GSH-Cr(III)	4.95 ± 0.01	0.238 ± 0.01	4.8%	< 3

[a] Acid (12.1N HCl) or base (10N NaOH) were titrated into samples until visible precipitate was formed on the bottom of the tubes.

[b] Samples were titrated at 23^{o}C and centrifuged before analysis by ICP/MS. Results are averages of triplicate analysis with standard deviations.

Figure 1. Absorbance spectra of soluble organo-Cr(III) complexes. Concentrations of Cr(III) are identical to those listed in Table 1. A) 3.4 mM cysteine-Cr(III) absorption peak 584 nm; B) 3.9 mM malate-Cr(III) absorption peak 595 nm; C) 4.8 mM GSH-Cr(III) absorption peak 604 nm; D) 3.6 mM serine-Cr(III) absorption peak 600 nm; E) 2.9 mM oxaloacetate-Cr(III) absorption peak 607 nm; F) 5 mM $Cr(NO_3)_3$, monomeric chromium, absorption peak 597 nm.

Figure 1.

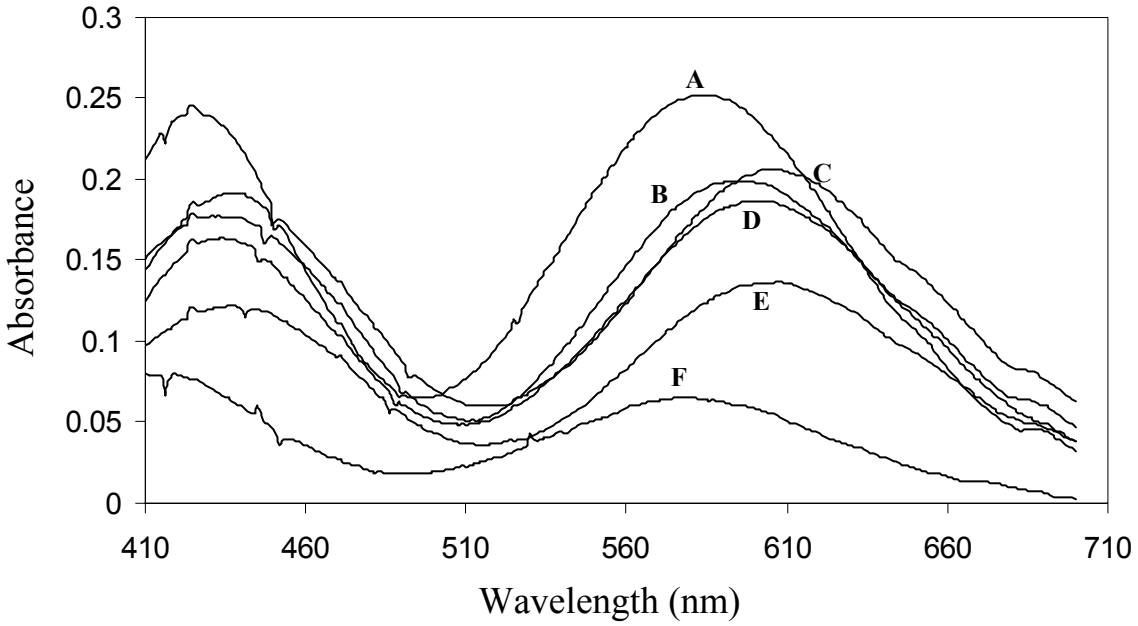
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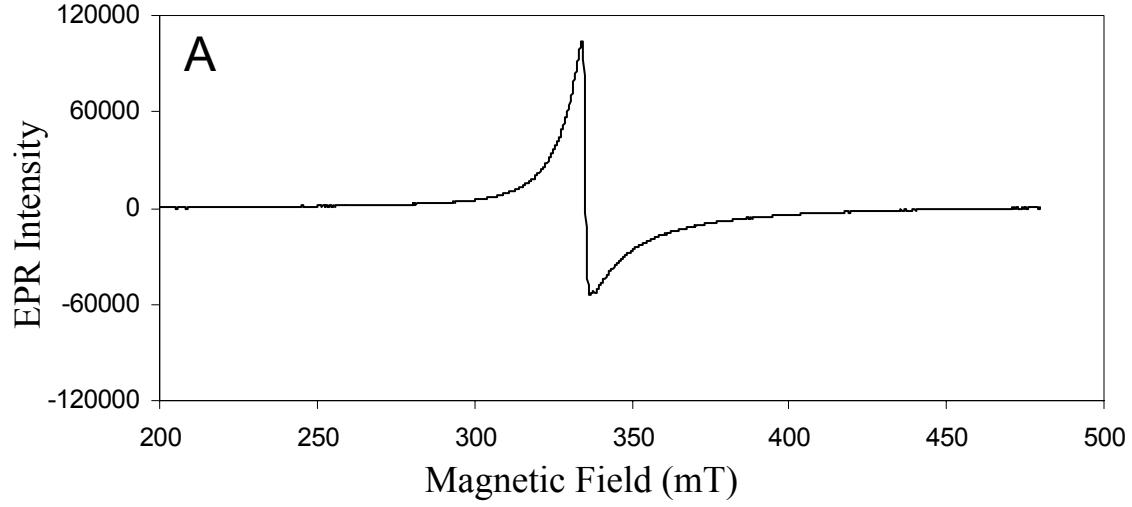
Figure 2. EPR analysis of organo-Cr(III) complexes. A) 5 mM monomeric Cr(III) standard; $Cr(NO_3)_3$; B) 3.6 mM serine-Cr(III); C) 3.4 mM cysteine-Cr(III); D) 3.9 mM malate-Cr(III); E) 4.8 mM GSH-Cr(III); F) 2.9 mM oxaloacetate-Cr(III).

Figure 2.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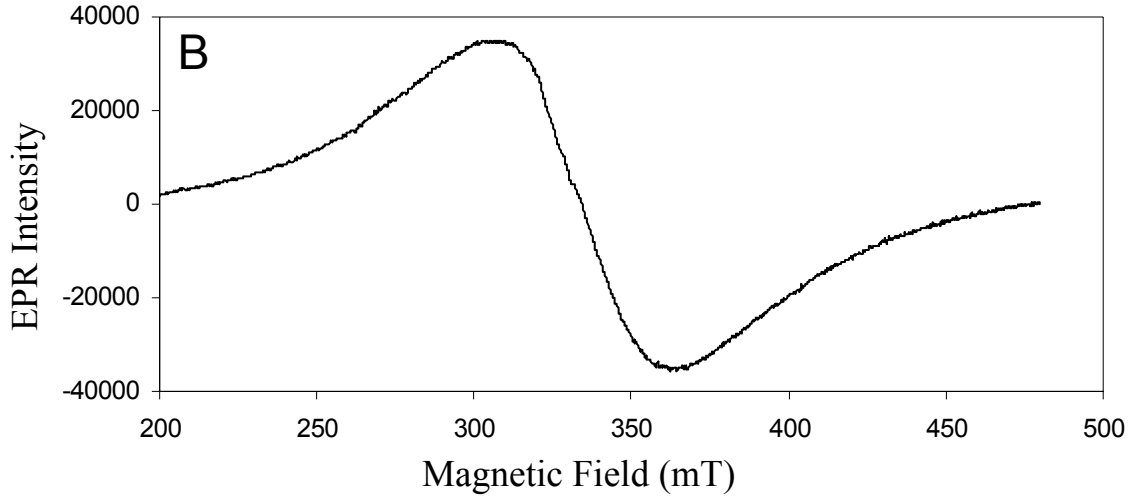
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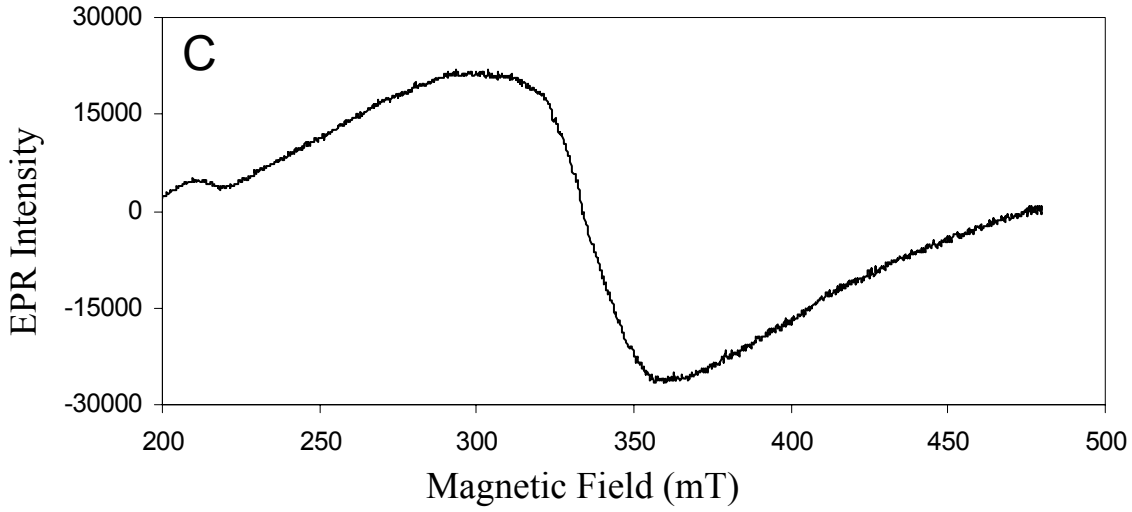
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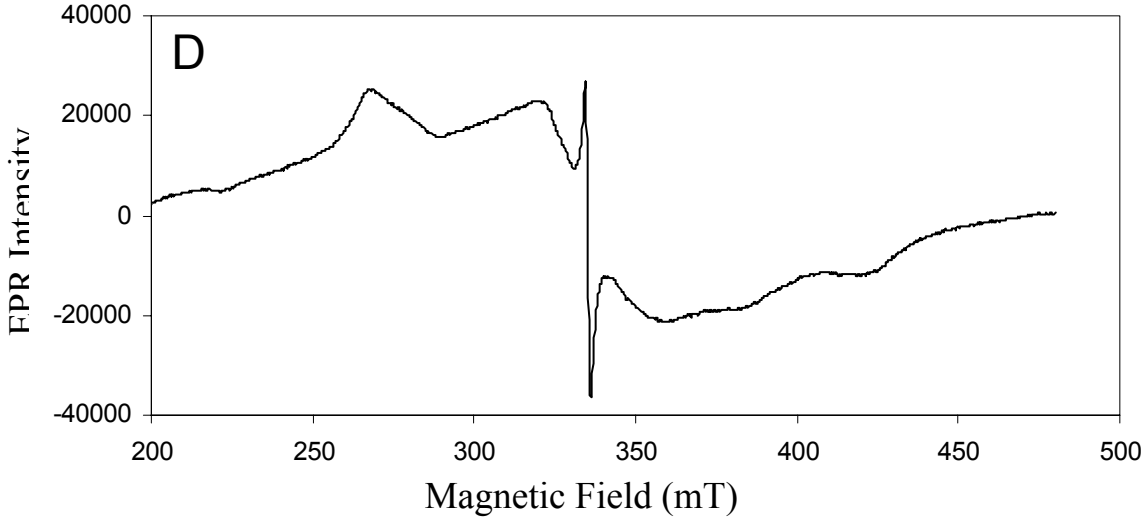
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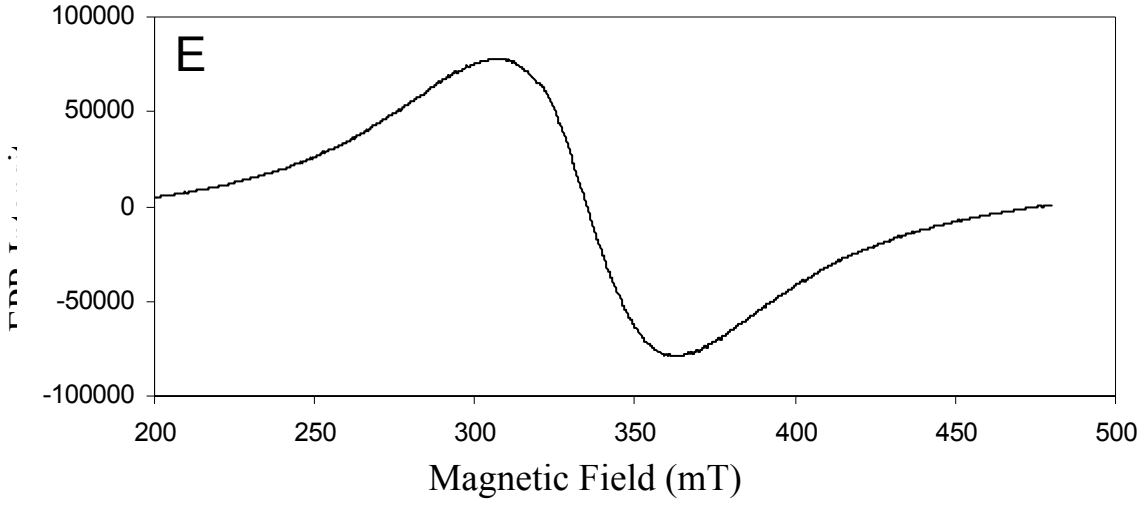
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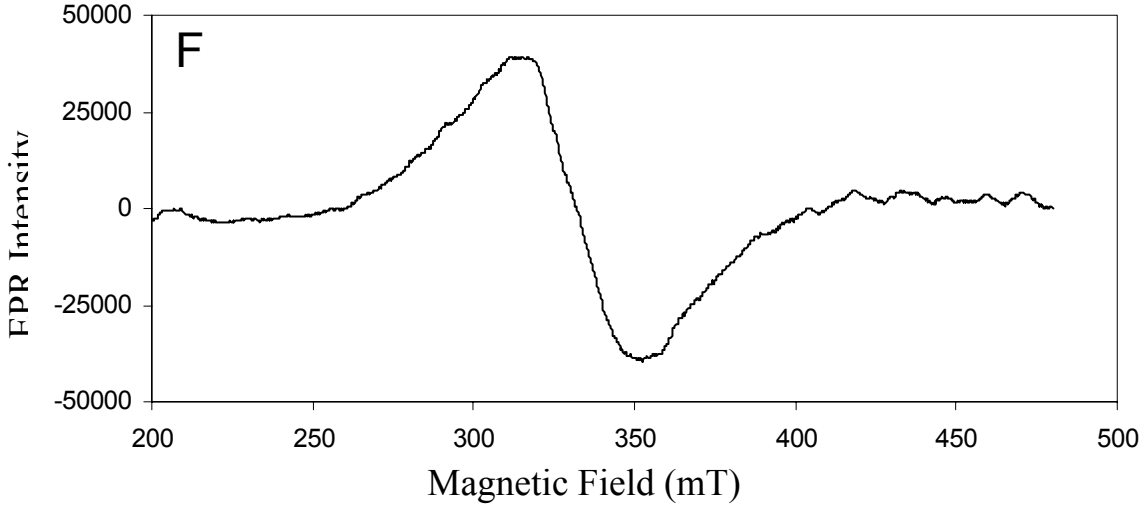
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CHAPTER FOUR

MINERALIZATION OF A SOLUBLE ORGANO-CHROMIUM(III) COMPLEX BY NOVEL BACTERIAL ISOLATES

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ABSTRACT

Microbial reduction of soluble hexavalent chromium [Cr(VI)] is thought to result in the production of an insoluble trivalent [Cr(III)] precipitate. Bioremediation methods based on this idea have been investigated as a method for clean up of Cr(VI) contaminated environments. However, recent evidence demonstrates that soluble organo-Cr(III) complexes are readily formed upon microbial, enzymatic, and chemical reduction of Cr(VI), instead of insoluble $Cr(OH)_3$ precipitates. The presence of soluble Cr(III) species has been detected in the environment, but their fate and recalcitrance remains unknown. In this study, two bacteria, PTX1 and PTX2, capable of mineralizing an enzymatically produced organo-Cr(III) complex, NAD^+-Cr(III), were isolated and characterized. The bacteria utilized both free NAD^+ and NAD^+ in NAD^+-Cr(III) complex as a carbon and energy source. Bacterial mineralization of the NAD^+-Cr(III) resulted in the precipitation of Cr(III) on the bacterial surface as detected by transmission electron microscopy. Phylogenetic classification based on 16s rDNA analysis placed PTX1 in the *Leifsonia* genus and PTX2 in the *Rhodococcus* genus. The new *Leifsonia* and *Rhodococcus* isolates highlight a novel aspect of the biogeocycle of chromium, where soil microbes mineralize soluble organo-Cr(III) complexes, leading to the production of insoluble Cr(III).

INTRODUCTION

The biogeochemical cycle of chromium predominantly follows the interplay of soluble carcinogenic hexavalent [Cr(VI)] and non-toxic trivalent chromium [Cr(III)] (5). Cr(III) is the predominant form of chromium in the environment while Cr(VI) is mainly introduced as a by-product of industrial use (20). Reduction of Cr(VI) in the environment can be done via both chemical and biological means. Bacteria were first demonstrated to reduce Cr(VI) in 1979 (21) and since then numerous bacteria have been characterized as been able to reduce Cr(VI) (13, 23, 27, 31, 37, 39, 40) under many different conditions, therefore providing possible methods for in situ bioremediation at contaminated sites. The microbially reduced Cr(III) end-product is hypothesized to result in an insoluble inorganic form (3). However evidence is beginning to highlight a more complex role for microbes in the Cr cycle: 1) reduction by several bacteria species has resulted in the production of soluble form of Cr(III) found in the supernatant (7, 25, 26, 37); 2) enzymatic reductions of chromate have formed organo-Cr(III) complexes, where Cr(III) is bound to reductants, such as NAD^+ (34) and cytochrome c_7 (2); and 3) reduction of Cr(VI) in the presence of common intracellular components, which are not chelating agents, results in the formation of several different soluble organo-Cr(III) complexes (35). In addition, it has been demonstrated that the presence of natural chelating agents, such as citrate, in Cr(III) contaminated soils results in the formation of soluble organo-Cr(III) complexes which are able to migrate through soils (18).

The biogeocycle of soluble organo-Cr(III) complexes remains largely unknown and the fate and recalcitrance of the complexes in the environment is not well understood. One possible fate is the re-oxidation of Cr(III) to Cr(VI) by manganese [Mn(IV)] rich

soils (4). Such a process would results in a secondary source of Cr(VI) contamination away from the original site. A second possible fate is the further transformation of the organo-Cr(III) complexes to a final insoluble Cr(III) form, which could occur via ligand exchange or by direct microbial mineralization of the organic portion of the complexes. Transformation to a final insoluble form of Cr(III) would complete the biogeochemical cycle of Cr(III) and further validate bioremediation as a viable means for Cr(VI) clean up.

We have investigated the microbial mineralization of organo-Cr(III) complexes as a means for transforming the soluble organo-Cr(III) complexes into an insoluble form. Here we report the isolation of two different bacteria able to grow on the soluble organo-Cr(III) complex, NAD^+-Cr(III). The bacteria were characterized based on their ability to utilize both the NAD^+-Cr(III) complex and NAD^+ for growth and the molecular phylogeny of their 16s rDNA.

MATERIALS AND METHODS

Chromium detection. Potassium chromate, K_2CrO_4 (Sigma, St. Louis, Mo.), was used as the Cr(VI) source in all experiments. Cr(VI) was measured by a reported method (36) using chromate as the standard and using ChromaVer powder pillows (HACH, Loveland, CO) containing diphenylcarbazide. NAD^+-Cr(III) complex was produced enzymatically as previously reported (34). Total soluble Cr(III) was calculated as the difference between total soluble chromium and Cr(VI). Samples were prepared for total soluble chromium by first centrifugation at 16,500 x g for 5 min at 23^{o}C and then filtered through a 0.22 μm MILLIPORE MILLEX^{TM}GP syringe filter to remove any precipitates and large complexes. Samples were analyzed using an HP 4500 Plus Series inductively coupled plasma/mass spectrophotometer (ICP/MS) (Agilent Technologies Inc., Palo Alto, CA). Total soluble Cr(III) in whole cell reduction cultures was prepared identically to the ICP/MS method, but analyzed using an Optima 3200RL inductively coupled plasma /optical emission spectrophotometer (ICP/OES) (Perkin Elmer Life and Analytical Sciences Inc, Boston, MA). Monomeric chromium (III), $Cr(NO_3)_3$ (Aldrich, Milwaukee, WI), in distilled water (pH <3) was used to generate a standard curve for ICP/MS and ICP/OES.

Media preparation. The two minimal media for PTX2 and PTX1 growth analysis were prepared as follows. The first medium was composed of the enzymatically produced organo-Cr(III) complexes which were dialyzed against ddH_2O to remove unbound NAD^+. After dialysis, samples were filtered and measured for total soluble Cr(III) by ICP/MS and total NAD^+ by absorbance spectrometry, OD_{260nm}. NAD^+-Cr(III) medium contained an initial ratio of NAD^+:Cr(III) of 2:1. PTX1 and PTX2 were

inoculated into medium at 2% final concentration. Cultures were sampled for changes in both NAD^+ and Cr(III) concentrations after a period of >4 months. The second minimal media (MM-NAD^+) was composed of the following components and prepared as follows: 3 g/L Na_2HPO_4, 1 g/L KH_2PO_4 were dissolved in ddH_2O and sterilized. After the mixture was autoclaved for 45 minutes, 1.5 mL trace mineral solution (composed of the following per liter, $MgSO_4$ 10 g, $CaCO_3$ 2 g, $FeSO_4*H_2O$ 4.5 g, $ZnSO_4*7H_2O$ 1.44 g, $MnSO_4*4H_2O$ 1.12 g, $CuSO_4*5H_2O$ 0.25 g, $CoSO_4*7H_2O$ 0.28 g, H_3BO_3 0.06 g, and concentrated HCl 10 ml was added. Before inoculation, filter sterilized NAD^+ was added to a final concentration of 1.5 mM and then medium was inoculated with either PTX1 or PTX2 at a final concentration of 10%. Samples were incubated at 30^OC and shaken at 250 rpm. Growth of bacteria was monitored by total protein analysis using Pierce Modified Lowry Protein Assay Kit (Pierce, Rockford, IL) following manufactures instructions. After growth in media cultures were periodically plated on LB agar to confirm viability and no contamination was present.

Bacterial chromate resistance and reduction. Luria Bertani (LB) medium was used in both Cr(VI) resistance and reduction experiments. LB was prepared as follows, yeast extract (0.5%) (Becton, Dickinson, and Co., Sparks, MD), NaCl (1%) (J.T. Baker, Phillipsburg, NJ) and tryptone (1%) (Becton Dickinson, and Co., Sparks, MD) were added to 1 L ddH_2O, pH was adjusted to 7.0 with 4 N NaOH. For LB agar plates, agar (Becton, Dickinson, and Co., Sparks, MD) was added to a final concentration of 1.5%. LB medium and agar then were sterilized by autoclaving for 45 minutes at 121^oC. After autoclaving, LB was cooled to room temp and then filter sterilized Cr(VI) was added to a final concentration of either 100 μM, 500 μM, or 15 mM. Controls containing LB with

no Cr(VI) were also used to measure growth under non-Cr(VI) conditions. PTX1, PTX2, and *E. coli* were inoculated into the media at a final concentration of OD_{600nm} = 0.1 – 0.15 and growth was monitored by increases in OD_{600nm}. Samples for Cr(VI) reduction were removed hourly, centrifuged 16,500 x g 5 min to remove cells, and then analyzed by diphenylcarbazide assay.

Electron microscopy. Preparation of samples for the transmission electron microscopy (TEM) was as follows: 10 μL of samples were added to 90 μL of ddH_2O and mixed, 3 μL of each was immediately placed on the disks to air dry. For samples that were negatively stained, 5 μL of Phosphotungstic Acid (PTA) dye was added and removed after 30 s. Samples were placed under a lamp for 15 min to dry the dye, and then viewed on the JEOL transmission electron microscope (JEOL JEM 1200 EX, Tokyo, Japan) at the Washington State University (WSU) Electron Microscopy Center.

16s rDNA analysis. Genomic DNA was purified using the Puregene™ DNA Isolation Kit (Gentra Systems, Minneapolis, MN) following the manufacturers instructions. After DNA purification, the 16s rDNA was amplified using a GeneAmp® System 2400 PCR thermocycler (Perkin Elmer Life and Analytical Sciences Inc, Boston, MA) with the primer GB-13 and GB-14, as listed in Table 1. Primers were purchased from Invitrogen (Invitrogen life technologies, Carlsbad, CA). The PCR reaction mixture were composed of 5 μL 10X PCR buffer, 2 μL 50 mM $MgCl_2$, 0.6 μL 25 mM dNTP's, 1.25 μL 12.5 μM Forward Primer, 1.25 μL 12.5 μM Reverse Primer, 1 μL Genomic DNA, 0.25 μL Taq, and ddH_2O to final volume of 50 μL. The following PCR cycle conditions were applied for 16s rDNA amplification: an initial denaturing at 95°C for 3 min, 25 cycles of denaturing at 95°C for 30 sec, annealing at 58°C for 30 sec, and

elongation at 72°C for 1:30, and a final elongation at 72°C for 7 min. The samples were held at 4°C after amplification of 16s rDNA. Products were checked 0.8% agarose gel and then purified using the Qiagen Gel Extraction Kit (Qiagen Inc, Valencia, CA), following the manufactures protocol. Purified products were sequenced using the ABI Prism BigDye™ terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) as follows: mixing together 40-90 ng DNA template, 3.2 pmol (0.25 μL) DNA primer, 4 μL Big Dye mix and 1.75 μL ddH_2O for a final volume of 10 μL. Primers are as listed in Table 1. The 16s rDNA cycle sequencing conditions were as follows, an initial denaturing at 94°C for 4 min, 30 cycles of denaturing at 96°C for 10 sec, annealing at 50°C for 5 sec, and elongation at 60°C for 4 min. Samples were cleaned using Performa™ DTR Gel Filtration cartridges (Edge Bio Systems, Gaithersburg, MD) and then analyzed at the WSU Laboratory of Bioanalysis and Biotechnology (LBBI).

Phylogenetic Tree. PTX1 and PTX2 16s rDNA sequences were compared to know sequence in the National Center for Biotechnological Information (NCBI) database (http://www.ncbi.nlm.nih.gov) using the BLASTN program (1). Sequences were then aligned using the CLUSTAL W (41) program (http://www.ebi.ac.uk/clustalw) on the European Bioinformatics Institute site. Phylogenetic trees were constructed from 10 equivalent 16s rDNA sequences selected for each bacterium, PTX1 or PTX2, using the PHYLIP phylogeny inference program package version 3.5c (12). Distance matrices were estimated using DNADIST. Bootstrap analyses were done using 1000 replicate alignments with the SEQBOOT program. Phylogenetic trees were then constructed using the distance matrices generated as inputs for the NEIGHBOR and CONSENE programs and viewed using TreeView 1.6.6 (30). The following bacterial nucleotide sequences

with their accession numbers were used to create the PTX1 phylogenetic tree: *Leifsonia shinshuensis* , AB028940; *Leifsonia xyli* strain S29n, AY509236; Naphthalene-utilizing bacterium IS1, AF531474; *Leifsonia* sp. V4.MO.14, AJ244675; *Leifsonia naganoensis*, AB028941; *Leifsonia poae*, AF116342; *Leifsonia aquaticum* (DSM 20146), X77450; *Leifsonia xyli* subsp. *xyli*, U96183; *Microbacterium* sp. VA8728_00, AF306834; *Agrococcus jenensis*, X92492. The following bacterial nucleotide sequences with their accession numbers were used to create the PTX2 phylogenetic tree: Arctic seawater bacterium R7851, AJ293819; *Rhodococcus* sp. YK2, AB070458; *Rhodococcus* sp. P_wp0233, AY188941; *Rhodococcus* sp. 5/14, AF181690; *Rhodococcus* sp. S9, AF260713; *Rhodococcus* sp. 5/1, AF181689; *Rhodococcus* sp. isolate E60, AY316681; *Rhodococcus fascians* (DSM 20669), X79186; *Rhodococcus* sp. A1XB1-5, AY512642; *Rhodococcus* sp. UFZ-B520, AF235011.

Enzymatic Analysis. Analysis of enzyme activity was done using the bioMerieux API ZYM kit (bioMerieux, Inc., Hazelwood, Mo) following the manufactures protocol. Bacteria were grown to an OD_{600nm} = 2.5, in LB media. Samples were removed, 10 ml, and centrifuged at 7,500 x g 10 min at 4^{o}C. Supernatant was decanted and samples re-suspended in 10 ml ddH_2O. The API ZYM strips, previously equilibrated to room temperature 23^{o}C, were prepared by distributing 5 mL of ddH_2O into the wells of the tray to create a humid atmosphere. PTX1 or PTX2 sample, 65 μL per well, was dispensed into the individual cupules and incubated at 37^{o}C for 4 hours. After incubation, 1 drop of ZYM reagent A and 1 drop of ZYM reagent B were added and incubated for 5 minutes. The strips were then exposed to direct sunlight for 2.5 min and the results recorded. Values ranging from 0-5 were assigned based on intensity of the colors. A positive result

(+) was an intensity of 3 - 5, a weakly positive result (w) was an intensity of 1 – 2, and a negative result (-) was an intensity of 0.

RESULTS

Growth of bacteria on NAD^+-Cr(III) and NAD^+. We have previously reported the formation of NAD^+-Cr(III) by a bacterial enzyme system and the complex is relatively stable (34). Filter sterilized NAD^+-Cr(III) solutions remained clear after approximately 1 year at room temperature, 23°C. Exposure of the filter sterilized NAD^+-Cr(III) solution to air resulted in bacterial growth in the solution after 1 year incubation at 23°C. The NAD^+-Cr(III) solution in which the bacteria were discovered was investigated to identify the carbon and energy source utilized by the bacteria for growth. The NAD^+-Cr(III) solution initially contained 16.4 mM of NAD^+ and 8.8 mM Cr(III) (34). After bacterial growth, the NAD^+ and soluble Cr(III) concentrations decreased to 6.22 mM and 5.73 mM, respectively. NAD^+ was hypothesized to be the carbon and energy source for the bacteria based on its decrease from solution. Using sterile technique, samples were removed and bacteria were spread onto LB agar plates. Two different colony morphologies were visible 3-4 days after incubation at 23°C. Both were further isolated from the mixed culture plate into individual pure cultures. The two bacteria were designated as isolates PTX1 and PTX2. Growth experiments were repeated with the two bacteria on dialyzed and filter sterilized NAD^+-Cr(III). The bacteria were grown individually in solution and as mixed cultures over a period of four months at 23°C (Table 2). Visible bacterial growth was not apparent until after an extended period of time, 3-4 months. Un-inoculated controls showed no change in either Cr(III) or NAD^+ concentrations and no precipitate formed in the tubes (Table 2). Cultures containing PTX1, PTX2, or both showed significant decreases in NAD^+ concentrations and minimal decreases in Cr(III)

concentrations. PTX1 cultures appeared to utilize the NAD^+ more readily than PTX2, while co-cultures showed the greatest decrease of NAD^+.

Since the bacteria were capable of extremely slow growth on NAD^+-Cr(III), attempts were made to grow the bacteria on MM containing NAD^+ as the sole carbon and energy source. Both PTX1 and PTX2 bacteria were grown in LB medium at 30^{o}C with constant shaking, 250 rpm, over night. Samples, 5ml, were removed from both cultures, washed 2x with 5 ml MM, re-suspended in a final volume of 1 ml of MM, and inoculated into 50 ml of freshly prepared MM with 1.5 mM NAD^+. Cultures incubated at 30^{o}C with constant shaking 250 rpm. Samples were removed daily to measure bacterial growth by total protein analysis (24). PTX1 showed the ability to grow on MM-NAD^+ as detected by increases in total protein (Figure 1), reaching a max of 231.5 $\pm$ 4.5 μg/ml after 12 days. PTX2 showed much slower growth and an overall lower growth yield (Figure 1), reaching a max of only 20.1 $\pm$ 3.0 μg/ml after 21 days. Microscopic analysis of PTX2 cultures over the 28 days growth showed the cells changing from rod to coccus morphology, and the latter were clumped together in aggregates. PTX2 was capable of growth when plated on LB, but appeared to remain in a very slow growing state in the MM-NAD^+ medium.

Bacterial morphology and transmission electron microscopy (TEM) analysis. PTX1 and PTX2 cultures were grown on LB agar plates for morphological and microscopic investigation. PTX1 colonies were yellow in color, round in shape with smooth edges, and a glossy sheen. While, PTX2 colonies were orange in color, round in shape with rough edges, a matte sheen, and raised fruiting like bodies when grown longer. Both strains were only capable of aerobic growth. PTX1 grew well in LB medium

at temperatures between 23-37^{o}C, while PTX2 grew well between 23-30^{o}C and did not grow at 37^{o}C. Microscopic analysis of bacteria grown in LB broth confirmed the two strains had different morphologies. PTX1 was a small to medium rod shaped bacterium, and PTX2 was a large rod shaped bacterium.

Both bacterial cultures were further visualized using the JEOL transmission electron microscope at the WSU Electron Microscopy Center to identify differences in size, shape, chromium(III) precipitation, and whether the bacteria were gram positive or negative. Samples from the original NAD^{+}-Cr(III) growth solution, replicate NAD^{+}-Cr(III) growth with pure cultures, and MM-NAD^{+} growth cultures of individual strains were diluted 1:10 with 3 μl dispensed onto carbon coated formvar grids. The original mixed culture was stained with PTA for 30 s. The bacteria were identified as gram positive with two predominate morphological phenotypes, rod and coccus (Figure 2). The rods were present in significantly greater numbers than the cocci. Both bacteria had electron dense material surrounding the outer wall, which was hypothesized to be Cr(III) precipitation on the bacterial cell wall. To identify whether the electron dense material was due to Cr(III) or PTA stain, samples were analyzed without PTA staining. TEM analysis of unstained pure cultures grown in the NAD^{+}-Cr(III) and MM-NAD^{+} medium showed significant differences. Both PTX1 and PTX2 bacteria grown in the NAD^{+}-Cr(III) medium had electron dense material surrounding the bacterial cell (Figure 3A & 4A respectively). Bacteria grown in MM-NAD^{+} had significantly less electron dense material surrounding the cell (Figures 3B & 4B, respectively). The difference between the TEM images signals that Cr(III) is probably precipitating on the cell well. This implies that as soluble organo-Cr(III) products are utilized by bacteria, the Cr(III) is

further transformed from its soluble state to an insoluble precipitate on the cell surface. Such a precipitation event would lead to the removal of soluble Cr(III) from the environment and Cr(III) residing in a final insoluble form.

Phylogenetic characterization and metabolic profiles. PTX1 and PTX2 were further characterized based on their 16s rDNA sequences. PCR amplified 1,388 base pairs of PTX1 and 1,373 base pairs of PTX2 16s rDNA. Sequences were compared to those in the NCBI database using the BLASTN program (1). The 10 most similar sequences were then selected and aligned using CLUSTAL W (41). Phylogenetic trees were created from the alignments using the PHYLIP programs DNADIST, NEIGHBOR, and CONSENSE. Bootstrap values were generated using SEQBOOT with 1000 replicates. Phylogenetic results placed PTX1 in the *Leifsonia* genus (Figure 5), on a distinct branch between naphthalene-utilizing bacterium IS1, identified as a *Leifsonia xyli* species, and *Leifsonia* sp. V4.MO.14. PTX2 was placed in the *Rhodococcus* genus (Figure 6), clustered with *Rhodococcus* sp. 5/1, *Rhodococcus* sp. 5/14, and Arctic seawater bacterium R7851.

Metabolic profiles of PTX1 and PTX2 were analyzed using the bioMerieux API ZYM enzymatic analysis text strips. The API ZYM kit tests for the presence of 19 different enzymes (esterases, aminopeptidases, proteases, phosphotases, and glycosidases). PTX1 tested positive for 10 enzymes and weakly positive for 4 while PTX2 tested positive for 8 enzymes and weakly positive for another 2 (Table 3).

Chromium reduction and resistance. Both PTX1 and PTX2 demonstrated resistance to elevated levels of soluble Cr(III) as represented by their ability to grow in the NAD^+-Cr(III) solution containing approximately 9 mM soluble Cr(III). The 2 isolates

were further investigated for resistance to elevated levels of Cr(VI) and their ability to reduce Cr(VI). Initially, PTX1 & PTX2 cultures were tested for growth in LB medium containing 500 μM Cr(VI). Both grew well and did not appear to be effected by Cr(VI) level of 500 μM (data not shown). PTX1 and PTX2 were then tested for growth in LB and LB+15 mM Cr(VI) (Figure 7A and B). *E. coli* W (ATCC 11105) was also grown in LB and LB+15 mM Cr(VI) for additional comparison (Figure 7A & B). Growth of all cultures was greatly effected by the presence of 15 mM Cr(VI). *E. coli* W was unable to grow in 15 mM Cr(VI) LB and 7 day old cultures were not viable when plated onto LB agar. PTX2 grew poorly in the 15 mM Cr(VI) LB medium, max OD_{600nm} = 0.239 after 24 h in comparison to cultures in LB which grew to an OD_{600nm} = 9.0 in the same 24 h. PTX2 did remain viable after 7 days as denoted by its ability to grow on LB agar plates. PTX1 grew well in the 15 mM Cr(VI) LB, max OD_{600nm} = 2.34 after 96 h, close to its growth maximum of OD_{600nm} = 3.48 after 24 h, and remained viable by growing on LB agar plates after 7 days. All cultures showed no apparent decrease in Cr(VI) suggesting that survival is due to a resistance mechanism and not reduction. Similar resistance to elevated Cr(VI) has been found in another *Microbacteriacea* family gram positive bacteria, *Microbacterium* strain MP30 at 15 mM Cr(VI) (32), as well as other gram negative bacteria, Pseudomonad (CRB5) at approximately 10 mM Cr(VI) (25).

The Cr(VI) reduction activity of PTX1 & 2 was assayed using actively growing cultures, initial OD_{600nm} = 0.1. Cr(VI) was added to a final concentration of 100 μM. Culture growth was very similar to that in the absence of Cr(VI) (Figure 8A). PTX1 reduced Cr(VI) faster that PTX2 with complete reduction within 24 h compared to approximately 48 hours (Figure 8B). Minimal abiotic reduction was noted in the control

composed of LB with 15 mM Cr(VI). Both cultures grew at a similar rate in the presence of 100 μM Cr(VI) as compared to LB with no Cr(VI) (Figures 8B and 7B). Analysis of PTX1 and PTX2 Cr(VI) supernatants immediately after chromate reduction and14 days post reduction showed no Cr(VI) and total Cr in solution remained unchanged, approximately 100 μM.

DISCUSSION

The role of microorganisms in the biogeocycle of chromium has long focused predominantly on the reduction of Cr(VI) to produce insoluble Cr(III). However, microbial reduction has shown that significant amounts of Cr(III) remain soluble in the supernatant after Cr(VI) reduction (7, 25, 26, 37) as well as the two isolates tested here. Recent evidence has pointed to a much more complex biogeocycle, in which insoluble Cr(III) is not the immediate reduction end-product (2) (34) (35). The fate and recalcitrance of these soluble organo-Cr(III) end-products, along with those formed chemically through complexation between Cr(III) and organic acids in soil (18), is not well understood. Re-oxidation of Cr(III) to Cr(VI) by manganese rich soils (4) has already been demonstrated as one possible fate. A second possible fate is bacterial mineralization of the organo-Cr(III) end products. Bacteria are noted to degrade organo-metal complexes formed with chelators, e.g. EDTA-metal (29) and citric-metal (19), but degradation of organo-metal complexes formed with non-chelating agents is not well documented. The two bacteria described here, PTX1 and PTX2, are capable of mineralizing organo-Cr(III) complexes formed with non-chelating agents. The source of the two bacteria is likely from the ambient air, derived from local soils. Both bacteria were isolated from a solution containing organo-Cr(III) complexes, NAD^+-Cr(III). The bacterial growth in these cultures was coupled to a decrease in the amount of NAD^+ present (Table 2). This signaled NAD^+ as the likely carbon and energy source for growth. Mineralization of NAD^+ for growth was confirmed for PTX1 by its ability to grow on both NAD^+-Cr(III) (Table 2) and NAD^+ (Figure 1). Complexation of NAD^+ with Cr(III) appears to strongly interfere with PTX1's ability to utilize the NAD^+ for growth as

demonstrated by the overall differences in growth rate on NAD^+-Cr(III) versus MM-NAD^+, months compared to days. Even though the growth rate is slowed, PTX1 is still capable of degrading the organo-Cr(III) complex. PTX2 growth on NAD^+-Cr(III) did not consume as much NAD^+ as PTX1 (Table 2) and growth on MM-NAD^+ was slower than PTX1 (Figure 1). PTX2's ability to mineralize NAD^+ is inferior compared to PTX1, with growth on NAD^+ extremely slow as detected by the mild increase in total protein (Figure 1). Hence the growth rate of PTX2 is over months instead of weeks. In addition, since PTX2's rate of growth is extremely slow in both media, inhibition due to Cr(III) complexation appears be more negligible than in PTX1.

The mechanism for utilization of NAD^+ by PTX1 and PTX2 is undetermined, but is probably via an NAD^+ glycohydrolase (NADase). NADases hydrolyze NAD^+ into ADP-ribose and nicotinamide. Such enzymes are present in both bacteria (8) and eukaryotes (15). *Streptococcus pyogenes*, a gram positive cocci, is known to secrete an NADase into the extracellular medium (8). PTX1 or PTX2 may use a similar system to release NAD^+ from the NAD^+-Cr(III) complex for utilization. Based on better NAD^+ consumption in pure culture, PTX1 probably is the organism able to breakdown NAD^+ extracellularly. The mechanism presents a means for the 2 isolates to grow in a synergistic relationship where PTX1, through the extracellular breakdown of NAD^+, is able to release an additional, more readily useable, carbon source possibly aiding PTX2's ability to grow in co-culture. PTX2 may also secrete factors that are beneficial to PTX1 into the co-culture medium.

Through the hydrolysis of the NAD^+ portion of the NAD^+-Cr(III) complex, Cr(III) could be free to either precipitate from solution or bind to the cell surface. Since

Cr(III) precipitate was not directly observed in the solutions the Cr(III) is most likely binding to the bacterial cell surface. TEM photos of the bacteria grown in the presence and absence of Cr(III) support the hypothesis that the Cr(III) precipitates on the cell surface. Unstained PTX1 and PTX2 cultures grown in NAD^+-Cr(III) medium (Figures 3A and 4A) are coated with an electron dense material that is not present when the cultures are grown on MM-NAD^+ (Figures 3B and 4B). Since absence of Cr(III) is the main difference between the two media, the electron dense material is most likely Cr(III). The electron beam does interact with material in the MM-NAD^+ growth medium (Figure 3B and 4B), creating bubble-like structures surrounding the bacteria, but is not similar to the electron dense material covering cultures grown in NAD^+-Cr(III) (Figure 3A and 4A). In addition, PTX1 cultures grown for over 1 year have an apparent crystalline Cr(III) precipitate surrounding the cells (Figure 9). No crystalline precipitate was apparent in the same PTX1 cultures at 4 months or in the PTX2 cultures. This signals that as bacteria gradually consume the NAD^+ portion of the NAD^+-Cr(III) complex, the overall ratio of NAD^+:Cr(III) decreases from the initial 2:1. Hence, the solubility of the complex is altered by the continued consumption of the NAD^+ ligand. The removal continues overtime until the complex decreases below a minimum ratio to maintain solubility and the Cr(III) precipitates in an insoluble form on the cell surface. The resulting precipitate appears to grow into a Cr(III) crystalline structure (Figure 9). Since no precipitation was seen at 4 months growth with a ratio of 1.6:1 of NAD^+ and Cr(III) in solution, a significant portion of the NAD^+ must first be consumed by the bacteria before the precipitation of Cr(III) occurs. Since Cr(III) precipitation on PTX1 took approximately a year before it was observed, bacteria in the environment may need extended periods of

time in order to degrade soluble organo-Cr(III) complexes to the point where Cr(III) precipitates. The resident time frame of soluble organo-Cr(III) complexes may be quite variable based on the subsurface flow rate in different environments. This could greatly affect the amount of organo-Cr(III) complex mineralized and Cr(III) precipitated. More research in this area is needed. Precipitation of Cr(III) on cell membranes has been noted in other bacteria (25) (28). The precipitation of Cr(III) on the cell surface represents an important pathway for the formation of an immobile Cr(III) form, which has been long postulated as the terminal point of Cr(VI) bioremediation.

PTX1 and PTX2 were identified as both being Gram-positive rods based on their morphology and lack of a periplasmic space in the TEM images. Phylogenetic analysis of the 16s rDNA sequence confirmed both as Gram-positive rods. Based on the 16s rDNA analysis PTX1 belongs to the *Leifsonia* genus (Figure 5). PTX1 shows 99-98% sequence homology to members of the *Leifsonia* genus with only 94% homology to *Agrococcus jenesis*. PTX1 also forms a distinct branch when aligned with other known *Leifsonia* species and probably represents a new species of *Leifsonia*. The 16s rDNA analysis of PTX2 placed it in the genus *Rhodococcus* (Figure 6), with 99% sequence homology to the 10 closest species used for the phylogenetic analysis. PTX2 was place in a monophyletic branch with *Rhodococcus* sp 5/1 (closest), *Rhodococcus* sp 5/14, and an Arctic seawater bacterium. Due to the overall high sequence homology and close alignment, PTX2 is closely related to *Rhodococcus* sp 5/1, although it may represent a new *Rhodococcus* species. Microscopic analysis of PTX2 showed both rod and coccus morphology, which is consistent with the *Rhodococcus* genus (16). Further investigation is needed to definitively classify both isolates. Biochemical analysis of PTX1 and PTX2

using API ZYM test strips demonstrated that both to have different metabolic capabilities, i.e. esterases, aminopeptidases, proteases, phosphotases, and glycosidases. PTX2 displayed similar API ZYM profiles to the closely related genus *Nocardia* (14) while no profile for the genus *Leifsonia*, formerly called *Clavibacter* (38), could be found for comparison of PTX1. Both PTX1 and PTX2 are strict aerobes and grow well on LB medium (Figure 7A). PTX1 was able to grow between 23-37^{o}C, with an apparent optimum at 30^{o}C. PTX2 grew optimally between 23-30^{o}C, with inhibited growth at 37^{o}C, similar to other *Rhodococcus* species (6). Colony morphology of PTX1 and PTX2 (see results section) were comparable to *Leifsonia* (11, 38) and *Rhodococcus* (6, 16) in the literature. Both *Leifsonia* and *Rhodococcus* are generally soil associated microbes with some having phytopathogenic traits (9, 22, 33). In addition, species of both genus have been noted to be able to degrade pollutants (6, 10), demonstrating an apparent ability to utilize atypical carbon sources for growth, similar to that described here. In addition to their catabolic capacities PTX1 and PTX2 both appear resistant to elevated concentrations of both Cr(VI) and Cr(III), 15 mM (Figure 7B) and 5 mM, respectively. This is similar to other strains (25, 32) and indicates that the bacteria should be able to withstand the elevated levels of chromium waste found at chromium contaminated sites. Both strains were also capable of reducing Cr(VI) (Figure 8B) and produced soluble Cr(III) reduction end-products. This indicates that PTX1 and PTX2 are capable of both production and consumption of organo-Cr(III) end-products.

Since soluble organo-Cr(III) complexes have been detected in the environment (17), these bacteria represent a method for further transforming the soluble organo-Cr(III) complexes into less soluble and less mobile forms. The process of mineralizing organo-

Cr(III) complexes is probably not unique to these two bacterial species, but is likely present in many soil microorganisms, contributing to the overall immobilization of soluble organo-Cr(III) complexes in the environment. We therefore propose a new Cr-cycle taking into account the biogeo-production and mineralization of organo-Cr(III) end-products (Figure 10).

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Table 1. Primers for 16s rDNA amplification and sequencing[a]

Primer Name	Primer Sequence
GB-13 (Forward)	GAGAGTTTGATC(C or A)TGGCTCAG
GB-14 (Reverse)	ACGG(C or T)TACCTTGTTACGACTT
LC2F	AAGAGTTCGTAGGCGGTT
LC2R	CGTTAGCTACGGCACAGA
SC2F	GAGCGTTGTCCGGAATTA
SC2R	GTTCTTCGCGTTGCATCG
LC3F	CGTGTCGTGAGATGTTGG
LC3R	CACCAACAAGCTGATAGG
SC3F	CGTGTCGTGAGATGTTGG
SC3R	CGTTGCTGCATCAGGCTT

[a] GB-13 and GB-14 were used for amplifying the 16s rDNA sequence and for the first round of DNA sequencing. F and R refer to forward and reverse primers, respectively. The number 2 and 3 refer to primers used in the second and third round of sequencing.

Table 2. Changes in NAD^+ and Cr(III) in PTX1 and PTX2 NAD^+-Cr(III) culture medium.

Sample[a]	Cr(III) (mM)[b]	NAD^+ (mM)[c]	NAD^+ : Cr(III)
Pre-incubation Control[d]	2.45 ± 0.17	5.15 + 0.04	2.10 : 1
No Bacteria Control	2.55 ± 0.04	5.41 ± 0.27	2.12 : 1
PTX1	2.56 ± 0.13	4.25 ± 0.16	1.66 : 1
PTX2	2.54 ± 0.05	4.83 ± 0.14	1.90 : 1
PTX1 & PTX2	2.38 ± 0.12	4.01 ± 0.05	1.67 : 1

[a]Cultures were grown in sterile NAD^+-Cr(III) medium for 4 months at 23°C on bench top.

[b]Cr(III) concentrations were measured by ICP/MS. Results are averages of triplicate analysis with standard deviations.

[c]NAD^+ concentration were measured by absorbance spectrometry at OD_{260nm} and compared to a standard curve. Results are averages of triplicate analysis with standard deviations.

[d]Pre-incubation control represents the initial concentration of the master solution before the 4 month incubation.

Table 3. API ZYM enzyme analysis[a]

Enzyme[b]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
PTX1	-	w	+	+	w	+	w	w	-	-	+	+	-	+	-	+	+	+	+	-
PTX2	-	+	+	+	+	w	-	w	-	-	+	+	-	+	-	+	-	-	-	-

[a] API ZYM test strips measure for the presence or absence of different esterases, aminopeptidases, proteases, phosphotases, and glycosidases.

[b]Enzyme: 1. Control; 2. Alkaline phosphatase; 3. Esterase (C 4); 4. Esterase Lipase (C 8); 5. Lipase (C 14); 6. Leucine arylamidase; 7. Valine arylamidase; 8. Cystine arylamidase; 9. Trypsin; 10. α-chymotrypsin; 11. Acid phosphatase; 12. Naphthol-AS-Bl-phosphohydrolase; 13. α-galactosidase; 14. β-galactosidase;15. β-glucuronidase; 16. α-glucosidase; 17. β-glucosidase; 18. N-acetyl-β-glucosaminidase; 19. α-mannosidase; 20. α-fucosidase. Results represent triplicate experiments. + = positive, w = weakly positive, and - = negative

Figure 1. Growth of PTX1 (■) and PTX2 (◆) on NAD^+ at 30°C for 28 days. Results are representative of multiple experiments (n>3).

Figure 1.

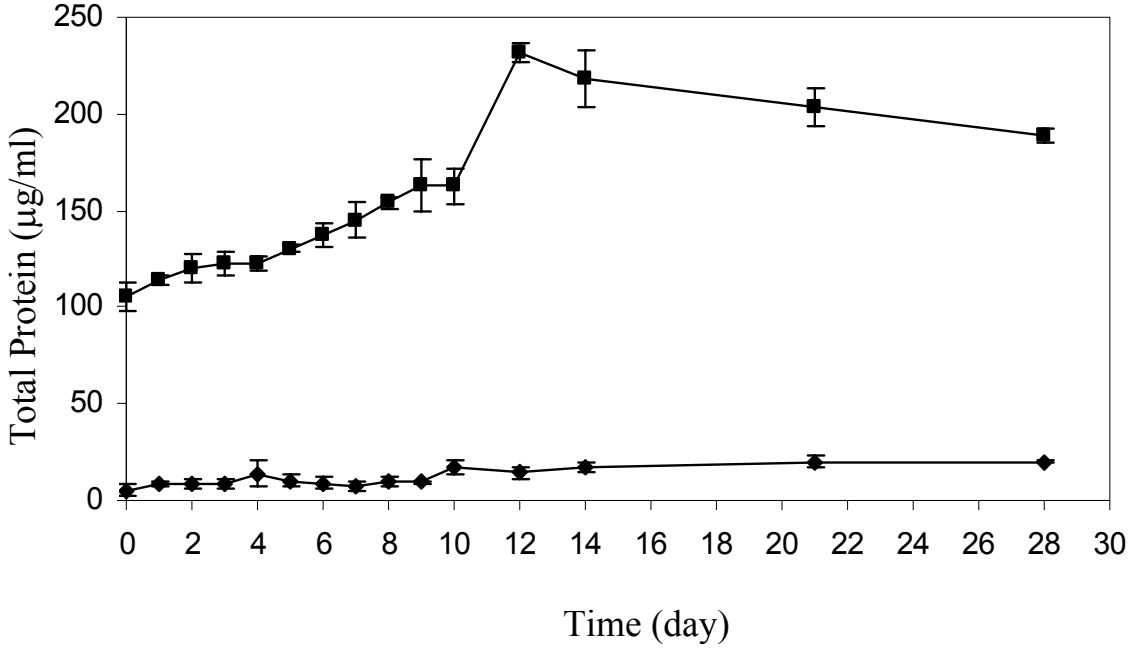

Total Protein (µg/ml)
250
200
150
100
50
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Time (day)

Figure 2. TEM photo of PTA stained PTX1 and PTX2 co-culture growing on NAD^+-Cr(III). Concentration of Cr(III) was approximately 9 mM.

Figure 2.

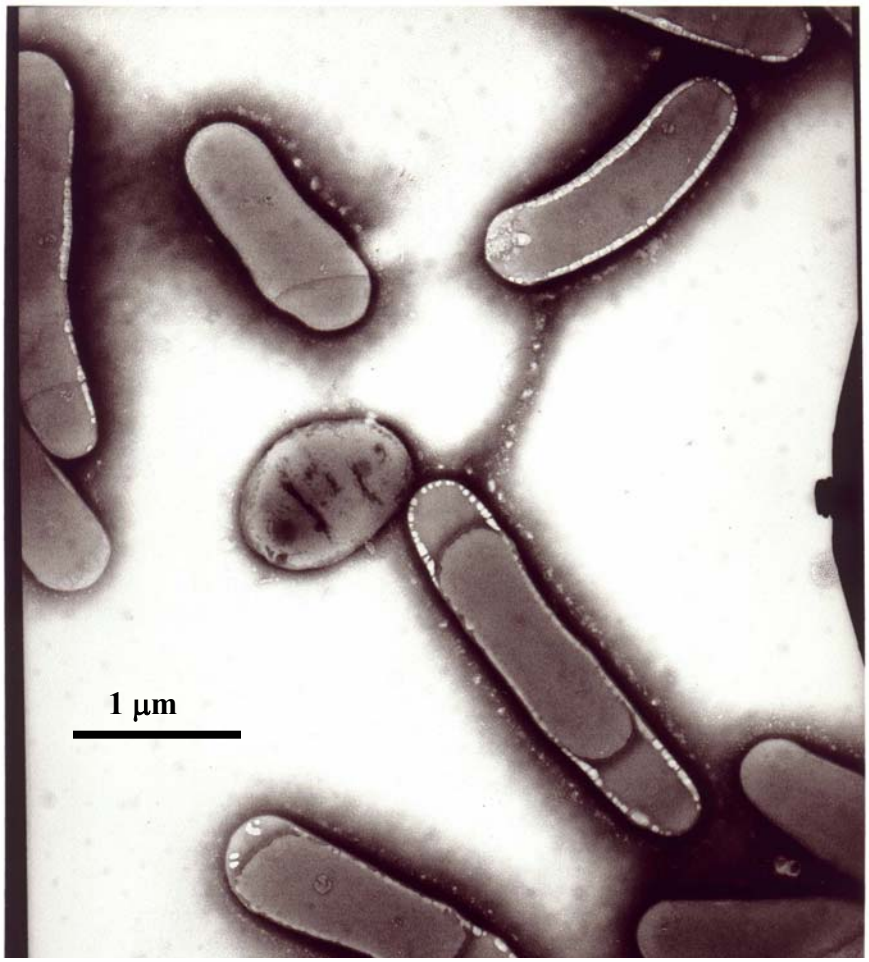


Figure 3. (A) TEM photo of PTX1 grown on NAD^+-Cr(III) at 23^oC for 4 months. (B) TEM photo of PTX1 grown on NAD^+ at 30^oC for 14 days.

Figure 3A.

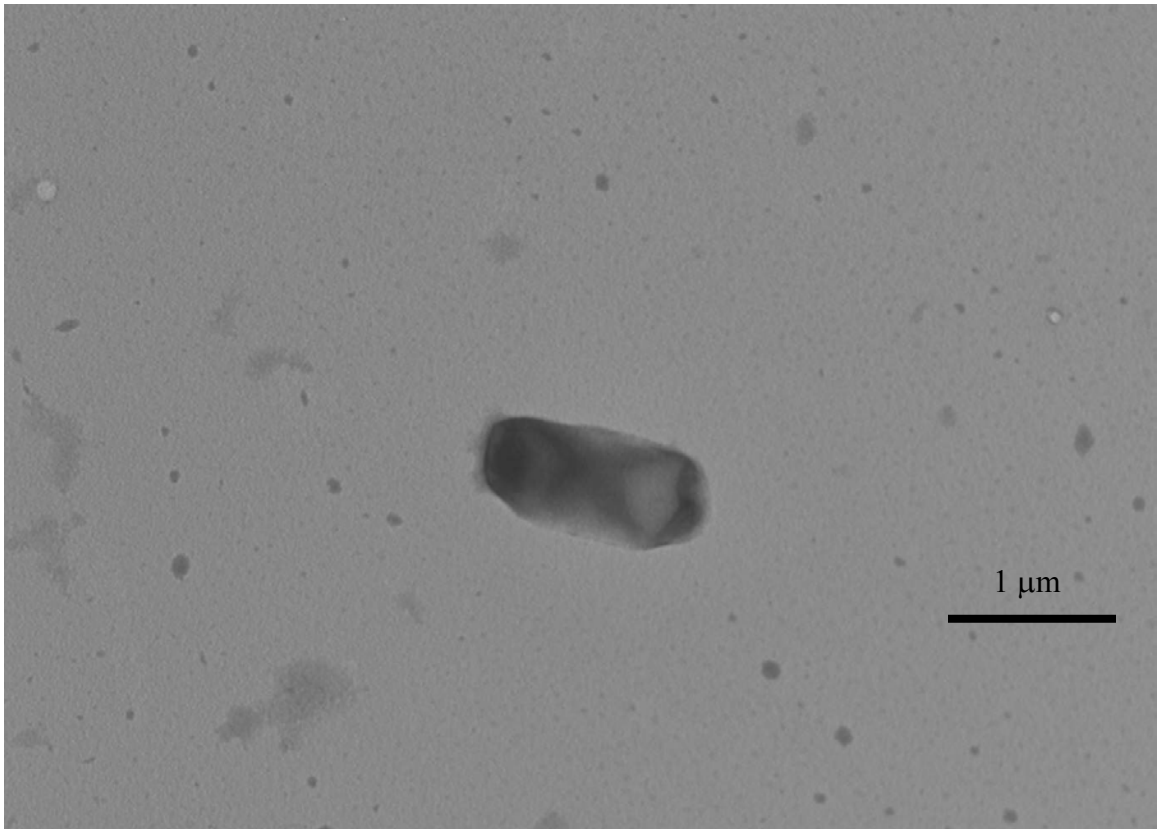


Figure 3B.

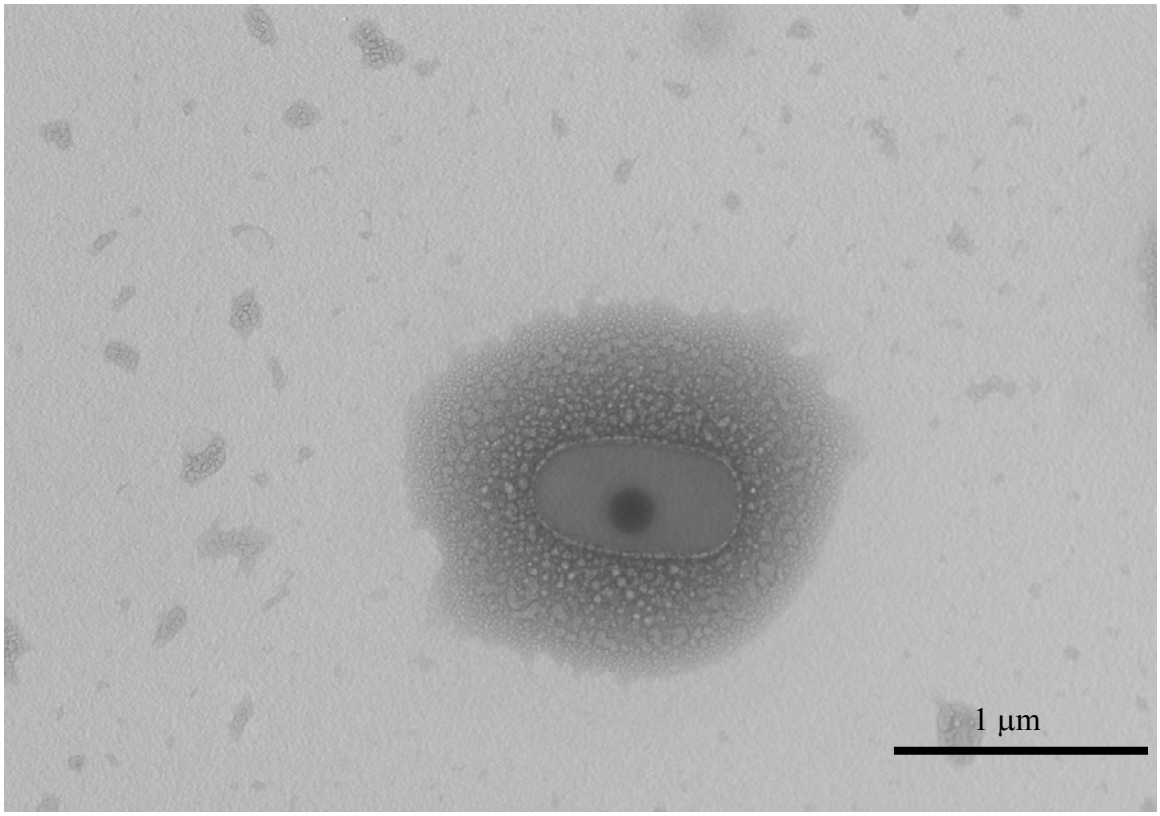


Figure 4. (A) TEM photo of PTX2 grown on NAD^+-Cr(III) at 23^{o}C for 4 months. (B) TEM photo of PTX2 grown on NAD^+ at 30^{o}C for 14 days.

Figure 4A.

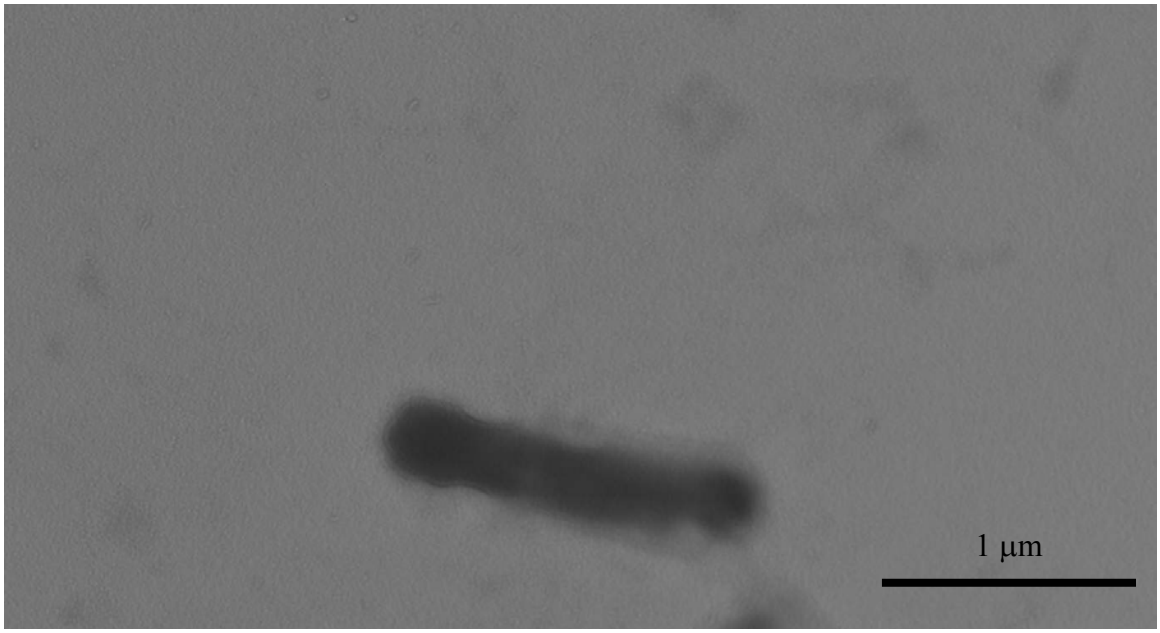


Figure 4B.

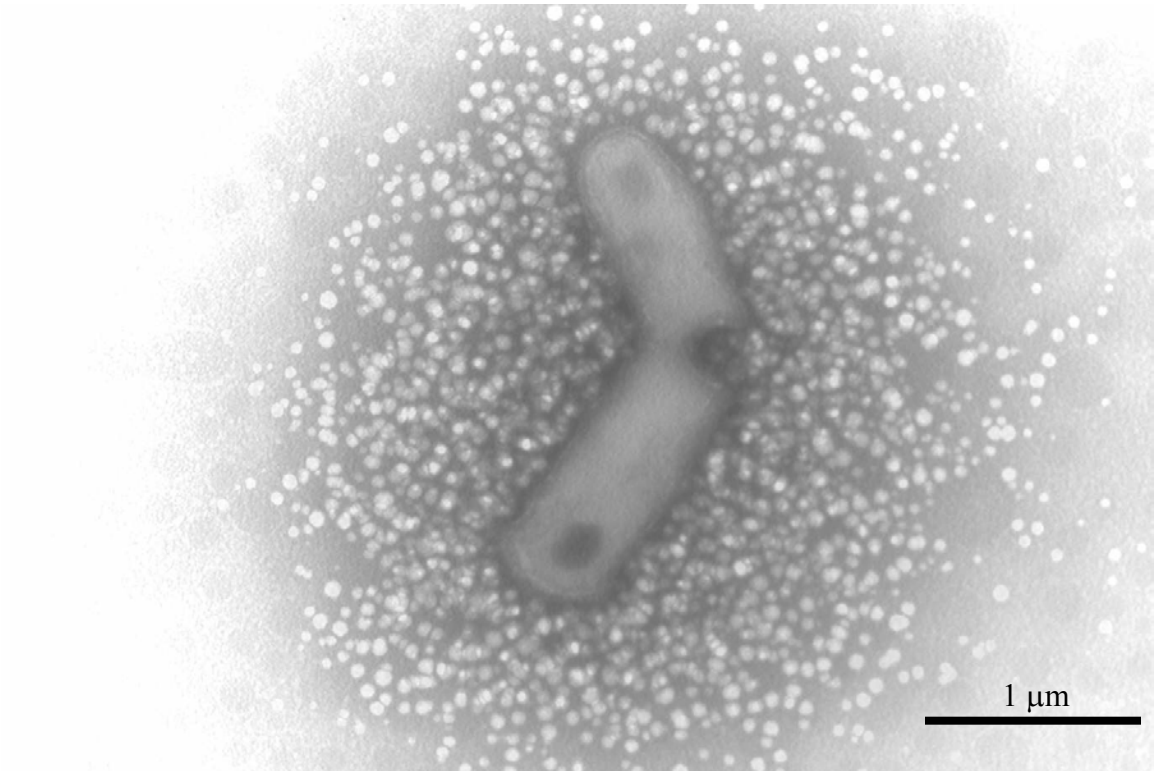


Figure 5. Phylogenetic tree of PTX1 bacterium. Bootstrap values determined from 1000 replicates are listed at the branch points.

Figure 5.

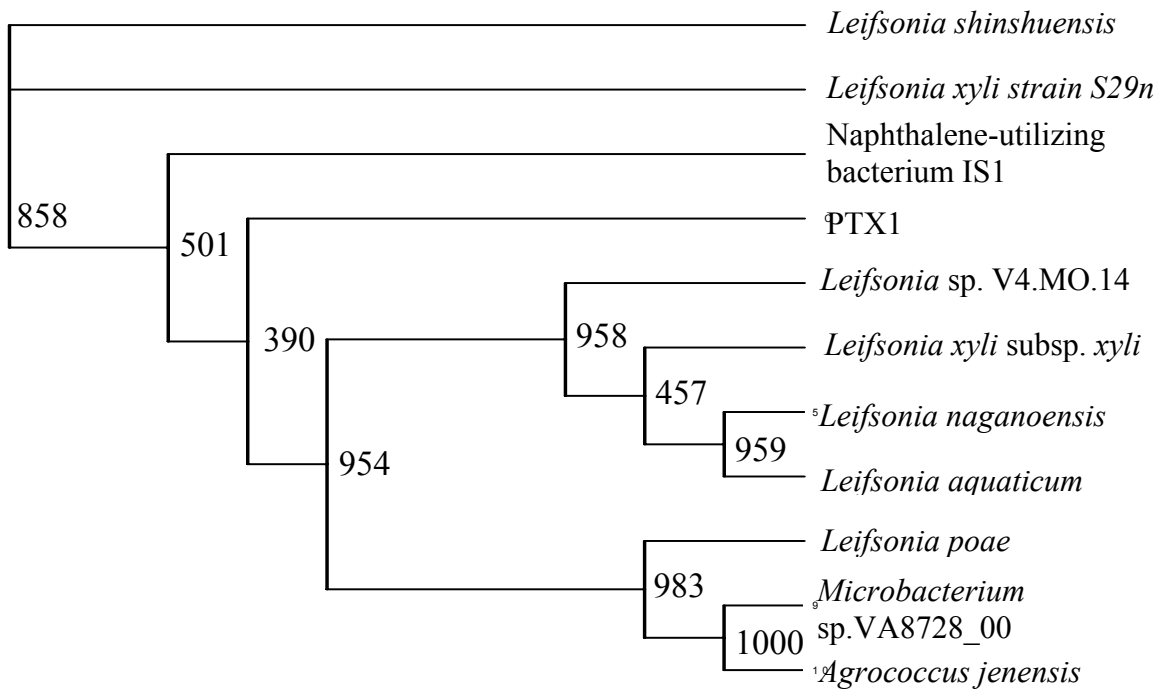

Leifsonia shinshuensis
Leifsonia xyli strain S29n
Naphthalene-utilizing bacterium IS1
PTX1
Leifsonia sp. V4.MO.14
Leifsonia xyli subsp. xyli
Leifsonia naganoensis
Leifsonia aquaticum
Leifsonia poae
Microbacterium sp.VA8728_00
Agrococcus jenensis
858
501
390
958
457
959
954
983
1000

Figure 6. Phylogenetic tree of PTX2 bacterium. Bootstrap values determined from 1000 replicates are listed at the branch points.

Figure 6.

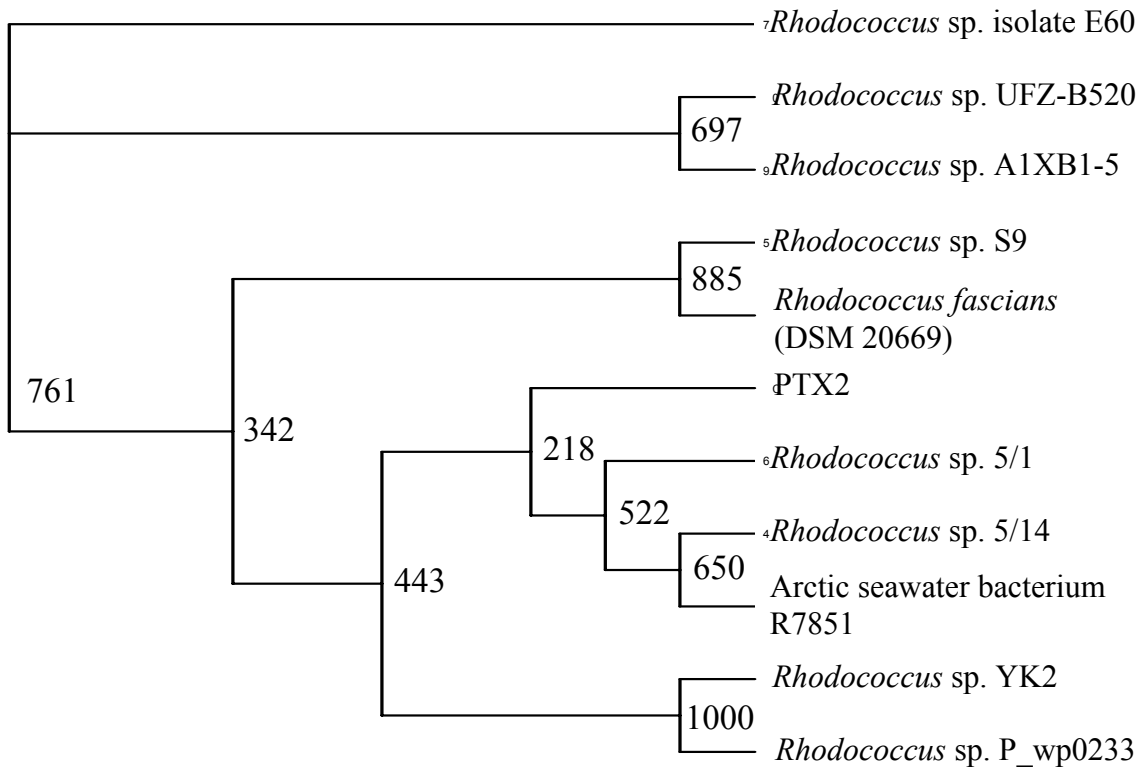

7 Rhodococcus sp. isolate E60
8 Rhodococcus sp. UFZ-B520
697
9 Rhodococcus sp. A1XB1-5
5 Rhodococcus sp. S9
885
Rhodococcus fascians (DSM 20669)
761
1 PTX2
342
218
6 Rhodococcus sp. 5/1
522
4 Rhodococcus sp. 5/14
650
443
Arctic seawater bacterium R7851
Rhodococcus sp. YK2
1000
Rhodococcus sp. P_wp0233

Figure 7. (A) Growth of PTX1 (■), PTX2 (◆), and *E. coli* W (▲) on LB at 30^{o}C for 72 hours. (B) Growth of PTX1 (■), PTX2 (◆), and *E. coli* W (▲) on LB with 15 mM Cr(VI) at 30^{o}C for 72 hours. Results are representative of triplicate experiments.

Figure 7A.

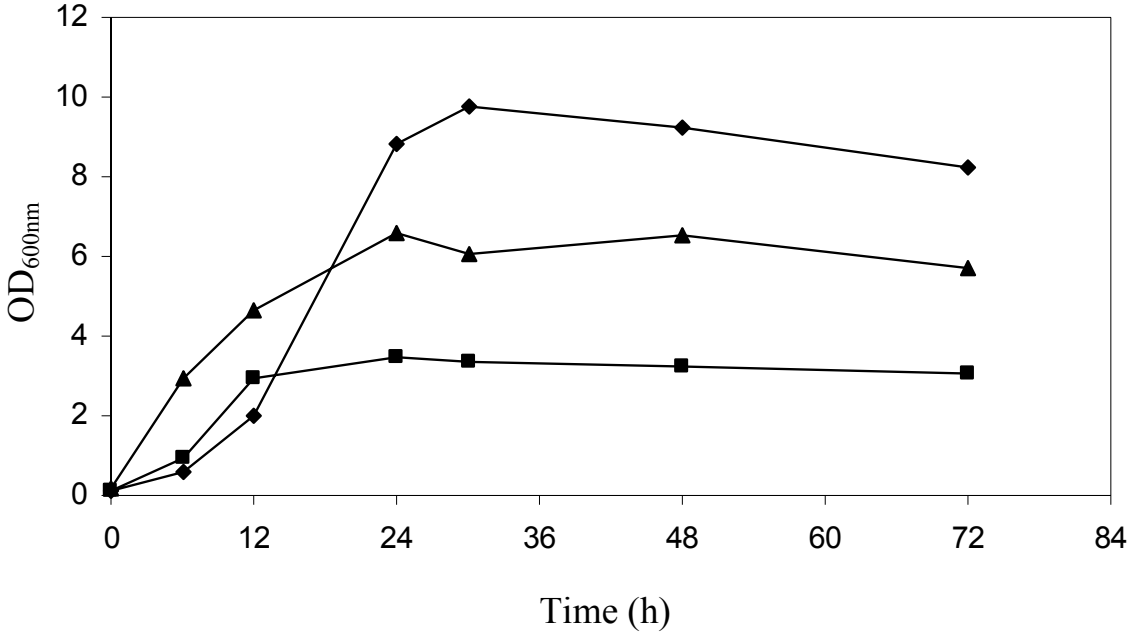


Figure 7B.

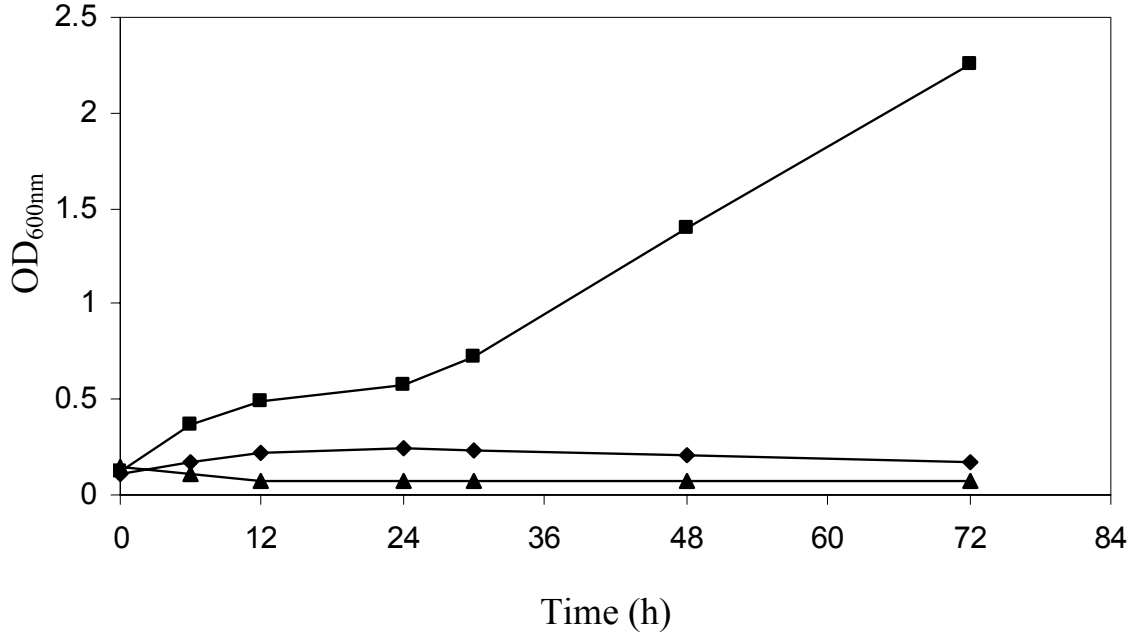


Figure 8. (A) Growth of PTX1 (■), PTX2 (◆), and *E. coli* W (▲) in LB with 100 μM Cr(VI) at 30°C. (B) Whole cell reduction of Cr(VI) by PTX1 (■) and PTX2 (◆). Mid-log phase cells were diluted to an approximate OD_{600nm} = 0.1 in LB with 100 μM Cr(VI). Abiotic reduction of Cr(VI) in control (×). Results are averages of triplicate analysis with standard deviations.

Figure 8A.

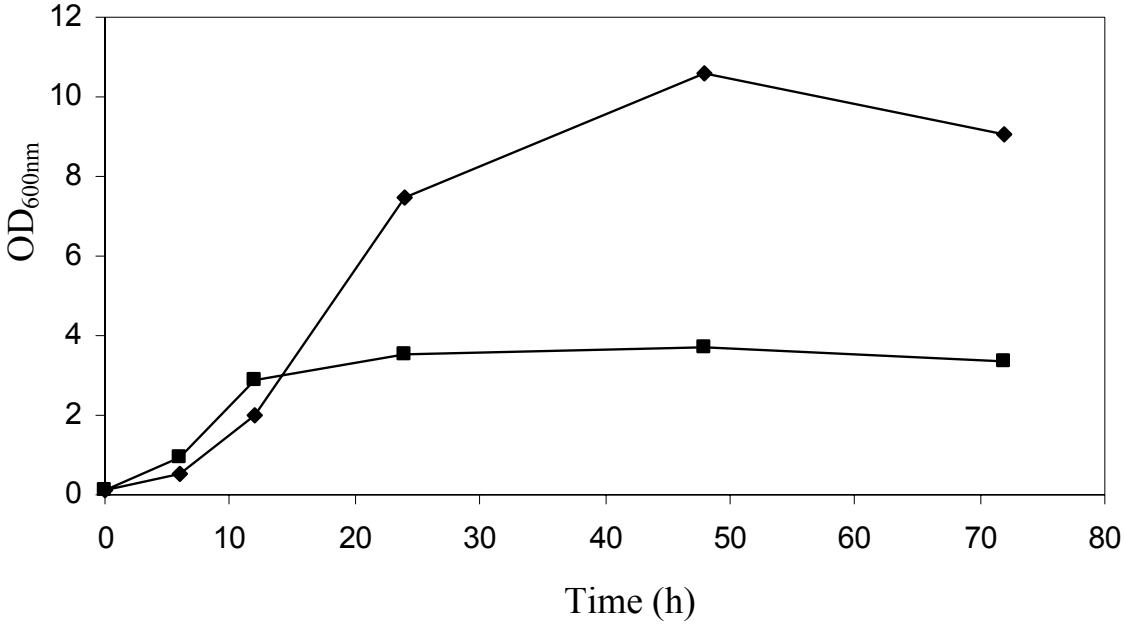


Figure 8B.

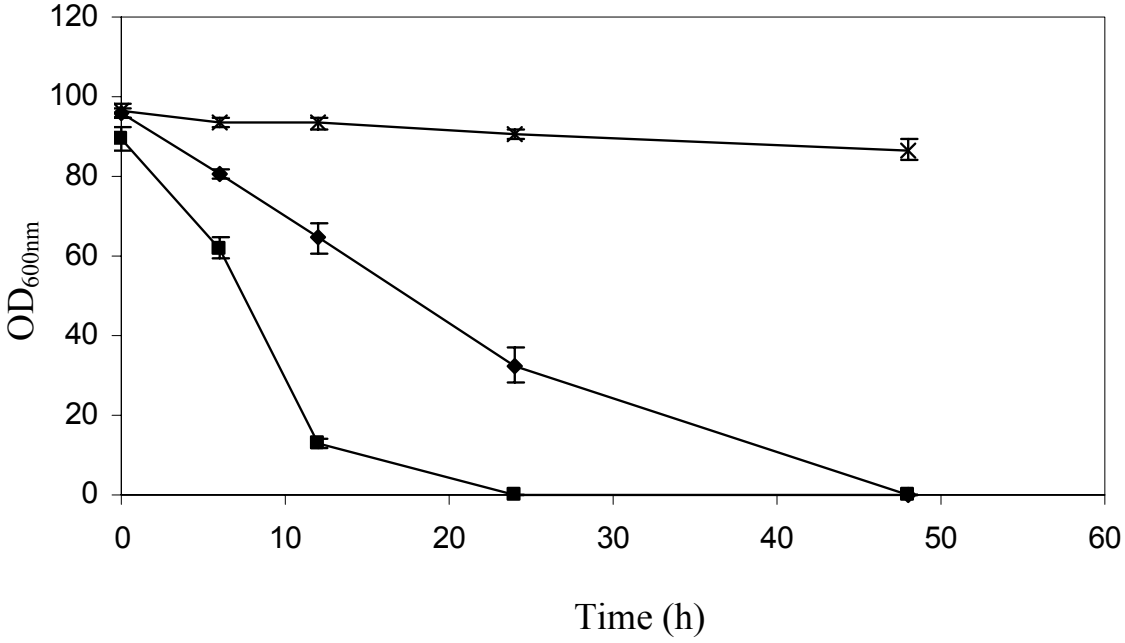


Figure 9. TEM photo of unstained PTX1 culture grown on NAD^+-Cr(III) for 1 year at 23^oC.

Figure 9.

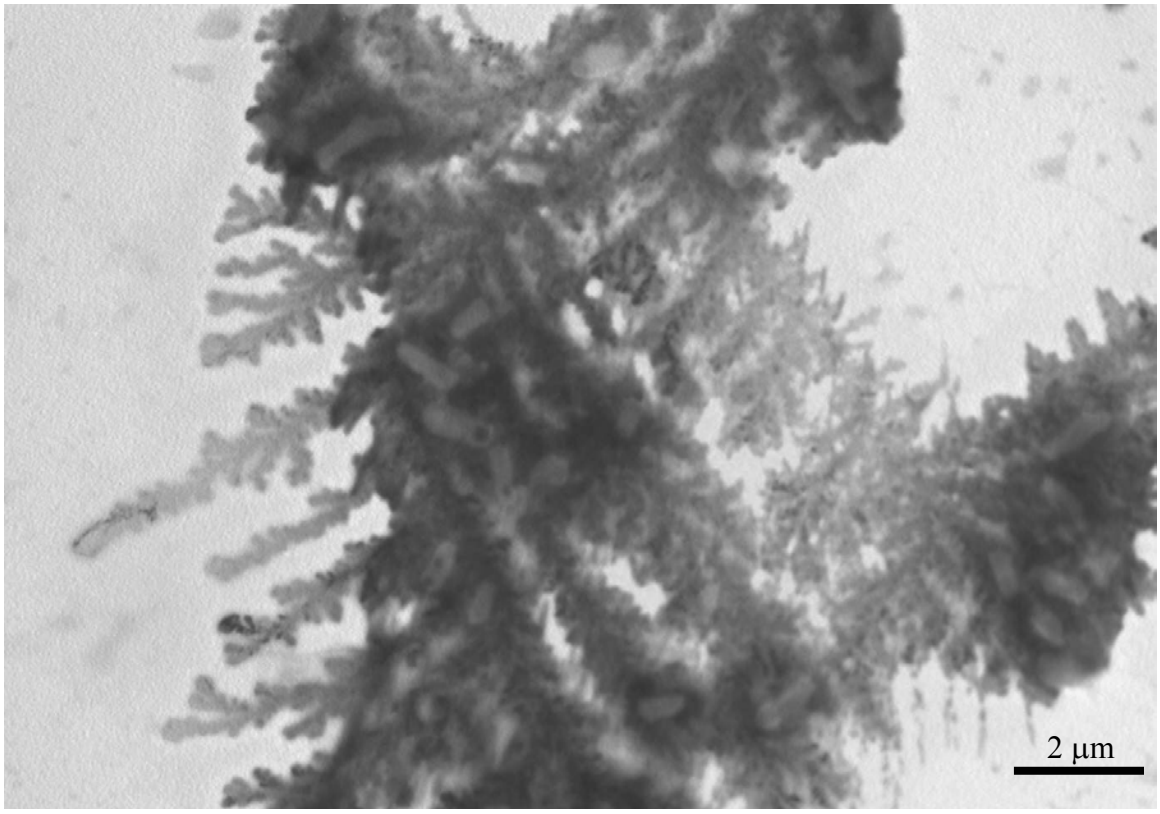
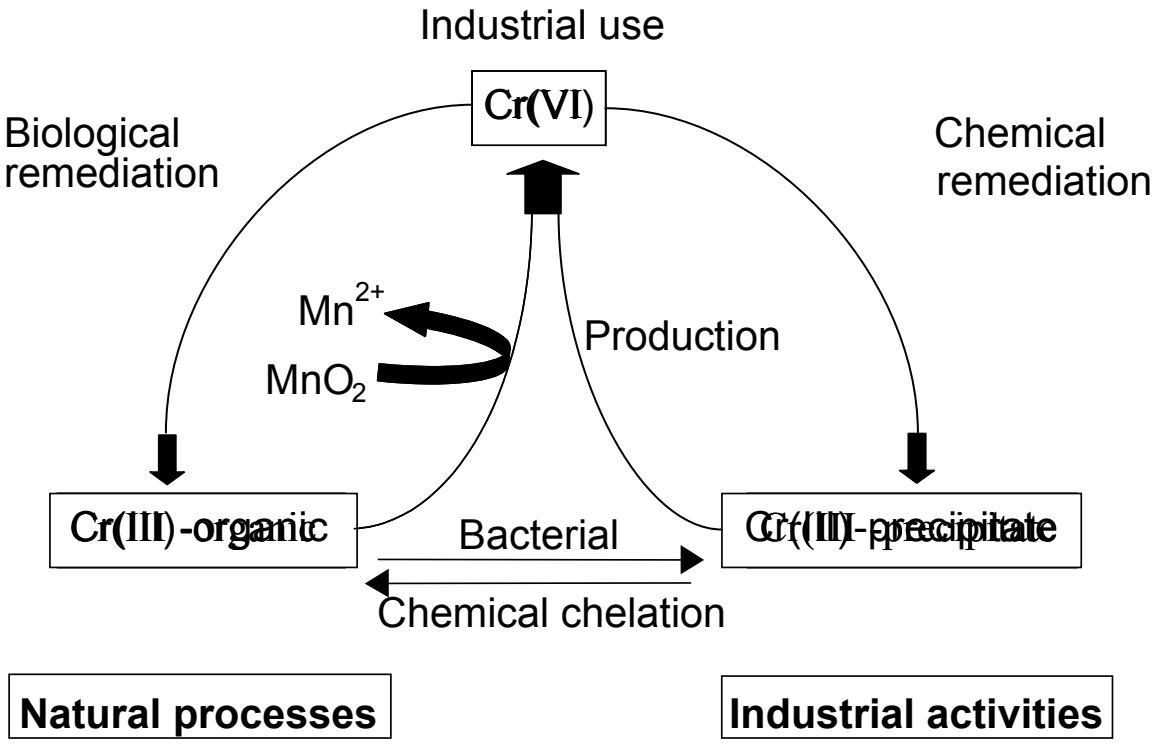


Figure 10. Chromium cycle updated to account for both the microbial production and mineralization of organo-Cr(III) complexes.

Figure 10.


Industrial use
Cr(VI)
Biological remediation
Chemical remediation
Mn2+
MnO2
Production
Cr(III)-organic
Bacterial
Chemical chelation
Cr(III)-precipitate
Natural processes
Industrial activities

CHAPTER FIVE

CONCLUSIONS AND FUTURE DIRECTIONS

Cr(VI) contamination has become a major environmental problem. Bioremediation of Cr(VI) is a promising method for remediating contaminated sites. Research focused on the mechanism of microbial Cr(VI) reduction and the conditions favoring reduction are important to the understanding and implementing of in situ remediation technologies. Additionally, understanding the fate of the reduced Cr(III) end-products is important to conceptualize the long term outcome (mobilization, re-oxidation, and recalcitrance) of Cr(III) at remediated sites.

Chapter two describes a general mechanism of Cr(VI) reduction. The mechanism utilizes a flavin reductase and reduced flavins, which are present in all bacterial cells, to reduce Cr(VI) to Cr(III). The specific reduction rates and enzyme kinetics are discussed in this section. The research also identified the reduced end-product as a soluble organo-Cr(III) complex. This was significant in that Cr(VI) reduction in the Cr biogeocycle is thought to result in precipitation of Cr(III) as an insoluble inorganic form, i.e. $Cr(OH)_3$. The complex is composed of multiple NAD^+ and Cr(III) in a ratio of 2:1, which remains soluble at neutral pH and intracellular physiological conditions.

Chapter three describes the formation of multiple soluble organo-Cr(III) end-products formed by the reduction of Cr(VI) in the presence of individual cellular organics. The end-products were identified as being composed of multiple Cr(III) ions with organic ligands formed into a complex similar to that discovered for the NAD^+-Cr(III). The complexes were further demonstrated to remain soluble and stable upon

dialysis in water and across a broad pH range representative of that found in the environment. This work further identified a mechanism for the production of soluble organo-Cr(III) complexes in the biogeocycle of Cr and represents a source for soluble organo-Cr(III) complexes, which have been detected in the environment. The work also raised the questions about the long term mobility and recalcitrance of soluble organo-Cr(III) complexes.

Chapter four addresses an aspect of the long term fate of the soluble organo-Cr(III) complexes in the environment. Cr(III) is noted to be substitution inert with very slow ligand exchange rates, hallmarking the soluble organo-Cr(III) complexes to remain present in the environment for long periods of time. Microorganisms capable of mineralizing the NAD^+-Cr(III) complexes were identified and characterized. These bacteria were both identified as being gram positive aerobes of the genus *Leifsonia* and *Rhodococcus*. After the bacteria mineralized the organic component, it was discovered that the Cr(III) precipitated onto the cell wall. This identified a pathway in the Cr biogeocycle for the formation of a final insoluble Cr(III) precipitate.

The work presented here represents a significant advance in the understanding of mechanism of Cr(VI) reduction and new aspects of the biogeocycle of Cr, formation and mineralization of soluble organo-Cr(III) complexes. Future work will need to address the microbial production, toxicity, re-oxidation, mobility, and structure of the organo-Cr(III) complexes. The research will be important to address possible contamination concerns and to properly model the *in situ* bioremediation process for future field application.

APPENDIX I

ATTRIBUTION PAGE

Numerous people contributed to the dissertation research discussed here, both in and out of the laboratory setting. Experiments were done primarily by me. Additional researchers are listed as coauthors. The following is a summary of my research contribution and those who helped complete the research.

Chapter 2.

The Fre purification was done with the help of Dr. Tai Man Louie. The EPR analysis was done by Drs. Arthur G. Roberts and David M. Kramer. The ICP/MS analysis was done with the help of Charles Knaack. All other experiments were completed by me. All the writing was done by me with significant input from Dr. Xun.

Chapter 3.

The EPR analysis was completed with the help of Dr. Arthur G. Roberts. The ICP/MS analysis was done with the help of Charles Knaack. All the writing was done by me with editing from Dr. Xun.

Chapter 4.

All work was done by me expect ICP/MS analysis was done with the help of Charles Knaack and ICP/OES analysis was done with the help of Paul Gibson. Electron Microscopy work was done with the assistance of Dr. Chris Davitt. All writing was done by me with editing from Dr. Xun.