Chromate Reduction by Desulfovibrio Desulfuricans ATCC 27774

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CHROMATE REDUCTION BY DESULFOVIBRIO DESULFURICANS ATCC 27774

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ABSTRACT

CHROMATE REDUCTION BY DESULFOVIBRIO DESULFURICANS ATCC 27774

By
Ning Zhang
May 2012

Dissertation supervised by Dr. John F. Stolz

Chromium has been used extensively in the industry process of metal refinishing and electroplating. It is also a byproduct of the processing of fissionable materials at United States Department of Energy facilities. Chromate (Cr (VI)) is soluble and readily absorbed by cells, while the reduced form of chromium, Cr (III), is insoluble. Thus means of reducing Cr (VI) to Cr (III) in the environment is a potential means of remediation. Desulfovibrio desulfuricans strain 27774 is a sulfate reducing bacterium that can reduce Cr (VI). It also can respire nitrate to ammonia. As some sites of chromium contamination also contain high concentrations of nitrate, an investigation of Cr (VI) reduction under nitrate reducing growth conditions by D. desulfuricans strain 27774 was conducted. A growth medium that was compatible with the colorimetric assay for Cr (VI) and did not itself reduce Cr (VI) was formulated. Cell assays determined that Cr (VI) reduction was
primarily in the supernatant, catalyzed by a secreted secondary metabolite. A genomics investigation identified two pathways as possible mechanisms.
DEDICATION

I would like to dedicate this work to my mother, Zhang YuFang, for all of the support and encouragement she has given me throughout my life. While others have come and gone in my life, they have always been there for me. I could not have done this without them.
ACKNOWLEDGEMENT

I would like to thank my advisor, Dr. John F. Stolz, for all of his guidance and extraordinary support. I have learned so much from him. I truly appreciate all of the knowledge he has shared with me. I will always be grateful to him for giving me the opportunity to work in his lab and for his encouragement and financial support on my research.

I would also like to thank my committee members, Dr. Skip H.M. Kingston and Dr. Michael J. Tobin, for their help and advice on this project. Their suggestions provided valuable new ideas and pointed me in the right direction. I want to further thank Dr. Skip H.M. Kingston for offering his instruction and use of his instruments to allow me to perform Inductively Coupled Plasma Mass Spectroscopy (ICP-MS).

I also owe a tremendous thanks to all of the faculty members, graduate students, and postdoctoral researchers who helped me learn various techniques that were necessary for this research. Dr. Partha Basu gave me many instructions on chemistry perspective of my experiment. Igor Pimkov generously shared his time, supplies, and knowledge of chemical purification, TLC, and NMR with me and helped me clarify my research direction. Peter Chovanec gave me instructions on basic biochemical experimental technics with great patience. His previous research on this project provided significant information that helped me begin my endeavor to further this work. Naudia Martone helped me to run ICP-MS in order to test SES-3 medium’s Cr (VI) reduction activity. The MS data was done by John Thomas.
In addition, I want to thank past and present Stolz Lab members for all of their assistance with instrument operations and for making this such an enjoyable lab to work in: Lucas Eastham; Robert Reiter; Jennifer Evans; Rishu Dheer; Shrabani Basu; Spencer Heaps, and all of the undergraduate researchers.

I also want to thank the Duquesne University Department of Biological Sciences and Department of Chemistry & Biochemistry for the use of their facilities. I would especially like to thank the Center for Environmental Research and Education for giving me the opportunity to be a part of the Environmental Science & Management program. Thanks Lisa Mikolajek and all CERE faculties especially Mr. Edward Schroth for their supports during my study at CERE. It was a memorable studying and life experience.
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LIST OF ABBREVIATIONS

BLAST-Basic local alignment search tool

CHR-Chromate ion transporter

Cr-DPC-Cr (VI) and diphenylcarbazide reaction

EA-Ethyl acetate

ICP-MS- Inductively Coupled Plasma Mass Spectroscopy

MR-membrane-bound reductase

MS-Mass Spectrometer

m/z-Mass-to-charge

NCBI – National Center for Biotechnology Information

NMR – Nuclear magnetic resonance

NRPS-Nonribosomal peptide-synthetase

PKS-Polyketide synthase

Rf-Retention factor

ROS-Reactive oxygen species

SR- Soluble reductase

SRB-Sulfate-reducing bacterium

TLC-Thin layer chromatography

TMS-Transmembrane segment

UV/VIS – Ultraviolet/visible

USEPA-United Stated Environmental Protection Agency
INTRODUCTION

Chromium

Chromium is odorless and tasteless and is naturally found in rocks, plants, soil and volcanic dust, humans and animals. It is the sixth most abundant element in the Earth’s crust. Chromium combines with iron and oxygen in the form of chromite ore (ATSDR, 2011). The most common oxidation states of chromium in the environment are trivalent (Cr (III)), hexavalent (Cr (VI)) and the metal form. Cr (III) occurs naturally in many vegetables, fruit, meats, grains and yeast (USEPA, 2011). Cr (VI) and (0) are generally introduced to the environment by industrial processes. Chromium is extensively used in the process of metal refining and electroplating (Bailar, 1997; USEPA, 1998; Ryan et al, 2002). Chromium compounds, in either the Cr (III) or Cr (VI) forms are used for chrome plating, dyes and pigments, leather and wood preservation (USEPA 1998; ATSDR 2000).

According to the Toxics Release Inventory (TRI), in 1997, the releases of chromium were 706,204 pounds to the air from 3,391 large processing facilities. Cr (III) and (VI) are released to the environment mainly from stationary point sources. Chrome-plating sources are estimated to contribute 700 metric tons of chromium per year to atmospheric pollution, 100% of which is believed to be Cr (VI) (ATSDR, 2000). According to the TRI, these same sources released 111,384 pounds to the water (ATSDR, 2000). Electroplating, leather tanning, and textile industries release a significant amount of chromium to surface waters. Furthermore, the estimated releases of chromium to soil were 30,862,235 pounds. This accounted for about 94.1% of total environmental releases (ATSDR, 2000). In addition, 939 soil and 472 sediment samples collected from 1,036
National Priority List (NPL) hazardous waste sites containing chromium (HazDat, 2000). Community exposure occurs in a variety of ways, including airborne particulates, drinking water, masonry walls, and contaminated soil. The general population is exposed to chromium by eating food, drinking water, and inhaling air that contain chromium. The mean daily chromium intake from air, water, and food is estimated to be <0.2-0.4, 2.0, and 60 micrograms respectively (ATSDR, 2000). The USEPA has a maximum contaminant level of total chromium including Cr (VI) in drinking water of 0.1 mg/L or 100 ppb for public water systems (USEPA, 1999).

**Chromium health effects**

Cr (III) is a nutritionally essential element in organisms. Cr (III) is widely distributed in the body and accounts for most of the chromium in plasma or tissues. On the other hand, Cr (VI) is more toxic and poses potential health risks to people. Cr (VI) is taken up by erythrocytes after absorption and reduced to Cr (III) inside the red blood cells after it is absorbed into the bloodstream. Cr (III) binds to transferrin, an iron-transporting protein in the plasma once absorbed into the bloodstream (EPA, 1998; ATSDR, 2000; Dayan and Paine, 2001).

Cr (VI) is able to travel via nonspecific anion transporters across the plasma membrane. Cellular enzymatic and other systems can reduce Cr (VI) to Cr (V), or (IV), or (III) species inside the cell (Figure 1).
Those species are non-diffusing and trapped inside the cell. Cr (VI) can be absorbed by the lung and gastrointestinal tract, and even by intact skin. Once inhaled, Cr (VI) compounds irritate the respiratory tract, resulting in airway obstruction, and lung, nasal, or sinus cancer. Dermal exposure to chromium can produce irritation and allergic contact dermatitis (Polak, 1983; Bruynzeel et al, 1988). The US EPA listed Cr (VI) as one of the 17 chemicals posing the greatest threat to public health (Marsh and McInerney, 2001). Cr (VI) is a potent carcinogen when inhaled. The USEPA has proposed classifying Cr (VI) as carcinogenic to humans via ingestion (USEPA, 2011).
Chromium environmental chemistry

Chromium is a transition metal located in group VI-B of the periodic table. Chromium can occur in a number of oxidation states, but (III) and (VI) dominate in the environment system due to the specific redox potentials and pH range. The environmental behavior of chromium is a function of its oxidation state. Speciation in groundwater is affected by the Eh and pH conditions (Figure 2). Cr (VI) exists in alkaline, strongly oxidizing environments while Cr (III) exists in moderately oxidizing and reduced environments. Chromate (CrO$_4^{2-}$) or dichromate (Cr$_2$O$_7^{2-}$) ions are the most common Cr (VI) species. H$_2$CrO$_4$ form is the dominant species below pH 0.6 (Cotton and Wilkinson, 1980). HCrO$_4^-$ dominates between the pH values of 1 and 6. CrO$_4^{2-}$ dominates at or above pH 6. Dichromate ion rarely exists in biological systems because the chromium concentration in environment is generally lower than 10$^{-2}$ M (Beas and Messmer, 1986; Losi et al., 1994).

Cr (III) in the form of oxides, hydroxides or sulfates is the most common form in the environment. The Cr (III) species dominate at pH < 3.6 (Francoise and Bourg, 1991). Cr (OH)$_4^{+}$ is the major form at pH higher than 11.5 (Rai et al, 1987). In acid media, Cr (III) exists as the hydrated cation or inorganic complexes. Cr (III) is oxidized to Cr (VI) form under alkaline conditions. Cr (VI) compounds are strong oxidizers and highly soluble, while Cr (III) compounds are more likely to form inert precipitates at near-neutral pH.

The oxidation state and solubility of chromium are particularly important for bioavailability. The redox reaction of chromium in soils depends on soil structure and redox potentials. Cr (VI) compounds can be taken up by plants and animals, be absorbed
or precipitated. Cr (VI) can be reduced to Cr (III) by inorganic electron donors such as Fe (II) and S$^{2-}$, or by bioprocesses involving organic matter. Cr (III) can be precipitated as oxides and hydroxides. Soluble Cr (III) complexes can undergo oxidation when they are in contact with manganese dioxide.

Figure 2–Eh-pH diagram for aqueous chromium species (Rai et al., 1989)

**Microbial responses to Cr (VI)**

The biological effects of chromium depend on its oxidation state and cellular localization. At the extracellular level in bacteria, Cr (VI) is more toxic than Cr (III) because Cr (VI) readily enters the cytoplasm (Wong and Trevors, 1988; Katz and Salem, 1993). In the cytoplasm, free radicals are generated in the transformation of Cr (VI) to Cr (III) damaging DNA and inducing adverse effects (Shi and Dalal, 1990; Kadiiska et al., 1994). Cr (III), on the other hand, is less able to cross cell membranes due to its insolubility (Wong and Trevors 1988; Katz and Salem, 1993). Inside the cells, Cr (III)
may generate toxic effects by binding to phosphates in DNA (Kortenkamp et al, 1991, Bridgewater et al, 1994, Plaper et al, 2002), as well as binding to carboxyl and sulfhydryl groups in proteins (Levis and Bianchi, 1982), and competing with ion transport by transferrin (Moshtaghie et al, 1992). In Saccharomyces cerevisiae, oxidative damage to proteins is considered to be the central mechanism of chromium toxicity (Sumner et al, 2005).

Microbes have developed different mechanisms to resist Cr (VI) toxicity including efflux of chromate ions from the cell cytoplasm, Cr (VI) reduction to Cr (III), and other mechanisms (Figure 3).

Figure 3 –Mechanisms of chromate transport, toxicity and resistance in bacterial cells. Mechanisms of damage and resistance are indicated by thin and heavy arrows, respectively. (A) Chromosome-encoded sulfate uptake pathway which is also used by
chromate to enter the cell; when it is mutated (X) the transport of chromate diminishes. (B) Extracellular reduction of Cr (VI) to Cr (III) which does not cross the membrane. (C) Intracellular Cr (VI) to Cr (III) reduction may generate oxidative stress and protein and DNA damage. (D) Detoxifying enzymes are involved in protection against oxidative stress, minimizing the toxic effects of chromate. (E) Plasmid-encoded transporters may efflux chromate from the cytoplasm. (F) DNA repair systems participate in the protection from the damage generated by Cr derivatives (Martha I. et al. 2008) (Reprinted with permission)

Efflux of chromate ions

Cr (VI) resistance is attributed to the decreased uptake and/or enhanced efflux of Cr (VI) by the cell membrane in Pseudomonas aeruginosa and Alcaligenes eutrophus CH34 (Bopp et al, 1983; Ohtake et al, 1987; Alvarez et al, 1999; Aguilera et al, 2004; Nies and Silver, 1989; Valls et al, 2000; Vaneechoutte et al, 2004). The Chromate Ion Transporter (CHR) superfamily of transporters was reported to be responsible for chromate resistance (Nies et al, 1998). The CHR protein family currently contains 135 sequences of homologs (Cervantes and Campos-Garcia, 2007). The ChrA proteins from P. aeruginosa and Cupriavidus metallidurans have been well studied. Chr A functions as a chemiosmotic pump that effluxes chromate from the cytoplasm using the proton motive force (Alvarez et al, 1999; Pimentel et al, 2002). Chr A is encoded by plasmid pUM505 of P. aeruginosa and pMOL28 from C. metallidurans (Cervantes et al, 1990; Nies et al, 1990). Chr A from P. aeruginosa has 416 amino acids (aa) and displays a topology of 13 transmembrane segments (TMS) (Jimenez-Mejia et al, 2006). The ChrA protein (401 aa) from Cupriavidus displays a topology of 10 TMS and shows 29% of identical amino acids with ChrA from Pseudomonas (Nies et al, 1998). In addition, the ChrA2 genes expression of the C.metallidurans also confers chromate resistance (Juhnke et al, 2002).
In *Shewanella oneidensis*, in response to chromate stress, the genes and proteins involved in iron transport and sulfur assimilation were observed to be strongly induced. These up-regulated genes include TonB iron transport system genes, assimilative as well as genes governing sulfate transporters such as cysP, cysW-2, and cysA-2 (Brown *et al.*, 2006). In *Escherichia coli*, Chr 3N and Chr 3C genes were required for chromate resistance (Amada Diaz-Magana, *et al.*, 2009). The molecular response to chromate in *Pseudomonas putida* F1 was found to involve proteins controlling inorganic ion transport and metabolism, and amino acid transport/metabolism (Dorothea *et al.*, 2010).

**Cr (VI) reduction to Cr (III)**

Most microorganisms cannot use chromate as an electron acceptor for growth. Cr (VI) reduction mechanisms generally fall into two categories: 1) chemical reactions associated with H\(_2\)S and compounds such as amino acids, nucleotides, sugars, vitamins, organic acids or glutathione or 2) enzymatic reactions associated with cytochromes, hydrogenases. Cr (III)’s impermeability to the cell membrane is the basis for several mechanisms involving the transformation of Cr (VI) to Cr (III) (Czako-Ver *et al.*, 1999). The search for Cr (VI) reducing microorganisms has been pursued after the discovery of the first microbe capable of reducing Cr (VI) in the 1970s (Romanenko and Korenkov, 1977). A number of chromium-resistant microorganisms were isolated, such as *Bacillus cereus*, *B.subtilis*, *Ps. aeruginosa*, *P. ambigua*, *P. fluorescens*, *E.coli*, *Achromobacter eurydice*, *Micrococcus roseus*, *Enterobacter cloacae*, *Desulfovibrio desulfuricans* and *D. vulgaris* (Lovely, 1993, 1994). Bacteria that have the capability to reduce Cr (VI) to Cr (III) are summarized in Table 1. Most of these bacteria are facultative anaerobes and
widespread in nature. Sulfate-reducing bacteria received significantly more attention among anaerobic bacteria.

Table 1—Bacteria with Cr (VI) reduction activity

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<th>Organism</th>
<th>Substrate/redox condition</th>
<th>Reference</th>
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<td><em>Achromobacter Eurydice</em></td>
<td>Acetate, glucose/anaerobic</td>
<td>Gvozdyak, <em>et al.</em>, 1986</td>
</tr>
<tr>
<td><em>Alcaligenes eutrophus</em> CH34</td>
<td>Sodium gluconate/aerobic</td>
<td>Nies and Silver, 1989</td>
</tr>
<tr>
<td><em>Aeromonas dechromatica</em></td>
<td>Galactose, mannose, melibiose, sucrose, fructose, lactose, cellobiose, arabinose, mannitol, dulcitol, sorbitol, glycerol/anaerobic</td>
<td>Kvasnikov, <em>et al.</em>, 1985</td>
</tr>
<tr>
<td><em>Agrobacterium radiobacter</em></td>
<td>Glucose, fructose, maltose, lactose, mannitol, glycerol/aerobic</td>
<td>Llovera, <em>et al.</em>, 1993</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>Acetate, glucose/anaerobic</td>
<td>Gvozdyak, <em>et al.</em>, 1986</td>
</tr>
<tr>
<td><em>Bacillus sp.</em></td>
<td>Glucose/aerobic</td>
<td>Wang and Xiao, 1995</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>Acetate, glucose/anaerobic</td>
<td>Gvozdyak, <em>et al.</em>, 1986</td>
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<tr>
<td><em>Desulfovibrio vulgaris</em></td>
<td>Hydrogen/anaerobic</td>
<td>Lovley and Phillips, 1994</td>
</tr>
<tr>
<td><em>Desulfovibrio desulfiticans</em></td>
<td>Anaerobic</td>
<td>Lovley, 1993</td>
</tr>
<tr>
<td><em>Desulfomicrobium norvegicum</em></td>
<td>Sodium lactate/Anaerobic</td>
<td>Chardin <em>et al.</em>, 2002; Michel <em>et al.</em>, 2001</td>
</tr>
<tr>
<td><em>Desulfotomaculum reducens</em></td>
<td>Lactate/Anaerobic</td>
<td>Tebo and Obraztsova, 1998</td>
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<tr>
<td><em>Deinococcus radiodurans</em> R1</td>
<td>Lactate/Anaerobic</td>
<td>Fredrickson <em>et al.</em>, 2000</td>
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<td><em>Enterobacter cloacae</em></td>
<td>Acetate, glycerol, glucose/anaerobic</td>
<td>Fujii and Toda, 1990</td>
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<td><em>Escherichia coli</em></td>
<td>Acetate/anaerobic</td>
<td>Kvasnikov, <em>et al.</em>, 1988</td>
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<td><em>Escherichia coli</em> ATCC 33456</td>
<td>Glucose, acetate, propionate/anaerobic</td>
<td>Shen and Wang, 1993</td>
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<td><em>Micrococcus roseus</em></td>
<td>Acetate, glucose/anaerobic</td>
<td>Gvozdyak, <em>et al.</em>, 1986</td>
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<td><em>Microbacterium sp.</em> MP30</td>
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<td>Pattanapiitpaisal <em>et al.</em>, 2001</td>
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<td><em>Pseudomonas aeruginosa</em></td>
<td>Acetate, glucose/anaerobic</td>
<td>Gvozdyak, <em>et al.</em>, 1986</td>
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<td><em>Pseudomonas dechromaticans</em></td>
<td>Peptone, glucose/anaerobic</td>
<td>Romanenko and Korenkov, 1977</td>
</tr>
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<td><em>Pseudomonas chromatophila</em></td>
<td>Ribose, fructose, fumarate, lactate, acetate, succinate, butyrate, glycerol</td>
<td>LebedewL and Lyalikova, 1979</td>
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Factors affecting chromate reduction

Chromate-reducing bacteria can utilize a number of electron donors for Cr (VI) reduction (Table 1). These electron donors are mostly natural organic substances such as low-molecular-weight carbohydrates, amino acids, and fatty acids. In *Desulfovibrio vulgaris*, hydrogen can be effective electron donor for Cr (VI) reduction (Lovley and Phillips, 1994). Studies have tried to establish a relationship between these electron donors and Cr (VI) reduction rate. It has been reported that the addition of electron donors such as glucose or formate, that increase hydrogen bioavailability can stimulate Cr (VI) reduction. Yeast extract or nutrient broth may have chromate reduction activity due to their organic compounds with sulfhydryl groups (Wang and Xiao, 1994).

Generally, high cell densities are required for Cr (VI) reduction in either aerobic or anaerobic condition. The rate of Cr (VI) reduction increased along with increasing cell density in *E.coli* ATCC 33456 (Shen and Wang, 1994). However, the Cr (VI) reduction rate is not necessarily proportional to the cell density. The concentration of Cr (VI) also affects the reduction rate. The time required for complete reduction of Cr (VI) increased progressively as the initial concentration increased in the culture of *En. cloacae* (Wang et
al., 1989), _E. coli_ (Shen and Wang, 1994), _P. fluorescens_ (Wang and Xiao, 1994), and _Bacillus sp._ (Wang and Xiao, 1994).

Oxygen presence can inhibit Cr (VI) reduction in facultative bacteria because oxygen is the preferred final electron acceptor compared to Cr (VI) (Wang and Shen, 1994). Cr (VI) reduction in _A. radiobacter_ EPS-916, _E.coli_ ATCC 33456, and _P. stutzeri_ CMG463 is partially inhibited in the presence of oxygen (Llovera, _et al_., 1993; Shen and Wang, 1993). Cr (VI) reduction is completely stopped on _E.cloacea_ HO1 under aerobic condition (Fujie, _et al_., 1990).

Inhibition of sulfate and nitrate on Cr (VI) reduction has been reported for anaerobic cultures. _P. putida_ can tolerate up to 1mM sulfate and 200 µM nitrate without compromising its chromate reduction (Ishibaschi, _et al._, 1990). _Bacillus sp._ is able to stand up to 10 mM sulfate and 16 mM nitrate (Shen and Wang, 1994). The optimal pH and temperature for microbial Cr (VI) reduction are aligning with the optimal pH and temperature for bacteria growth. _En. cloacea_, the optimal conditions for Cr (VI) reduction took place at pH 6.0-8.5 and 20-40 °C (Komori _et al._, 1989). _E.coli_’s maximum initial Cr (VI) reduction rate was observed at pH 7 and 36 °C.

**Cr (VI) reduction by aerobic microbes**

Cr (VI) reduction activities have been found in a number of aerobic bacteria including _P. aeruginosa, A. eutrophus_ CH34, _P. fluorescens, P. synxantha, Bacillus, E. coli_ ATCC 33456, and _Shewanella alga_ BrY-MT (Bopp _et al._, 1983; Ohtake _et al._, 1987; Alvarez _et al._, 1999; Aguilera _et al._, 2004; Nies and Silver, 1989; Valls _et al._, 2000; Vaneechoutte _et al._, 2004; Bopp and Ehrlich, 1988; Gopalan and Veeramani, 1994; McLean _et al._, 2000; Shen and Wang, 1994; Wang and Xiao, 1995; Shakoori _et al._, 1999, 2000; Guha _et al._, 2001;
Camargo et al, 2003). Aerobic bacteria Cr (VI) reduction usually occurs as a multi-step process with Cr (VI) reduced to the short-lived intermediates Cr (V) and/or Cr (IV) before further reduction to the more stable end product, Cr (III) (Figure 4).

Figure 4—plausible mechanisms of enzymatic Cr (VI) reduction under aerobic and anaerobic conditions. Under anaerobic conditions, soluble and membrane-associated Cr (VI) reducing enzymes have been reported. SR and MR represent soluble and membrane-associated reductase, respectively (Wang and Shen, 1995). (Reprinted with permission)

Electron donors for Cr (VI) reduction include NADH, NADPH from the endogenous reserve (Appenroth et al, 2000). Little is known about chromate reduction mechanisms for aerobic bacteria. Some enzymes are shown to catalyze the reaction in vitro such as nitrate reductase-NfsA (Kwak et al, 2003), flavin reductase (Puzon et al, 2002), ferrireductase (Mazoch et al, 2004), and flavoproteins like YieF (Ackerley et al, 2004). E.coli ATCC 33456 has a NADH or NADPH dependent Cr (VI) reductase which is
different with NfsA and YieF (Woo-Chul Bae et al, 2005). Bacillus sp. RE was found to have a soluble Cr (VI) reduction enzyme which can be enhanced by NADH and NADPH (Elangovan, et al, 2006). The enzyme activity was optimal at pH 6.0 and 30 °C. A soluble NADH or NADPH-dependent enzyme was purified from P. putida showing chromate reduction activity (Park et al, 2000). Some metabolites and enzymes have been shown to be responsible for the modulation of yeast cell tolerance to chromate such as glutathione and glutathione reductase (Pesti et al, 2002; Jamnik and Raspor, 2003; Gazdag et al, 2003), glucose-6-phosphate dehydrogenase, mitochondrial Mn-dependent superoxide dismutase (Gazdag, et al, 2003), cytosolic Cu, Zn-superoxide dismutase and methionine sulfoxide reductase (Sumner et al, 2005).

Cr (VI) reduction by anaerobic bacteria

In the absence of oxygen, microorganisms are proposed to reduce Cr (VI) through action of a soluble reductase (SR) using NADH or endogenous electron reserves as an electron donor. Microorganisms can also reduce Cr (VI) by the mediation of a soluble reductase (SR), a membrane-bound reductase (MR), or both reductases with or without involving cytochrome contents of b, c and d (Figure 4). These enzymes commonly show NADH: flavin oxidoreductase activity (Gonzalez et al, 2005).

Sulfate-reducing bacteria (SRB) and iron-reducing bacteria (IRB) account for a significant percentage of known Cr (VI) reducing microorganisms. Shewanella oneidensis MR-1 is able to enzymatic dissimilatory reduce Cr (VI) (Daulton, et al, 2002; Myers, et al, 2000; Viamajala, et al, 2002) and chemically reduce Cr (VI) (Caccavo, et al, 1996; Nyman, et al, 2002; Wielinga, et al, 2001). Studies indicated that S. oneidensis has multiple nonspecific Cr (VI) reduction mechanisms and metal resistance mechanisms.
Deinococcus radiodurans is the most radiation-resistant organism discovered to date. Cr (VI) can be directly reduced in its anaerobic cultures with lactate. Higher rates of Cr (VI) reduction were observed in the presence of anthraquinone-2, 6-disulfonate (AQDS) (Fredrickson, et al, 2000).

Most research on the metabolism and biochemistry of SRB has been done on the genus Desulfovibrio, a member of the δ-proteobacteria (Hansen, 1994; Postgate, 1984). It has been proposed that SRB are able to reduce heavy metals via the production of H₂S, which is the end product of dissimilarly sulfate reduction, and via enzymatic processes involving polyhemic cytochromes. Most SRB cannot grow by using chromate as the sole electron acceptor except for Desulfotomaculum reducens and D. vulgaris UFZ B490 (Tucker, 1996, Pietzsch et al, 1999, Tebo and Obraztsova, 1998).

It has been reported that soluble and membrane-associated enzymes are involved in the Cr (VI) reduction process under anaerobic conditions. The Cr (VI) reduction activities are associated with electron transfer systems catalyzing the electron shuttle along the respiratory chains (Wang and Shen, 1995). Cytochrome families such as cytochrome b and cytochrome c are involved in enzymatic anaerobic Cr (VI) reduction. D. vulgaris str. Hildenborough (ATCC 29579, NCIMB 8303) is a model organism for studying the energy metabolism of sulfate-reducing bacteria and for understanding the bioremediation of toxic metals. D. vulgaris str. Hildenborough can dissimilarly reduce chromate by c₃ cytochrome (Lovley and Phillips, 1994), [Fe] hydrogenase, or [NiFeSe] hydrogenase with H₂ as the electron donor (Chardin, et al, 2003). The optimum conditions for Cr (VI) reduction were reported to be pH 7, 30-37 °C, in the presence of 25 mM sodium formate and 25 mM sodium citrate (Humphries and Macaskie, 2002). The loss of the cell
viability is due to Cr (III) toxicity (Klonowska, et al, 2008). Desulfomicrobium norvegicum was reported to have the highest Cr (VI) reduction rate among sulfate-reducing bacteria (Michel, et al, 2001). This strain grew in the presence of up to 500 µM chromate. The Cr (VI) presence induced morphological changes and leakage of periplasmic proteins into the medium. Cr (VI) reduction in D. norvegicum was found to involve a hydrogenase and a c-type cytochrome (Chardin, et al, 2002; Michel, et al, 2001). Tetraheme cytochrome c₃ from D. norvegicum showed twice as much activity as tetraheme cytochrome c₃ from D. vulgaris str. Hildenborough (Michel, et al, 2000). D. reducens strain MI-1 is a spore-forming sulfate-reducing bacterium which is the first SRB reported to grow with metals as sole electron acceptors (Tebo and Obraztsova, 1997). The metal reduction mechanism is not clear so far. It is suggested that cytochromes b and c were involved (Tebo and Obraztsova, 1997). It was reported that the spores of Desulfotomaculum reducens str. MI-1 was responsible for the U (VI) reduction when grown fermentative on pyruvate with hydrogen gas as electron donor (Junier, et al, 2009). Anaerobic Cr (VI) reduction by resting E.coli cells was reported to be enhanced by quinone redox mediators, higher initial cell density, added glucose and Mn²⁺ and Pb²⁺ (Liu, et al, 2010).

Multipheme cytochromes c (Table 2) in the SRB showing Cr (VI) reduction activity generally contain four hemes with a bishistidinyl iron coordination and redox potentials in the range from -200 mV to -400 mV. Others from Desulfovibrio spp. include octaheme cytochromes c₃ (Czjzek, et al, 1996), dodecaheme cytochrome Hmc (Bruschi, et al, 1992), and a nine-heme cytochrome (Saraiva, et al, 1999). It was reported that only cytochromes that have low redox potential (in the range -200 to -400 mV vs standard
hydrogen electrode) and have bishistidinyl heme iron coordination can act as metal reducing biocatalysts (Chardin, et al, 2003). Hydrogenases which catalyze the reversible oxidation of molecular hydrogen are important in the anaerobic metabolism of chemotrophic and phototrophic bacteria. In Desulfovibrio and Desulfomicrobium, [Fe], [NiFe], and [NiFeSe] hydrogenases were found to reduce chromate (Chardin, et al, 2003). The [Fe] hydrogenase from Desulfovibrio vulgaris str. Hildenborough has the highest chromate reduction rate (Chardin, et al, 2003).

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Cr (VI) reduction rate (µmol Cr (VI) min⁻¹ mg⁻¹ enzyme)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c3(Desulfovibrio vulgaris Hildenborough)</td>
<td>391 ± 18</td>
</tr>
<tr>
<td>Cytochrome c3(Desulfomicrobium norvegicum)</td>
<td>739 ± 56</td>
</tr>
<tr>
<td>Cytochrome c7(Desulfuromonas acetoxidans)</td>
<td>557 ± 25</td>
</tr>
<tr>
<td>Cytochrome c355(Desulfovibrio vulgaris Hildenborough)</td>
<td>No activity</td>
</tr>
<tr>
<td>Cytochrome c3 H35M (D.vulgaris Hildenborough)</td>
<td>400 ± 19</td>
</tr>
<tr>
<td>Cytochrome c3 H2M (D.vulgaris Hildenborough)</td>
<td>341 ± 23</td>
</tr>
<tr>
<td>[Fe] hydrogenase (D. vulgaris Hildenborough)</td>
<td>7679 ± 4</td>
</tr>
<tr>
<td>[NiFeSe] hyrogenase (Desulfomicrobium norvegicum)</td>
<td>161 ± 3</td>
</tr>
</tbody>
</table>

Table 2—Chromate reductase activity of purified proteins (Michel et al, 2000)

Cr (VI) is reduced to Cr (III) which can form precipitates or soluble complexes with appropriate substances (Pourbaix, 1966). Reduced Cr (III) precipitates were found both on the cell surface and in the membranes of D.vulgaris str. Hildenborough (Florence Goulhen et al, 2005). Cr (III) complexes were identified both extracellular and in the cytoplasm of Shewanella oneidensis MR-4 cells (Middleton, et al, 2003). In 2002, Puzon and colleagues showed that a soluble Cr (III)-NAD+ complex was formed after Cr (VI) was reduced by a flavin reductase from E. coli (Puzon, et al, 2002). In the presence of organic metabolites, Cr (VI) reduction can form both soluble and insoluble organo-Cr (III) complexes (Puzon, et al, 2005). Shewanella MR-4 has hindered growth due to Cr (VI), which was hypothesized that the chromium toxicity was a consequence of
chromium as Cr$^{3+}$, Cr(OH)$^{2+}$, and/or Cr(OH)$_2$ (Bencheike-Latmani, et al, 2007). The growth lag of Desulfovibrio vulgaris is reported to be associated with the Cr (III) toxicity (Klonowska, et al, 2008).

Protection against oxidative stress

A number of microorganisms respond to the oxidative stress brought by chromate. Oxidative stress plays a major role in chromate toxicity in E. coli. The SOS response was activated and proteins such as SodB and CysK which counter oxidative stress were increased in response to chromate stress on E. coli K-12 (Ackerley, et al, 2006).

Caulobacter crescentus responded to chromate or dichromate stress by up-regulating genes that are known to be involved in response to oxidative stress (Ping Hu, et al, 2005). These genes include genes encoding superoxide dismutase (SOD), glutathione-s-transferase (GST), thioredoxin, and glutaredoxin compounds (Ping Hu, et al, 2005). ChrR protein from Pseudomonas putida and YieF from E. coli were found to be dimers and reducing chromate efficiently to Cr (III) ($K_{cat}/K_m = 2 \times 10^4$ $M^{-1} \cdot s^{-1}$). The ChrR dimer generated a flavin semiquinone during chromate reduction and transferred >25% of the NADH electrons to ROS (Ackerley, et al, 2004). Plasmid pMOL28 from C. metallidurans encodes ChrC and ChrE which may be involved in chromate resistance (Juhnke, et al, 2002). In P. aeruginosa, stress proteins such as chaperonins and proteins involved in free radicals detoxification by the glutathione system were observed to be overexpressed (Kocberber Kilic, et al, 2010). The Chaperonin system DnaK/DnaJ/GrpE is known to protect cells against thermal and oxidative stress (Vlamis-Gardikas, 2008) and its expression level increased when Euglena gracilis was exposed to Cr (VI) ions (Ferreira et al, 2007). Glutathione (GSH) synthetase, an antioxidant molecule, and glutathione-S-
transferase (GST) help prevent the toxic effect of free radicals. GSH interacts with chromate to generate redox-cycling Cr (VI), which enhances Cr (VI)-induced DNA strand breaks and causes the development of glutathione-Cr (III)-DNA adducts (Ackerley et al., 2006). In P. aeruginosa, the interaction of GSH and Cr (VI) in the cytoplasm leads to the formation of GSSG (glutathione disulphide) and Cr (III) (Kocberber Kilic et al, 2010). GST was also found in bacteria such as E. coli, Proteus mirabilis, Pseudomonas sp. and Streptomyces griseus to help metabolize naturally occurring toxins and ROS (Traversolo et al, 2008). The lack of these free radical-preventing enzymes causes yeast increased metal sensitivity (Bai et al, 2004; Helbig et al, 2008). Upregulation of glutaredoxin compounds genes was also reported in C. crescentus (Ping Hu et al, 2005).

Other Cr (VI) resistance mechanisms

Protection from DNA damage is another microbial chromate response. The SOS response was activated in response to chromate stress in E. coli K-12 (Ackerley et al, 2006). P. aeruginosa RuvB and RecG helicases are involved in repairing DNA damage caused by chromate stress (Miranda et al, 2005). In S. oneidensis, the SO0368, uvrD, and hrpA genes were induced after 24 h Cr (VI) exposure (Chourey et al, 2006). Endonucleases and RecA protein were observed to be up-regulated in response to chromate stress in C. crescentus (Ping Hu et al, 2005). In P. aeruginosa, upregulation of MucD (role in expopolysaccharide production) and outer membrane proteins were observed with Cr (VI) exposure (Kocberber Kilic et al, 2010). The sulfate uptake pathway was also suspected to be involved in chromate resistance response. C. crescentus down regulates a sulfate transport system reducing chromate uptake (Ping Hu et al,
2005). Nitroreductase in *E.coli* and *P. ambiguа* was found to have chromate reduction activity (Kwak *et al*, 2003).

**Desulfovibrio desulfuricans ATCC 27774**

*Taxonomy*

Species of *Desulfovibrio* are typically Gram-negative, non-spore forming about 0.7 um in cell diameter. The growth of sulphate-reducing bacteria is common found in anaerobic regions of marine and estuarine sediments and saline ponds because of their high sulphate content (Truper *et al*, 1969). *Desulfovibrio desulfuricans* belongs to the domain of Bacteria, Proteobacteria, Delta, Deltaproteobacteria, Desulfovibrionales, Desulfovibrionaceae, *Desulfovibrio*, *Desulfovibrio desulfuricans*, *Desulfovibrio desulfuricans* subsp. *Desulfuricans* (NCBI, 2011).

Figure 5—Distance tree of 16 rRNA genes from *Desulfovibrio* species with complete genome. Bar is the number of nucleotide substitutions. (JGI, 2011)
Description

*Desulfovibrio desulfuricans* ATCC 27774 is a sulfate-reducing bacterium. Sulfate-reducing bacteria (SRB) are anaerobic microorganisms that use sulfate as a terminal electron acceptor in the degradation of organic compounds. SRB receive broad attention due to their capabilities to remove sulfate and heavy metals from waste streams.

*Desulfovibrio desulfuricans* can use thiosulfate, tetrathionate and sulfite as electron acceptors besides inorganic electron acceptors (Postgate, 1951; Cypionka, 1987). Major electron donors consist of volatile fatty acids including acetate, propionate, butyrate, C3 and C4 fatty acids including lactate, pyruvate, malate, alcohols including ethanol, propanol, H2/CO2, and sugars and longer chain fatty acids.

*Desulfovibrio desulfuricans* ATCC 27774 was isolated from the rumen of a sheep. *D. desulfuricans* ATCC 27774 is commonly found in soils. It is the first well documented sulfate-reducing bacterium capable of growing by dissimilatory nitrate reduction. This organism grows anaerobically and utilizes a wide variety of electron acceptors, including sulfate, sulfur, nitrate, and nitrite. This organism can reduce a number of toxic metals including U (VI), and Fe (III). This organism is responsible for the poisonous hydrogen sulfide gas in marine sediments and in terrestrial environments such as drilling sites for petroleum products. The complete genome was sequenced by the US DOE Joint Genome Institute (JGI-PGF) in 2009 (Table 5). *D. desulfuricans* typically grow anaerobically. *D. desulfurican* ATCC 27774 is reported to be able to grow at nearly atmospheric oxygen levels (Lobo et al, 2007).
Table 5—Desulfovibrio desulfuricans ATCC 27774 Genome Information (JGI, 2011)

<table>
<thead>
<tr>
<th>DNA, total number of bases</th>
<th>Number</th>
<th>% of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA coding number of bases</td>
<td>2418169</td>
<td>84.16%</td>
</tr>
<tr>
<td>DNA G+C number of bases</td>
<td>1668667</td>
<td>58.07%</td>
</tr>
<tr>
<td>Genes total number</td>
<td>2443</td>
<td>100.00%</td>
</tr>
<tr>
<td>Protein coding genes</td>
<td>2382</td>
<td>97.50%</td>
</tr>
<tr>
<td>RNA genes</td>
<td>61</td>
<td>2.50%</td>
</tr>
<tr>
<td>Protein coding genes with enzymes</td>
<td>687</td>
<td>28.12%</td>
</tr>
<tr>
<td>Protein coding genes with COGs</td>
<td>1808</td>
<td>74.01%</td>
</tr>
</tbody>
</table>

Recent studies on Desulfovibrio desulfuricans ATCC 27774

*Desulfovibrio* strain ATCC 27774 is one of a relative small group of sulfate-reducing bacteria that can grow with nitrate as an alternative electron acceptor. It grew more rapidly and to higher yields of biomass with nitrate than with sulfate. Although thermodynamically a less favorable electron acceptor, sulfate is used preferentially by *D. desulfuricans* ATCC 27774 in the presence of both sulfate and nitrate (Angeliki Marietou, *et al.*, 2008). Most attention has been paid on the illustration of this strain’s nitrate and sulfate dissimilatory pathways.

Soluble nitrate reductase was isolated and purified to illustrate nitrate reduction activity. A monomeric periplasmic enzyme was found to be nitrate reductase by Bursakov and colleagues. Electron Paramagnetic Resonance (EPR) signals due to one [4Fe-4S] cluster and Mo (V) were identified in dithionite reduced samples and in the presence of nitrate (Bursakov, *et al.*, 1995). To illustrate the sulfate respiratory pathway, a membrane, DsrMKJOP, from *D. desulfuricans* ATCC 27774 was purified and characterized. The complex has hemes of the c and b types and several iron-sulfur centers. The isolated Dsr complex displays an EPR signal with similar characteristics to the catalytic [4Fe-4S]^{3+} species observed in heterodisulfide reductases (Richardo *et al.*, 2005). The primary and
three-dimensional structures of a [NiFe] hydrogenases isolated from *D. desulfuricans* ATCC 27774 were determined. In *D. desulfuricans* ATCC 27774, the soluble, periplasmic [NiFe] hydrogenase is the only hydrogenase reported in this organism (Matias, *et al*., 2000).

Relatively less attention has been paid to the heavy metal reduction capability of *D. desulfuricans* ATCC 2774 although it has been broadly reported that other *Desulfovibrio* species actively reduce heavy metals including U (VI), Cr (VI).
Specific Aims

Microbial metal detoxification has brought great attention due to its potential bioremediation application. Metal reducing microbes have been discovered among different genera. Sulfate-reducing bacteria have received particularly attention and account for a significant percentage of heavy metal reducing microbes. Previous studies indicate that the electron transport system is involved in the metal reduction process. Cytochrome and hydrogenase were discovered to be responsible for Cr (VI) reduction in a few microbial strains such as *D.vulgaris*. Previous studies led us to explore Cr (VI) reduction activity induced by *D. desulfuricans* ATCC 27774. We will test the suitable grow culture for this strain in the laboratory condition. Further experiment will be done to test *D. desulfuricans* ATCC 27774’s Cr (VI) responses and elucidate its mechanism. We were expecting similar enzymatically Cr (VI) reduction activity as had been seen with other *Desulfovibrio* species. Our previous results suggested *D. desulfuricans* ATCC 27774 possessed a secondary metabolite that had Cr (VI) reduction activity. The specific aim of this work was to determine the mechanism of Cr (VI) reduction and verify that it involved a secondary metabolite.
**Experimental Approach**

Previous results in the lab have shown that Cr (VI) inhibits the ability of *D. desulfuricans* strain 27774 to grow using nitrate as the terminal electron acceptor. Once the Cr (VI) has been reduced, the cells are able to grow. Our hypothesis is that *D. desulfuricans* strain 27774 produces a secreted secondary metabolite that reduces Cr (VI) to Cr (III). In order to test this hypothesis cell cultures were grown under different growth conditions and the activity for Cr (VI) reduction determined using both a colorimetric assay and ICP-MS. Different media formulations were tested to eliminate background Cr (VI) reduction activity. Genomics and bioinformatics were used to identify potential biosynthetic pathways in the genome of *D. desulfuricans* strain 27774.
MATERIALS AND METHODS

Growth media and cell culture

A culture of *D. desulfuricans* strain 27774 was obtained from the American Type Culture collection.

SES-3 medium was prepared as reported in Stolz et al. (1997). GS-15 medium was prepared as reported in Lovley and Phillips (1988). Both media were amended with 20 mM sodium nitrate and 20 mM sodium lactate. The media were adjusted to pH 6.8 using HCl, dispensed into bottles, then degassed under a N₂-CO₂ atmosphere (80:20%) for 5 to 10 min depending on the volume. The media were autoclaved for 30 min for 100 ml and 45 min for 900 ml. Stock cultures of *D. desulfuricans* strain 27774 were maintained in either SES-3 or GS-15 medium in 100 ml volumes. Larger volumes (900 ml) were used for the experiments.

Cell growth was monitored by optical density at 600 nm using the Perkin Elmer Lambda 2 UV/Vis spectrophotometer. Cultures were harvested in early stationary phase (as determined by OD₆₀₀ nm).

Cr-Diphenylcarbazide (DPC) assay color reaction was monitored by optical absorbance at 540 nm using the Perkin Elmer Lambda 2 UV/Vis spectrophotometer. Quantitative measurements were based on Beer-Lambert law (*A = ec l*). A=absorbance; e=molar absorbance or absorption coefficient; c=concentration of the compound in the solution; l=path length of light in the sample. Concentrations were then calculated by comparing the results with a calibration curve (standard curve).

For 100 ml volume of bacteria culture, a sterile 50 ml centrifuge tube was weighed. A 20-ml syringe was used to transfer desired amount of *D. desulfurican* ATCC 27774 into
the 50-ml centrifuge tube under anaerobic conditions. Samples were centrifuged in the Eppendorf Centrifuge 5810 R at 9600 rpm, 26 °C for 10 minutes. The cell-free supernatant was then filtered through a 0.2 µm VWR Sterile Syringe Filter. The cell pellet was kept and washed with 10 ml of degassed 2.5 g/l sodium bicarbonate buffer (pH 6.8) twice. The suspended cells in bicarbonate buffer were centrifuged again to collect the cell pellet. The wet weight of the cell pellet was calculated by subtracting the centrifuge tube weight from the total weight.

**Chromium determination**

Cr (VI) was routinely measured using the diphenylcarbazide (DPC) assay as reported in Saltzman (1952). The Cr-DPC color reaction was determined by UV/Vis spectroscopy on the Perkin Elmer Lamda 2 spectrophotometer at 540 nm and the concentration was calculated based on the Beer-Lambert Law (A= ecl).

Selective samples were analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) with isotope dilution using US EPA method 6020a. Samples were analyzed on an Agilent 7700/7500 series ICP-MS. Stock solutions of Cr (VI) and Cr (III) were prepared by dissolving 1g/L of potassium dichromate and sodium chromite respectively, in DI water. To develop a standard curve, 10 ml of the stock solution was diluted to concentrations of 0, 20, 40, and 60 ppb.

To test the effects of the growth medium on the stability of chromium species, the culture media (SES-3 medium, GS-15 medium) were amended with 10 ppm (192 µM) of potassium chromate and incubated for 12 hours. A 25 µl sample was diluted with sterile DI water to a final volume of 10 ml, and then each sample was run in triplicate.
Diphenylcarbazide Assay

Cr (VI) concentration was analyzed using the diphenylcarbazide method (Urone, P.F., 1955). The reagent diphenylcarbazide forms strong red-violet color with Cr (VI) in acidic solution. Ethyl acetate, high purity acetone, or acetone meeting ACS specifications which does not have a tendency to be basic can be used to make solutions that give chromium diphenylcarbazide colors of consistently high absorbance (Urone, 1955). The 2.5% diphenylcarbazide was made by dissolving 0.025 g s-diphenylcarbazide in 9.67 ml acetone and 330 µl 3M sulfuric Acid. The stoichiometric amount between Cr (VI) to diphenylcarbazide is 3 to 2 mole ratio (Zittel, 1963).

To determine Cr (VI) in the samples, obtain 1.5-ml eppendorf centrifuge tubes and label them. Withdraw 1 ml sample into the test tube. To each tube, inject 50 µL 3M H₂SO₄ to acidify the sample. A 10 or 15 µl 2.5% diphenylcarbazide was then injected into each test tube for color development. Cr (VI) was then determined by either visually comparison with standard solutions or tests the absorbance at 540 nm with Perkin Elmer UV/VIS spectrometer. The concentration of Cr (VI) has a linear relationship with the absorbance at 540 nm. Experimental results were expressed either as absorbance at 540 nm or percentage of residue Cr (VI) after reduction. Anaerobic environment was provided when color development is not stable.

Desulfovibrio desulfurican ATCC 27774 Cr (VI) response

Chromate resistant experiment

Desulfovibrio desulfurican ATCC 27774 was grown for 48 hours (OD₆₀₀=0.055) in GS-15 medium. Twenty micro liter bacteria were transferred to five 25-ml serum bottles
under anoxic condition. Different concentrations of potassium chromate (0, 25, 50, 75, 100µM) were then added to each bottle. The same concentration of potassium chromate was also injected into GS-15 medium without cells as a control experiment. Cell density was monitored at 600 nm absorbance with Perkin Elmer UV/VIS spectrometer. Aliquot of samples was withdrawn during regular time interval to run the Cr-DPC reaction. The assay contains 940 ml of sample, 50 ml of 3 M H₂SO₄, and 10 µl 2.5% DPC in acetone.

*Chromate reduction activity detection*

Cells grown of GS-15 medium were harvested by centrifugation then re-suspended in 2.5 g/l sodium bicarbonate (pH 6.8). The cell suspensions are equally split to nine anoxic 25-ml serum bottles. An aliquot of 10 mM sodium lactate was injected into the 20ml cell suspension as electron donor. The bottles were labeled “cell suspension plus sodium lactate”. Equal amounts of sodium lactate were injected into 20 ml bicarbonate buffer (pH 6.8) in the absence of oxygen without cell as the control. Another electron donor, H₂, was provided by replenishing the 50-ml serum bottle with hydrogen gas after adding a 20 ml cell suspension and the bottle was labeled “cell suspension plus H₂”. Three bottles with 20 ml bicarbonate buffer (pH 6.8) were filled with equal amounts of hydrogen gas without cell in order to provide a control experiment for “cell suspension plus H₂”. A 20 ml cell suspension in bicarbonate buffer without any electron donor was injected to the 50-ml serum bottle as “cell only” experiment. A 20 ml bicarbonate solution with 100 µM chromate was injected to the 50-ml serum bottles as positive control. The serum bottles were sealed with thick butyl rubber stoppers and aluminum cap. A 200 µl 10 mM potassium chromate was added to start the reaction. Aliquots (935 µl) were taken at regular time intervals in 48 hours and were analyzed for Cr (VI) reduction using the
diphenylcarbazide method (Lovely and Phillips, 1994). The Cr (VI)-DPC reaction experiment contains 935 µl of sample, 50 µl of 3 M H₂SO₄, and 15 µl 2.5% DPC in acetone solution.

**Cr (VI) reduction by supernatant**

A 30 ml volume of supernatant was collected and restored in anaerobic serum bottle as described above. A number of 10-ml serum bottles were acquired. Different concentrations of supernatant were then made by mixing the supernatant with DI-water in anaerobic condition. The total volume of each bottle is 10 ml. The concentration of 100 µM chromate was achieved by injecting 100 µL of 10 mM potassium chromate stock solution. Aliquots (1ml) were taken at T1, T2, and T3 and analyzed using the diphenylcarbazide method (Lovely and Phillips, 1994).

**Secondary metabolism pathway detection using bioinformatics approach**

The genome of *Desulfovibrio desulfuricans* ATCC 27774 was acquired from the DOE Joint Genome Institute (JGI) website. The whole genome was uploaded to NPsearcher.com website to detect secondary metabolite gene clusters (Li. et al., 2009). The predicted chemical in SMILE form was then drawn using Pubchem drawing software.

**Extracellular active compound extraction, purification and characterization**

*Extraction of chromate reducing active compound from the supernatant*

For larger quantities of cells, *Desulfovibrio desulfuricans* ATCC 27774 was cultured in one liter bottles. The cells were harvested by centrifuging at 6000 rpm for 10 minutes at 4 °C. The clear supernatant was then transferred to a 1 liter extractor. 100 ml ethyl acetate
(EA) was added to the extractor and well mixed. The upper layer ethyl acetate after 30 minutes sitting was collected. The procedure was repeated three times. Sodium chloride was then added to the collected crude extraction and filtered to remove organic matter. Sodium sulfate was added to the filtrate to dry the mixture. The crude extraction was concentrated using a BUCHI R-3000 Rotavapor at 40 °C. The concentrated crude extraction was stored in a 1.5-ml clean glass vial in the refrigerator.

Purification of a chromate reduction active compound

Purification of the Cr (VI) reducing compound was done by preparative thin layer chromatography (TLC). The stationary phase was Silica G TLC Plates w/UV 254 (Lot #041013 L; Catalog #1624126). The ethyl acetate based crude extraction was collected and freeze dried. The extraction was dissolved in dichloromethane (DCM) for preparative TLC. For preparative TLC, 100 ml of the mobile solvent system (Methanol:DCM=1:9) was poured into the preparative TLC chamber and allowed to saturate for 10 minutes. DCM based crude extraction was spotted using Wiretrol II microcapillary tubes (Drummond, Catalog #5-000-2005). The TLC plate was carefully placed in the container and developed until the solvent front reached about 2 cm from the top. TLC plate was pulled out and dried in air. Each fraction was outlined with a pencil under UV light. Each fraction was scratched off and transferred to clean glass vial. Six fractions were observed and marked. Each fraction was dissolved in a mixture of methanol and DCM in order to extract the compounds from the silica gel. The glass tubes were centrifuged and dissolved fractions were freeze dried and dissolved in water. The diphenylcarbazide assay was conducted to test Cr (VI) reducing ability of each fraction. Fraction 3 with Rf value of 0.428 was identified to reduce Cr (VI). Fraction 3 was then dissolved in chloroform and
run on preparative TLC with a solvent system of 50% ethyl acetate and 50% DCM. Eight fractions were observed and each fraction was dissolved in water in order to conduct the diphenylcarbazide assay. However, Cr (VI) reducing activity was not detected in either of the eight fractions.

Characterization of active compounds

Nuclear magnetic resonance (NMR) spectroscopy was performed on a Bruker Avance 400 spectrometer operating at 400 MHz for 1H spectra for each fraction. Each fraction was dissolved in chloroform.

NanoESI (nano-electrospray ionization) was utilized to analyze metabolites produced during bacterial growth. The mixture of fraction No. 1 and No. 2 after the second preparative TLC purification was spiked with formic acid for a final concentration of 0.1% formic acid. The system used for the analysis was a HPLC-Chip Cube MS interface (Agilent Technology), 6520 Accurate Mass Quadrupole Time of Flight mass spectrometer (Agilent Technology), and an infusion chip, FIA-Chip II (Agilent Technology). The flow rate of the syringe pump was set to 1.6 µL/min. Total run time for analysis was 1 minute. Data was acquired using the Mass-Hunter Workstation (version) in positive and negative ionization mode for samples dissolved in acetonitrile and only positive ionization mode for water and 0.1% formic acid samples. Gas temperature, drying gas volume, fragmentor voltage, skimmer voltage, and octopole RF were set to 325°C, 5 L/min, 180 V, 65 V, and 750 V respectively. In each cycle MS spectra were acquired at 1 Hz (1 spectra/sec) (m/z 250-1500). Medium isolation (4 m/z) window was used for precursor selection. Collision energy was set to a slope of 3V/ (100
Da) with a 2V offset. Precursor’s exclusion was set to exclude after the first spectra and released after 0.25 minutes
RESULTS

Cell growth

Bacteria were grown at 26 °C. Aliquots (2 ml) of *Desulfovibrio desulfuricans* ATCC 27774 are injected to 100 ml GS-15 medium to start the cell growth. It usually took 72 hours for *Desulfovibrio desulfuricans* ATCC 27774 to reach the highest cell yield in GS-15 medium anaerobically. The cell density was checked on spectrometer and the readings of the highest yield ranged from 0.5 to 0.6 at 600 nm. In the presence of oxygen, *Desulfovibrio desulfuricans* strain ATCC 27774 indicated a 12-24 hour delayed growth (data not shown). No significant morphological change was observed after *D.desulfuricans* ATCC 27774 was grown in the presence of 50µM Cr (VI) for several generations. The growth of *D.desulfuricans* ATCC 27774 on the medium with chromate as the sole electron acceptor was not successful.

*Desulfovibrio desulfuricans* ATCC 27774 medium selection

IC ICP-MS results indicate that approximately 30% of Cr (VI) was reduced to Cr (III) in SES-3 medium in 12 hours but only 14% of Cr (VI) was reduced to Cr (III) in the GS-15 medium.

<table>
<thead>
<tr>
<th></th>
<th>GS-15 medium</th>
<th>GS-BACT</th>
<th>BACT-DPC</th>
<th>DI-DPC</th>
<th>SES-3 medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr(VI) (ppm)</td>
<td>12.80</td>
<td>3.59</td>
<td>0.84</td>
<td>0.74</td>
<td>15.31</td>
</tr>
<tr>
<td>Cr(III) (ppm)</td>
<td>2.5</td>
<td>15.83</td>
<td>30.13</td>
<td>7.52</td>
<td>7.07</td>
</tr>
</tbody>
</table>

Table 3–Relative Cr (VI) and Cr (III) quantity in five samples—GS-15 medium, bacteria culture on GS-15 medium, bacteria supernatant and DPC reaction, DI-water and DPC reaction, and SES-3 medium

GS-15 medium has less Cr (VI) reducing interference than SES-3 medium. The diphenylcarbazide assay also confirmed that GS-15 medium had less Cr (VI) reduction than the SES-3 medium. The result also indicates that Cr (VI) was reduced to Cr (III) by
the supernatant. Experiment was also conducted that the active compounds in supernatant did not react with the reagents in the diphenylcarbazide assay.

![Calibration curve for Cr(VI)](image1)

![Calibration curve for Cr(III)](image2)

Figure 6–IC ICP-MS Quantitative calibration curve for Cr (VI) and Cr (III)

**Cr (VI) effect on cell growth**

*Desulfovibrio desulfuricans* strain ATCC 27774 was exposed to different concentrations of potassium chromate (0, 25μM, 50 μM, 75μM, 100 μM) in order to examine Cr (VI)’s effect on cell growth. There was a corresponding longer delay of cell growth with increasing Cr (VI) concentration (Figure 7). The Cr (VI)’s effect on cell growth depends
on the initial concentration of the bacteria and Cr (VI) concentration. A higher initial bacterial concentration resulted in less delayed growth. At 25 μM chromate concentration level, the cell yield was half of the cell yield without chromate. At a higher chromate concentration (50 μM, 75μM, 100 μM), cell growth was further inhibited (Figure 9). With an initial cell density of 0.055 at absorbance of 600 nm, the Cr (VI) reduction rate showed an exponential decrease in bacteria growth (Figure 9). With an initial cell density of 0.069 at absorbance of 600 nm, the Cr (VI) reduction was complete in 24 hours at 25 μM, 50 μM, 75 μM concentration levels. The Cr (VI) reduction was complete within 48 hours at the 100 μM concentration level.

![Graph showing cell growth inhibition](image)

**Figure 7—** *D.desulfuricans* strain ATCC 27774 cell growth is inhibited with exposure of different chromate concentrations
Figure 8---25 µM Cr (VI) along with bacteria growth ♦ cell density is expressed by absorbance at 600 nm; 25 µM Cr (VI) was achieved in the cell growth culture at 48 hour as well as GS-15 medium. The higher the A540 is, the higher the Cr (VI) concentration.

Figure 9---Effect of 50 µM Cr (VI) exposure to Bacteria growth ♦ cell density is expressed as absorbance at 600 nm; 50 µM Cr (VI) was achieved in the cell growth culture at 48 hour as well as GS-15 medium. The higher the A540 is, the higher the Cr (VI) concentration.
Figure 10---Effect of 75 µM Cr (VI) exposure to Bacteria growth ♦ cell density is expressed by absorbance at 600 nm; 75 µM Cr (VI) was achieved in the cell growth culture at 48 hour as well as GS-15 medium. The higher the A₅₄₀ is, the higher the Cr (VI) concentration.

Figure 11---Effect of 100 µM Cr (VI) exposure to Bacteria growth ♦ cell density is expressed by absorbance at 600 nm; 100 µM Cr (VI) was achieved in the cell growth culture at 48 hour as well as GS-15 medium. The higher the A₅₄₀ is, the higher the Cr (VI) concentration.
Cr (VI) reduction by whole cells

Previous investigations on several Desulfovibrio species indicated that they can enzymatically reduce Fe (III) and U (VI) (Lovley and Phillips, 1992; Coleman, et al, 1993). This led us to investigate whether Desulfovibrio Desulfubrican ATCC 27774 might also act as Cr (VI) reducer. There was no chromate reduction activity observed in cell suspension without added electron donor (Figure 12). Cr (VI) reduction activity was observed when Sodium Lactate or hydrogen gas was provided in cell suspensions as electron donor.

Figure 12--- Cr (VI) response by cell suspension only ♦. Cr (VI) control—20 ml sodium bicarbonate buffer (pH 6.8) with 100 µM Chromate exposure. ■. Cell suspension in 20 ml bicarbonate buffer (pH 6.8) with 100 µM Chromate exposure.
Figure 13-- Cr (VI) reduction activity by cells suspension with 10 mM sodium lactate ●. Cr (VI) control, 20 ml bicarbonate buffer with 100 µM Chromate. ■ 20 ml cell suspension with 10 mM sodium lactate ▲ 10 mM sodium lactate 20 ml sodium bicarbonate buffer (pH 6.8) (The below-100µM Cr(VI) zero time point was possible due to the Cr (VI) reduction by lactate and/or other factors.)

Figure 14--- Cr (VI) reduction by cell suspension with H₂ ●. Cr (VI) control, 20 ml bicarbonate buffer with 100 µM Chromate ■ 20 ml cell suspension in bicarbonate buffer (pH 6.8) with H₂ as electron donor ▲ 20 ml bicarbonate buffer (pH 6.8) with H₂
**Cr (VI) reduction by cell-free supernatant**

The experiments indicate that *Desulfovibrio desulfuricans* ATCC 27774 has hexavalent reduction activity mainly due to its cell-free supernatant. Hexavalent chromate reduction is rapid and reaches its limit depending on the initial quantity of the supernatant and Cr (VI) concentration. One micro liter of supernatant from a well grown bacteria culture can reduce up to 400 μM Chromate in less than 25 seconds. The reduction activity shows an exponential decrease (Figure 15).

![Figure 15](image_url) —Cr (VI) concentration from total 100 μM Cr (VI) reduction by different supernatant concentrations in DI-water
Extracellular active compound extraction, purification and characterization

Crude extracts from Desulfovibrio desulfuricans ATCC 27774 on SES-3 medium were purified by preparative TLC twice. The first preparative TLC used a mobile solvent system of 10% methanol in DCM. The second preparative TLC used mobile solvent system of 50% ethyl acetate and 50% DCM. Fraction No. 3 with Rf value of 0.428 in the first preparative TLC showed Cr (VI) reduction activity. The secondary preparative TLC was run for fraction No. 3. Another eight fractions were acquired. However, none of them showed significant Cr (VI) reduction activity. One possible reason is that the active compound lost its activity due to oxygen in the air and organic solvents. Another possible reason is that the quantity after purification was not enough to present significant Cr (VI) reduction activity.

<table>
<thead>
<tr>
<th>(+)</th>
<th>Fraction No.1</th>
<th>Fraction No.2</th>
<th>Fraction No.3</th>
<th>Fraction No.4</th>
<th>Fraction No.5</th>
<th>Fraction No.6</th>
</tr>
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<tbody>
<tr>
<td>Rf</td>
<td>0.786</td>
<td>0.643</td>
<td>0.428</td>
<td>0.3</td>
<td>0.229</td>
<td>0.143</td>
</tr>
<tr>
<td>Cr (VI)-DPC, Abs 540</td>
<td>1.338±0.002</td>
<td>1.321±0.004</td>
<td>1.305±0.001</td>
<td>0.340±0.001</td>
<td>1.331±0.001</td>
<td>1.349±0.001</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.355±0.003</td>
<td></td>
</tr>
</tbody>
</table>

Table 4–DPC assay of fractions eluted from preparative TLC

Predicted secondary metabolite pathway and possible chemical structures

The NPsearcher.com website predicted one mixed modular NRPS/PKSs and one non-mevalonate terpenoid mep gene cluster located from Ddes_1666 to ddes_1680 (2001768-2022596) about 20828 bp. The NRPS/PKS gene cluster coded proteins can be found in the Appendix I. The predicated compounds synthesized by the pathways fall into two categories by molecular weight—383 and 765. Diffusion MS indicated a dominate chemical with molecular weight of 339 in fraction No. 1 and 2 of the second preparative TLC crude extract.
Figure 16—Predicted secondary metabolism gene cluster in *Desulfovibrio desulfuricans* ATCC 27774 by NPsearcher.com

Figure 17—Predicted secondary metabolites structures by PubChem from NPsearcher.com

Figure 18—Mass Spectrometry scan on the crude extract after the second preparative TLC
DISCUSSION

A number of Cr (VI) reducing bacteria have been studied over the last two decades. Sulfate-reducing bacteria especially *Desulfovibrio* family have been extensively studied. Cr (VI) reduction mechanisms mainly fall into two categories: 1) chemical reduction by H$_2$S and organic compounds and 2) enzymatically reduction mainly by cytochrome and hydrogenases. These discoveries led our research to investigate the Cr (VI) response by *Desulfovibrio desulfuricans* ATCC 27774 which is genetically close to a number of extensively studied *Desulfovibrio* species with Cr (VI) reducing activity.

*Desulfovibrio desulfuricans* ATCC 27774 was initially grown on SES-3 medium. Bacterial growth reached its highest yield on this medium in 72 hours. However, SES-3 medium showed significant Cr (VI) reduction activity itself which greatly compromised the data reliability. IC ICP-MS experiment indicated that more than 30% of 10ppm Cr (VI) was reduced to Cr (III) by SES-3 medium in twelve hours. It was speculated that the organic compounds in yeast extract have chromate reduction ability. GS-15 medium was then chosen to grow *Desulfovibrio desulfuricans* ATCC 27774. The bacteria grew slower on GS-15 medium than on SES-3 medium. With the presence of 10 to 20 ml air, bacteria showed a 24 to 48 hour growth delay. It has been reported that *Desulfovibrio desulfuricans* ATCC 27774 can grow at nearly atmospheric oxygen levels (Lobo *et al.*, 2007). The oxygen response phenomena and mechanism was well reported and studied among *Desulfovibrio* species. Attempts to grow *Desulfovibrio desulfuricans* ATCC 27774 on chromate as sole electron acceptor were unsuccessful. This indicates that the *Desulfovibrio desulfuricans* ATCC 27774 cannot use chromate as sole electron acceptor.
for growth. The Cr (VI) reduction activity is a resistant mechanism and does not provide energy for cell growth.

Cell suspensions in bicarbonate buffer showed no Cr (VI) reduction activity. This indicates that *Desulfovibrio desulfuricans* ATCC 27774 does not have the capability to efflux Cr (VI) ion out of its cells. Higher initial Cr (VI) exposure concentration resulted in a longer growth delay. Sodium lactate was not effective electron donor for cells to enzymatically reduce Cr (VI) if this strain possesses such ability. Approximately 40 µM Cr (VI) was reduced when hydrogen gas was provided as electron donor. This suggests that this strain may have enzymatically Cr (VI) reduction ability due to its hydrogenases. It has been found that a number of *Desulfovibrio* species reduce Cr (VI) enzymatically. In *D. vulgaris* Hildenborough, chromate reducing proteins include cytochrome c₃, [Fe] hydrogenase (Lovley and Phillips, 1994; Michel et al. 2001). Through searching *Desulfovibrio desulfuricans* ATCC 27774 genome, five cytochrome c₃ proteins were identified to be similar with the cytochrome c₃ proteins in *Desulfovibrio vulgaris* str. Hildenborough. Four cytochrome c₃ proteins were found to be similar with the cytochrome c₃ proteins in *Desulfovibrio alaskensis* G20. *Desulfovibrio vulgaris* Hildenborough contains at least 17 periplasmic or membrane-bound c-type cytochromes (Matias et al., 2005; Pereira et al., 2007a, b). There are generally two types—monoheme c-type cytochrome and multiheme c-type cytochromes. A monoheme c-type cytochrome, cytochrome c₅₅₃, is present in *Desulfovibrio vulgaris* str. Hildenborough. This cytochrome contains a single heme coordinated by a histidine and a methionine residue. It has a redox potential in the range of 0-50 mV. Cytochrome c₅₅₃ was reported to have no Cr (VI) reduction activity in *Desulfovibrio vulgaris* (Michel et al., 2000). Among
multiheme c-type cytochromes, tetraheme cytochrome c\textsubscript{3} is the predominant cytochrome in SRB (LeGall and Fauque, 1988). Tetraheme cytochrome c\textsubscript{3} is found in all \textit{Desulfovibrio} species (Fauque \textit{et al}, 1991; Matias \textit{et al}., 2005). There are two types of tetraheme cytochrome c\textsubscript{3}. The first class has a molecular mass of 13 kDa and contains four low redox potential hemes (-120 to -400 mV). The second class represents another group of tetraheme cytochrome c\textsubscript{3} with genetic, structural and reactivity characteristics different from the first class. It is believed that tetraheme cytochrome c\textsubscript{3} with low redox potential plays a key role in reducing Cr (VI). Our genome comparison between three bacteria strains found similar gene-coding cytochrome c\textsubscript{3}. However, the experiment indicated that the cytochrome c\textsubscript{3} in \textit{Desulfovibrio desulfuricans} ATCC 27774 was not involved in Cr (VI) reduction process. It suggests that other factors are affecting metal reduction activity. It would also be an interesting experiment to explore cytochrome c family’s role in Cr (VI) response by \textit{Desulfovibrio desulfuricans} ATCC 27774.

In sulfate-reducing bacteria of the \textit{Desulfovibionaceae} family, three structurally distinct groups of hydrogenases have been identified based on the number and composition of their redox centers: [NiFe] hydrogenases which contain two [4Fe-4S] clusters, one [3Fe-4S] cluster and a nickel-iron binuclear site; [NiSeFe] hydrogenases which have a third [4Fe-4S] cluster instead of the mesial [3Fe-4S] center and have selenium coordinated to the nickel-iron binuclear site; [Fe] hydrogenases which contain a di-iron binuclear site. [Fe] hydrogenase and [NiFe] hydrogenase were found Cr (VI) reduction active in a close strain \textit{D.vulgaris} (Lovley and Phillips, 1994). Through protein searches on NCBI, [NiFe] hydrogenases were found in \textit{Desulfovibrio desulfuricans} str. ATCC 27774 genes. A soluble, periplasmic [NiFe] hydrogenase was purified and its three-dimensional structure
was determined by Pedro M. Matias and colleagues (Matias et al., 2000). Our experiments suggest that hydrogenase in *Desulfovibrio desulfuricans* ATCC 27774 reduces Cr (VI).

The cell-free supernatant showed great Cr (VI) reduction activity. The Cr (VI) reducing active compounds is heat stable, which excluded the possibility that the compound is an enzyme or protein. Purification of the active compound was conducted on the SES-3 medium with *Desulfovibrio desulfuricans* ATCC 27774. The active compound is soluble both in organic solvents and water. Through bioinformatics approach, a hybrid NRPS/PKS gene cluster was found in this strain. Predicated secondary metabolites fall into two categories by molecular weight—383 and 765. MS spectra on crude extract indicated the existence of a chemical with molecular weight 339, which positively supported the existence of the predicted secondary metabolites. However, NMR spectra and further purification are required to give a more positive confirmation.

Most chromate reducing activity on SRB are focusing on their enzyme system particularly c-type cytochrome and hydrogenase. Cell-free supernatant’s Cr (VI) reducing activity is rarely reported. This experiment indicates that *Desulfovibrio desulfuricans* ATCC 27774 reduces Cr (VI) mainly by its extracellular secondary metabolite. Secondary metabolite research was previously focused on discovering new drugs. It has been only recently that secondary metabolites in microbes are investigated for bioremediation. Further elucidation of its mechanisms will provide another effective way for bioremediation.

*D.desulfuricans* ATCC 27774 was found in Hanford superfund site in Washington State. Hanford superfund site is now the world largest and most challenging environmental
cleanup effort due to five decades of plutonium production. The mixture and distribution of different contaminants increased the complexity. Nitrogen gas was used for cooling purpose during production process. Nitrate is also a major contaminant in Hanford site. Nitrate generally exists in alkaline and more oxidizing environment which is similar with Cr (VI). *D. desulfuricans* ATCC 27774 is able to use nitrate as electron acceptor as well as reduce Cr (VI). This microbe has great advantage in bioremediation in Hanford superfund site as nitrate and Cr (VI) were often found to coexist.
In vitro, Desulfovibrio desulfuricans ATCC 27774’s growth was inhibited with Cr (VI) presence. Longer inhabitation period was observed with higher Cr (VI) concentration. Cell suspension in sodium bicarbonate buffer (pH 6.8) showed no Cr (VI) reduction activity. With 10 mM sodium lactate as electron donors, very limited Cr (VI) reduction activity was observed in 48 hours. Approximately 40 µM Cr (VI) was reduced when H₂ was provided as electron donor during 48 hours. This suggested that Desulfovibrio desulfuricans ATCC 27774 might possess enzymatically Cr (VI) reduction activity. Hydrogenases have been broadly reported to be Cr (VI) reductase. Hydrogenases were found in this strain by searching NCBI and proved by previous literatures. The cell-free supernatant was the main source of Cr (VI) reduction, however. Through bioinformatics approach, a NRPS/PKS gene cluster was found in this strain. The predicted secondary metabolites generally fall into two categories by molecular weight 383 and 765. Crude extracts from the spent culture medium was purified. Mass spectrometry on the bioactive compounds indicate a dominate chemical with molecular weight of 339. It positively supported the presence of the predicted chemical. However, NMR spectra and previous proteomics data could not give an equally positive support.

In summary, Desulfovibrio desulfuricans ATCC 27774 is able to reduce Cr (VI) by excreting secondary metabolites into the extracellular environment. Enzymatically reduction mechanism exists due to its hydrogenase proteins. The bioinformatics approach and crude extraction purification indicate a chemical with molecular weight 339 or 383. However, further purification and NMR spectra are required to elucidate its chemical structure.
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APPENDIX I--Predicted secondary metabolite amino acid sequences from NPsearcher.com for *Desulfovibrio desulfuricans* ATCC 27774

Ddes_1666
>GC00-1897-MONOMER 4Fe-4S ferredoxin iron-sulfur binding domain-containing protein (complement(2002415..2001909)) Desulfovibrio desulfuricans desulfuricans ATCC 27774
MSKYVVTTHNS AECIGCKACE VQCRLHNGG AGAFFCRIL TVEQSEPRPGL GFVVYTSCHC ENPVCVKACP TGAMRRREDG IVYVEKKFCV GCKACITACP WAAPOQNPDT GKVKCDLCP DRIDQGLLP ACVTTCAMSCL GFTVPSEASQ ERRQHFAEQI QKLRTTPTR

Ddes_1667
>GC00-1898-MONOMER Formate dehydrogenase (complement(2004614..2002500)) Desulfovibrio desulfuricans desulfuricans ATCC 27774
MNGNQLTHSV CGMCSCARPI TVETCNDTVK MLYGNLQSPL KGALCARGVA GKLLEDNER POSPLRQGA RGERKSWRAVS WDEALDHAQ KITEAQNRYG RQTVLWDSRE GPFTDLSRGF MRGLGSPNVSC SHSPSCDLNA HHACKAVLGL GRGMTVYDFA NAKHHIVLQTR NIFEAINGE ARTVMQALRK GCKLTVIDIR QNVTSKADK FHIIRPGTDY AFNLAVINTL ISENLYNKEY VRAHTTGFD LAAFVAPYTA QWAQQECGIE PRAITDLHA LAAAAQQVIW HPGWMTSRYA DSFQVGRALT VITALLGGTG AYKGGIVPGRT PKDCGKSGLK KFVDLYPAVK LPRADGGLGE NKAFFDGKGL LHKAFAISS PPEGVPVVKV YMAWRHDPLQ GFPDPDALKK KLDGLDLLVS TTFSWSTTAW YADVVLPMST YLERESIIAG KNLKPKQFFV RRRAVQPRYD TRADVTEIISG LSRRGLDLS VRDFAAEAVWN FQLEGTLTI EDFDAKGFI SLDTADLYVDQ STYAFPTSGS KVESSSESYG KGFAENAGIS MLPPYISPQ5 PPEGFTFFTIF GRVAVHTQGH TVNNPLLLEQ VPENTVWWT DSARAGLRK GDVRVLDAR GGNMGEAGIK ITAFIHIPA FVVHGFGHD PCESLAVDKG IADNKCLKGG LDLQDQGGGG LSLQEHFVSL EKVG

Ddes_1668
>GC00-1899-MONOMER 4Fe-4S ferredoxin iron-sulfur binding domain-containing protein (complement(2006203..2005802)) Desulfovibrio desulfuricans desulfuricans ATCC 27774
MYMLKNVLRN LSGKPATRLY PLEEREPFPS YRGVITNEVE KCIFCNSCAR VCPTDAILTV AKAGHHYDP FLCVYCSACV EKCPKCLVQ VPTHRKPSVT KFRVLRGTQP RVKKSAAEKG KEESGIEKKA EKE

Ddes_1669
>GC00-1900-MONOMER NADH-ubiquinone oxidoreductase chain 49kDa (complement(2007289..2006207)) Desulfovibrio desulfuricans desulfuricans ATCC 27774
>GCCO-1901-MONOMER NADH dehydrogenase (ubiquinone) 30 kDa subunit
(complement(2007753..2007286)) Desulfovibrio desulfuricans desulfuricans ATCC 27774
MMFEAKDVTP DTLLAEVQRL ANAKCRFVTM SQTVVDENTL RLFYHFDENL TMSDLRHNP E MVWSPTDAK GMVHLRMDV RNMPISS IYFCAVL IEN ETQDQFGVRF AGLPLDYQGG MYLEGTVTHA PYFTMTTVRR PAASA KGA EA KGDQA

>GCCO-1902-MONOMER NADH ubiquinone oxidoreductase 20 kDa subunit
(complement(2008329..2007772)) Desulfovibrio desulfuricans desulfuricans ATCC 27774
MGFVDKMIKR SLRKS PVWV HFDCGSCNGCD IEVLACLTPM YDVERF GV VM AGNPKHADVL LT VTVNRH RNHVLKQIEY MPSPKAVISL GACNLSGVF KDTYNVNLGA HNVI P VVFV PGCPPKPEAI IDGVVEALGV LKAKMGMPGV PQT FMPGDE DGTPAGARDE EAPASEGDKSLQNAG

>GCCO-1903-MONOMER respiratory-chain NADH dehydrogenase subunit 1
(complement(2009193..2008342)) Desulfovibrio desulfuricans desulfuricans ATCC 27774
MLSILSAVGG LILSPLVGG LTGVDRRLTA RLQSRLGPPL LPQFYDVFKL FGKEASVTA WLVSAYMTL ISSALALLIF F MGDLLLLE FVLT VGA F QV VGALCVPS YSNVL QGREL LT IKLRKSPF DISASHHGHQ ELVKGVQTEY SGYPYLIEIL AHWDLVII L GLC AMF WHTS VIGMATLVAA SLFTEILDN ITARLTTWQM VQQSKSLLLG G LALVNLWL YVA

>GCCO-1904-MONOMER NADH dehydrogenase, NADH dehydrogenase
(complement(2011148..2009193)) Desulfovibrio desulfuricans desulfuricans ATCC 27774
MNTLVFCCVA LPFAVALVY FTQDL DTRKL LVPAAVAVMA LAAVIMGAHG TFRLEAETFM GLPLDLSFLS LD LLLLYIL GLGWKLGSR T VMGMTVLQI GLYY LKVFLA DG SAPI AF PDGLSLIMV I ITVVGGLIT IYGLGYMDIH EEHLHLR VSR KPRFFAIIFC FLGAMNGLVL SNNLSW MFF WEVTTL CSYL
LISHDQTQEA NANAYRALWM NVLGGLAFVS AMLFIQKSLG TLSTEVLHK
MTAMDVKTTA MLLPFAAFFCL AFTKSAQVP FESWLCGAMV APTPVSELLH
SATMVKAGTY LLLRMAPAFA DTTMSTIVAL FGAFTFVGTC ILAVSQSNAK
KILAYSTIAN LGLIIACVGI NTAAASMAAT TIIYHSVK GLLFMCVGAI
EQRIGSRDIE DMRGLYSKMP RTAIITAIGI FTMLPPFGM LGKWMIAEA
IARATQAMTP IFFIALFVWARA GILVSSANL DRPPHGNPKA
TVMFALRSLC GLAVVFSFIS PLVLETFVEP SVAGVYARLG LKTEGFIPGA
SLTGGAGYFW IYLLFILLGL GAWIAWKAAR KVSNSAHAQP YFSGLTQEQA
GQIGFKGPMN AEFPVRLSNF YLTQYFGE GT ITRAIDIIST AFLIVLVGGL L

Ddes_1674
> GCCO-1905-MONOMER putative diguanylate cyclase 2011822..2013303
Desulfovibrio desulfuricans desulfuricans ATCC 27774
MYRSQQQTKK IYIAAFLVSG IFLVCSSLILL RNATWQIEQT SILHLYETTS
QLRVLQLQRLK TQNFQALRGL STAVVVYQPPQ ETSALFKELN SNRSFIRIGM
ADTSGMAAMM DRSGPSPQHT DFSDEDFRNN ALAGRAFFSS PRKDPHPGPQ
VIYCAVPVEQ EGGVQGLVVG VIRIENLLDI LDEPLFNANG FAGIIDANGH
FVLCSGTNST GTAASIFTLQ QIDREDLDAV RADLGNRRRN YFLYKTQDKQA
HLAGAFPITH NGWFLFCSAP LNDLALVPGR LLGGGTYVII LALVFLLLA
WRTVHCQNAKD DRQLQTMALT TLLTDAVNH SFRLEGTALL HNNPDITFIAI
WLDIKNFNF YSKMLKEAG NSELRRIAIRM LGKWPQGPLT RHTHIVGNTF
AGILFANQ DLAERFDSDAA QVEEHGAYRF SQVFPLRLYA GYVTNDMVEE
KDLTFMDMLN RAGIAMQVAK TFEAISTRFY SEEICEEALHL D

Ddes_1675
> GCCO-1906-MONOMER GCN5-ike N-acetyltransferase 2013534..2014082
Desulfovibrio desulfuricans desulfuricans ATCC 27774
MPIAIPRSGA DDRPIVPDVH ISDLKSLVIR PATVADVHGM SALINQYASA
NVMLARGPQY LYQHIQDYMV ATAPAVNDGH DVIVACGAVH VLWADLGEIR
SVAHPSCQG QFNGKRLVSQ LVNCRSRAL PRVFVTLVDP DFFARCIRGTE
FNKDDMPPSV WVECSKCPKF YCCEIAMMLHL

Ddes_1676
> GCCO-1907-MONOMER ornithine carbamoyltransferase 2014197..2015216
Desulfovibrio desulfuricans desulfuricans ATCC 27774
MNRLQHRDFL KEIDFTPEDL TYLLDLAANL QAKKSRREP EFLKGRNIVI
LFKEKDSSTR CSFVEVAAYD GARVTLYGPS GQSMGKESL ADTARLVSRF
YDGIEYRGFG QERVEALAE ASVPVWNGLT NEWHPTQFLA DMLTMRECCS
KPLNRQTLAY MGDARYNMGN SLMIGSALLG LDFRSVPاكA LWTADEVYEM
ACHIASGTTA RISRTENVV GQVGCFDST DWVSMGEPD DVWKERIELL
TPYRVTGDTM RQTNDDCKF LHCLPSFHRN DTMGEEIFQ RFGIECMENV
DEVFESPDRV AFEEAENRLH TIKAIVMATL AESPLVFN

Ddes_1677
> GCCO-1909-MONOMER potassium-transporting ATPase A 2016004..2017755
Desulfovibrio desulfuricans desulfuricans ATCC 27774
MENIWIQCLL YLSLTLALAW PLGKYIGKVM DGEFPWLQRA LACPERALYR LMGVDPAEQM GWKCYMACVI AFSAVSLVAL TALLMAQHLL PNPRNVSQA SWHLALNTAI SFVTNTNWQS YAGESTMSYL SQMAGLTVQN FVSAAVGIAV LFALIRGVRA SGGIGLGNFW ADATRAVLYI LLPLSLVMSL LLEIQGPVQF TFDYRAAALL EPLAEDGAP ITHQLVPMGP QASQVSPQKL GTNGGGFNGV NSAHIPHENT PASNMLELAD LLLIPAAALCFTFGAKIGDMR QGVAIFAAMF ILLTSAVSFT VQAELNATPQ IAQQGQVQVA PQSGLQGGAG GNMAGKEITRF GIAGSALWAS ATAAVSNGSV NAMHDSFTPL GGMIPVMLMQ LGEVIFGGVG SGLYGMLAFV LTTLFGALML VGRTPYELGK KIEPFEMKMA VVVCALTTVV ILAGGGMLCL APQIVDSLNN PLPHGFSEIL YAATSAGANN GSAFAGLNAN TPFLLNVLGLV LMLAGRFAPI AAILAMADGL AGKICPPGA GTLSTCNGLF IFLLIFVILL VAGALSFPAL ALGPLAEHLQ MTR

Ddes_1678
>GCOCO-1910-MONOMER K+-transporting ATPase subunit B 2017809..2019887 Desulfovibrio desulfovibicans desulfovibicans ATCC 27774 MSAKTSNPAN TNCILWQALA ASLVKLSPRD QVRNFVMFTV YLSAILATGL TVLGAANILS PATAQTGFAA IAAILWFTV FFANFAESIA EGRKGAQAES LKRARRNWTA RKLADRTA EHEIPSTTL KPGDSLYVA EEQPADGDY TEAGAAEVRK AITGESAPVI RESGGRDCAV TGGTTVLSDW LVIRTSEVQ RSFLDRIAM VEGAARQKTP SEVALNLLV ALTVIFILVT VSLWCARFT AGQEHVANPV GFAALVALFV CLAPTTILGAL LSAIAGIAAMS RLNRAVNLAM SGRIAEAAADVD VDVLDDKTTTLTTTGLRQAV EFPVGDHGSG EELADAAQLA SLADETPETG SRIVLARDQF TRCMDSLHGK DAVFIFPSAQ TRMSGVDAVG KHIRKGAADA VRAFVEKNNQ SESLACEEAV NAIARQGGTP LVVARESSIL GVIHLKDIKD GQVGERFAEL RRMTGIRTVMV TGGNPLTAAA IAAMAGVDVDY LAGATPETKL SLIREFQARG HLAVMTGDTNG NDAPALAQAD VAVAMNVTQ AAKEAGMVLD LDSSTKLLD IVRGKQQLM TRGSLTTFSL ANDAAKFYAI IPALFVSLYP GLAALNIMNL HSPQAVLSA TIYNAIIVA LIPLALRGVK YREESSQQLL RRNLVGYGLG GLAAPFAAIK LIMCLTGLD LV

Ddes_1679
>GCOCO-1911-MONOMER Potassium-transporting ATPase 2019897..2020553 Desulfovibrio desulfovibicans desulfovibicans ATCC 27774 MLITLRLRS ALFALMTAV VIAYTLVTA AGKALFPFQA GGSIIKAGGK EYSSLLLGQPF SEPHHLWGGR MQPDTTYSK NGKPLLYGGP SNSSPATTNY GTRALERDV SRRAPHAEKAP APVVELYVT SGSGLDPHIS PAAADFOVER LARATGFTPD EVRTIATMYT DGRSLGLLGE PRVHLKVNL ALDGPLLPGH TTSKTATVPP GSTAPHTR

Ddes_1680
>GCOCO-1912-MONOMER osmosensitive K+ channel signal transduction histidine kinase 2020588..2023473 Desulfovibrio desulfovibicans desulfovibicans ATCC 27774 MTDKHTRPDP DALLAQLRQT GTPQGIQADE LSGGHPVRG TLKIFGGYAA GVGKTYAML ARAAHTAEGGQ DIVVGVYEPH PRPEAALLQ GLESLAPRLR EYRGIPLHEL DVDAVLDRKP EIALVDELAY SNAIGCRNRK RYQDVEELLQ
GGISVWTTVN VQHLESNDV VAAMTGVAVR ERVPDSVFDP ADHVHLDLDE
PDELMLRE GKIYQAQAQAQ HALGHFFLPAL NLIALRETAL RRMDRINRR
AIPAEGPSRE AARQVKEHIL ICLSGAPNAAA RVVTAARMA EAFHADFTAL
FVRNNTSGLN DTQSTGTRLK NTRLAEQLGA AIVTAQGEDI PVQIAEYARM
SGVSKIVIGR SPAGWLRRG KTLVERLAEL APEMETYIIP DAEPISRNAS
RPGTLYRMVR AFRGTPRPSW RQWGATAALL AICTMTGLIM SSMGMPNAVI
TGLYMLGVGLG VSILTGTGPWY GVTASILGVA LFDFLAVRAPR FSLTVYDDGY
LSLFAAMEML SAAASAITGR ARSRQARQSAARALHLTELLLG NSRRLQKAEN
ENAILAEAR QFGTLHCDA VLYPVPYNGHR ETARRIFPYT AAQASREPGR
PHTLPRQLAK GQTECTHRTE KDELAVAQQVW AKNGRPAAGAG TDSLPGARLC
YPISSRTTV LAVAGLDVSP GSASPLNAAD SKNIVLALAECEMAVEKER
LARANADIAV RAQQEKLRAD VLRSVSHDLR TPLTAICGNA AMLAAGGDDIE
MQRRKALARA IEDARYLVE MVENLLALTR LEQYGFTLRL APELMEIIR
EALAVTRRRRA AAHNIRVDMA DALLMARMDA LRMVQLVVLNL LDNAVKYTPA
GTTRISAQA DGARARLEVA DNGPISAEKDTRFDMHSA SVMIKGDSSR
GMEVGLALCR SIVQAHGGDL RVNDNATRGA VFSLDLQREI EEGVVPESLR
ETPPTGTGARE
APPENDIX II

Media, Buffer, Reagent

GS-15 medium (per liter):

980 mL distilled water (dH₂O)

1.5 g (NH₄) Cl

0.6 g NaH₂PO₄

0.1KCl

2.5 g NaHCO₃

0.85 g sodium nitrate (electron acceptor)

2.72 ml 60% sodium lactate solution (electron donor)

2 ml 500x trace elements

2 ml 500x vitamin mix

Adjust pH to 6.8 with HCl

SES-3 nitrate medium (per liter):

980 ml dH₂O

0.23 g K₂HPO₄ (dibasic)

0.23 g KH₂PO₄ (monobasic)

0.46 g NaCl

0.23 g (NH₄)₂SO₄

0.12 g MgSO₄& 7H₂O

10 g yeast extract

2.8 ml sodium lactate (60% Solution)
4.2 g NaHCO₃
2 ml 500x trace elements
2 ml 500x vitamin mix
1.7 g NaNO₃
Adjust pH to 7.2 with HCl

**Vitamin mix (500x):**
980 mL of dH₂O
0.01 g Biotin
0.01 g Folic Acid
0.05 g Pyridotine Hydrochloride
0.025 g Riboflavin,
0.025 g Thiamine
0.025 g Nicotinic Acid
0.025 g Pantothenic Acid
0.025 g P-Aminobenzoic Acid
0.025 g Thioctic Acid
0.0005 g Vitamin B-12

**Trace elements (500x; pH 6.5):**
980 mL of dH₂O
7.55 g Nitriloacetic Acid (NTA)
15 g Magnesium sulfate
2.55 g Manganese Chloride

5 g Sodium Chloride

0.5 g Ferric Sulfate

0.5 g Calcium Chloride

0.5 g Cobalt Chloride

0.5 g Zinc Sulfate

0.05 g Cupric Sulfate

0.05 g Aluminum Potassium Sulfate

0.05 g Boric Acid

0.125 g Sodium Molybdate

0.12 g Nickel Chloride

0.125 g Sodium Tungstate

Bicarbonate buffer (per liter):

2.5 g sodium bicarbonate,

Adjust to pH 6.8 with HCl

2.5% Diphenylcarbazide:

0.025 g s-diphenylcarbazide

9.67 ml Acetone

330 µl 3M Sulfuric Acid (Urone, 1955)