MICROBIAL Cr(VI) REDUCTION: ROLE OF ELECTRON DONORS, ACCEPTORS, AND MECHANISMS, WITH SPECIAL EMPHASIS ON *CLOSTRIDIUM* spp.

By

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For my parents, whose support and understanding has helped culminate my dream into a reality.

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LIST OF ABBREVIATIONS AND ACRONYMS

16S ..... 16 Svedburg unit

AQDS ..... Anthraquinone di-sulfonic acid

ATP ..... adenosine triphosphate

BM ..... Basal media

Bp ..... base pair

CRB ..... chromium reducing bacteria

FRB ..... Fe(III) reducing bacteria

G ..... Glucose

GAF ..... Glucose +AQDS +Fe(III)

GC ..... Glucose+Cr(VI)

GCA ..... Glucose +Cr(VI) +AQDS

GCAF ..... Glucose+ Cr(VI)+AQDS +Fe(III)

GCF ..... Glucose +Cr(VI) +Fe(III)

PCR ..... Polymerase chain reaction

rDNA ..... ribosomal Deoxyribonucleic acid

RNA ..... Ribonucleic acid

X-gal 5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside

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Cr(VI) has been designated as a priority pollutant by the US Environmental Protection Agency (USEPA) due to its ability to cause mutations and cancer in humans. The risk associated with soil and groundwater contamination of chromium waste generated by many industries is high, and therefore Cr(VI) remediation is of critical importance. Using chemical and biological methods conjointly can decrease the cost of remediating contaminated sites. Microbial reduction of Cr(VI), an important aspect of biological remediation, requires the knowledge of microorganisms capable of reducing Cr(VI) and the mechanisms involved in the reduction processes.

The overall objective of this study was to investigate the effect of various electron donors and acceptors on chromate reduction by indigenous Cr(VI)-reducing bacteria isolated from Cr(VI) contaminated sites and to understand the mechanism of Cr(VI) reduction by enriched bacterial consortium and the pure isolate. A series of bacterial enrichment cultures were established with a range of electron donors such as acetate,

benzoate, lactate, citrate, and glucose, and electron acceptors such as Fe(III) and an humic acid analog, anthraquinone di-sulfonate (AQDS), to study their effects on the rates of Cr(VI) reduction. Results from this study demonstrated that the rates of Cr(VI) reduction in glucose and citrate enrichments were higher when compared with those of other electron donors. Enrichments amended with AQDS and Fe(III) showed enhanced rates of Cr(VI) reduction. Glucose- AQDS-Fe(III)-Cr(VI) enrichments (now on referred as GCAF) yielded the highest diversity of strains, which were distributed within the low G+C and high G+C groups of gram-positive bacteria. Phylogenetic analysis based on 16S rDNA studies revealed that isolates clustered with *Bacillus*, *Cellulomonas,* and *Clostridium* groups. Several strains were isolated from the consortium. Detailed kinetic studies with bacterial consortium and the pure strain GCAF-1 obtained from GCAF enrichment demonstrated an iron-promoted reduction of chromate. The presence of AQDS accelerated reduction of Cr(VI) only when Fe(III) was present in the medium. Analysis of fermentation metabolites produced by strain *Clostridium* sp. GCAF-1 revealed that the presence of Cr(VI) alters the acetate: butyrate and acetate: lactate ratios. Based on the overall results, direct and indirect (Fe (III) mediated) methods of reduction of Cr(VI) by *Clostridium* sp. GCAF-1 are proposed.

CHAPTER 1
GENERAL INTRODUCTION

Chromium, the 24th element on the periodic table, was first discovered in Siberian red lead ore (crocoite) in 1798 by the French chemist Nicholas-Louis Vauquelin. He named this new mineral *chrom* from the Greek word *χρωμα*, owing to the brilliant hues of the compound. Since then, chromium has found a variety of uses in the industries that exploit these colors and other characteristics such as its strength, hardness, corrosion resistance, and the oxidizing capabilities of certain chromium species (34).

Chromium in Environment

Chromium is found in many environments, including air, water, soil and all biota. It ranks 21st among the elements in crustal abundance (74). The average concentration of chromium in the continental crust has been reported as 125 mg/kg (108). Concentrations in freshwater generally range from 0.1 to 6.0 µg/L with an average of 1.0 µg/L, while values for seawater average 0.3 µg/L and range from 0.2 to 50 µg/L (23). Freshwater chromium concentrations are dependent on soil chromium levels in the surrounding watershed areas. In addition, drainage water from irrigated agricultural areas with elevated amounts of soil chromium levels can have high chromium concentrations (as high as 800 µg/L), as observed at various locations within San Joaquin Valley, CA (38, 50).

Chromium is extracted from chromite ore [$(Fe,Mg)O(Cr, Al, Fe)_2O_3$] that has largest deposits in South Africa, the Philippines, Southern Zimbabwe, and Turkey (100). The major users of chromium are the metallurgical, chemical, and refractory brick

industries (78). Other industries that employ chromium include pigment manufacture, metal finishing, corrosion inhibition, organic synthesis, leather tanning, and wood preservation (57, 34, 174). Extensive industrial usage of chromium leads to generation of large volumes of chromium-containing wastes that are discharged into the environment. In addition to this waste, leakage due to improper handling and faulty storage containers also adds to the accumulation of chromium in the environment.

Nutrition and Toxicity: Risks to Human Health

Chromium is designated by the U.S. Environmental Protection Agency (USEPA) as a priority pollutant due to its ability to cause genetic mutations and cancer. Chromium is unique among regulated toxic elements in the environment because different species of chromium, specifically Cr(III) and Cr(VI), are regulated in different ways. Relying on the chemical, toxicological, and epidemiological evidence, regulation of Cr(VI) concentration is different from that of Cr(III). Trivalent chromium is the nutritionally useful form, while the hexavalent form is toxic and mutagenic. Cr(VI) is both a powerful epithelial irritant and confirmed human carcinogen (77, 120). On the contrary, Cr(III) is an essential element in animal physiology and plays a role in glucose and lipid metabolism (5, 103).

Animals

Cr (VI) is highly mobile in some soils, and contact with Cr(VI) maybe inevitable for aquatic and terrestrial organisms, including humans. In trace amounts, chromium is an essential component of human and animal nutrition (65, 102). It is associated with glucose metabolism (102) and has been shown to be an integral component of glucose tolerance factor (GTF), a factor required for maintaining normal glucose tolerance. Chromium functions by regulating and potentiating insulin action by increasing insulin

binding to cell (4). Chromium is also known to be of importance in fat metabolism in animals (5).

The biotoxicity of chromate is largely a function of its ability to cross biological membranes and its powerful oxidizing capabilities (NAS 1974). Humans can absorb Cr(VI) compounds through inhalation, dermal contact and ingestion. Human health effects of Cr(VI) include lung cancer, respiratory irritation, dermatitis, kidney and liver damage, and damage to various proteins and nucleic acids, leading to mutation and carcinogenesis (18).

Plants and Algae

Pratt (123) reported that low concentrations of chromium stimulated the growth of plants. However, a few years later it was demonstrated conclusively that chromium is not an essential component in plant nutrition (61). The effect of Cr(VI) was apparent on seed germination when more than 80% of the reduction in seed germination was observed in the presence of Cr(VI) when compared with those germinated in the absence of Cr(VI) (133). Cr(VI) concentrations of 5 to 60 mg/kg soil have been shown to retard plant growth due to root damage (7).

Cr(VI) has been shown to affect the growth, photosynthesis, morphology, and enzyme activities in algae. Cr(VI) concentrations shown to be toxic to algae vary from 20 ppb to 10,000 ppb (7, 131, 135,143, 154).

Microorganisms

Hexavalent chromium is toxic and mutagenic to most bacteria. Among the visible effects reported in bacteria are cell elongation, cell enlargement, and inhibited cell division, which eventually leads to cell growth inhibition (31, 153). Changes in morphologies of gram-positive and gram-negative bacteria were also observed by

Bondarenko et al (21). Few colonies of bacterial species such as *Staphylococcus aureus*, *S. epidermidis*, *Bacillus cereus,* and *Bacillus subtilis* were formed with degenerate cells that were reduced in size (20). Cr(VI) concentrations of 10-12 ppm were inhibitory to most soil bacteria in liquid media and, in general, gram-negative bacteria were more sensitive to Cr(VI) than were gram-positive bacteria (132). Increased content of Cr(VI) in soil was toxic to saprophytic and nitrifying bacteria. Lowered microbial biomass in soil was observed in the presence of high Cr(VI) in soil when it was determined using adenosine triphosphate (ATP) method (2, 178). Other bacteria such as *E coli*, *Serratia marcescens* and *Enterobacter aerogenes* were unable to grow in Cr(VI) concentrations of 1 mM.(8). .Metabolic effects of Cr(VI) on bacteria were evident by the observed changes in electron transport systems (124).

Cr(VI) has been shown to cause mutagenic effects in *Escherichia coli*, *Bacillus subtilis*, and *Salmonella typhimurium* (113, 120, 162). The mutagenic effects of chromium are effective only when chromium crosses the cell membrane. Cr(VI) can easily diffuse across the cell membranes, unlike Cr(III) which can do so only under extreme conditions such as long incubations and high concentrations. Cell culture studies have shown that cellular uptake of chromate is at least 10 times greater than that of Cr(III) from equimolar solutions, (33, 55, 80). However, once inside the cell, most of Cr(VI) is reduced to Cr(III) by several reducing agents such as ascorbic acid, sodium sulfite, glutathione, NADPH and NADH (121). Based on several studies, it was concluded that trivalent chromium causes DNA-strand breaks (18, 19, 37, 80, 155, 156, 157). Cr(VI) causes genotoxic effects on bacterial cells, including frameshift mutations and base pair substitutions (120). DeFlora et al. (37) reported a more general effect of

unbalanced nucleotide pools. These studies suggest that although Cr(III) form is the major agent responsible for molecular events leading to mutagenicity, it is Cr(VI) that poses the greater risk to human life due to its ability to easily enter the cell.

Environmental Chemistry

Chromium can exist in oxidation states ranging from 0 to 6^{+}. The various chemical and biological changes that chromium undergoes in the environment depend on the conditions that govern its speciation and other activities. The solubility and adsorption by soil and sediments depend on the form of chromium species. Within the ranges of redox potentials and pH commonly found in soils, chromium exists predominantly as oxyanions of Cr(III) and Cr(VI).

Cr(VI) is a strong oxidizer and exists only in oxygenated species that are very soluble and pH dependant according to the following equilibria (111).

$H_2CrO_4 \Leftrightarrow H^+ + HCrO_4^-$ $\qquad K_{a1} = 10^{0.6}$

$HCrO_4^- \Leftrightarrow H^+ + CrO_4^{2-}$ $\qquad K_{a2} = 10^{-5.9}$

$H_2CrO_4^-$ is a strong oxidizing agent and dominant species below pH -0.6 (32). Monohydrogen chromate, $HCrO_4^-$, exists between the pH values of 1 and 6. CrO_4^{2-} predominates at or above pH 6. $Cr_2O_7^{-2}$ dichromate ion is formed by dimerization of $HCrO^-_4$ ion at Cr(VI) concentrations above 10^{-2} M (17, 86)

$Cr_2O_7^{2-} + H_2O \Leftrightarrow 2HCrO_4$ $\qquad K=10^{-2.2}$

Existence of dichromate ion is unlikely in the biologic systems as typical chromium concentrations in nature are considerably lower than 10^{-2} M, especially at physiological pH 7. Trivalent chromium is the more stable form. Due to its lower affinity for oxide and hydroxide ions, Cr(III) is known to form numerous complexes with both organic and inorganic ligands (99, 144). Due to chemical inertness, complex species of Cr(III) tend to

be more stable in solution and can be isolated. The main aqueous Cr(III) species include Cr^{3+}, $Cr(OH)^{2+}$, $Cr(OH)_3$, and $Cr(OH)^{4-}$(16), (14, 40). The Cr^{3+} species predominate at pH <3.6 (46), whereas $Cr(OH)^{4-}$ predominates at the pH >11.5 (126). At a slightly acidic to alkaline pH, ionic Cr(III) species precipitates as amorphous $Cr(OH)_3$ (126) or as a solid solution (Fe, Cr)$(OH)_3$ if Fe^{3+} is present (46) Cr(III) can also be chelated by organic molecules that are adsorbed to mineral surfaces (63, 64). In contrast, Cr(VI) compounds CrO_4^{2-}, $HCrO_4^-$, $Cr_2O_7^{2-}$ are very mobile in surface sediments because they are not strongly adsorbed to soils.

Both oxidation and reduction of Cr(VI) can occur in geologic and aquatic environments (Figure 1-1).The oxidation and reduction of chromium in soils depends on soil structure and on the redox conditions of the soil (73). Studies conducted to investigate the effect of adsorption of chromate and Cr(VI) on the clay sand mixture showed that clay was a suitable absorbent for chromate due to its high cation exchange capacity (CEC) and strong binding capability.(2). Chromium speciation in groundwater is affected by the pE (redox) and pH conditions (Figure 1-2).

Reduction of Cr(VI)

Reduction of Cr(VI) in soils depends largely on the presence of other electron acceptors such as the oxygen, nitrate, iron, and manganese that can act as electron sinks and accept electrons from the reactive organic and inorganic electron sources. Conditions will favor Cr(VI) reduction when electron donors are in excess and electron acceptors such as those mentioned above are low. Hexavalent chromium is a oxidizing agent and is readily reduced in the presence of appropriate electron donors, as shown in this equation:

$$HCrO_4^- + 7H^+ + 3e^- \Leftrightarrow Cr^{3+} + 4H_2O$$

Oxidation of Cr(III) in Soils

Cr(III) is chemically more stable than Cr(VI). Initial studies convincingly showed that, in most cases, oxidation of Cr(III) does not occur in soils, regardless of the conditions (16). This was explained with chemical inertness of the Cr(III) and its complexes in the range of pH that normally exists in soils. However, it has since been determined that some Cr(III) can be oxidized to the hexavalent form in the presence of Mn(IV). The amount of Cr(III) oxidized to Cr(VI) was shown to be proportional to the amount of Mn(IV) reduced to Mn(II) (3, 15). Oxidation of chromium occurs in soils that are high in Mn(IV) and oxides and low in organic matter content (73). The conditions required for chromium oxidation are fairly specific and only a few cases of oxidation of Cr(III) oxidation are reported in literature.

Oxidation of aqueous Cr (III) to Cr(VI) in soils does not occur over such a wide range as the reduction of aqueous Cr(VI). Also, Cr^{3+} precipitates almost completely as $Cr(OH)_3$ often in conjunction with iron at pH values from 5.5 and 12.0 (40, 126).

$$Cr^{3+} + 3H_2O \Leftrightarrow Cr(OH)_3\,(s) + 3H^+ \qquad Keq = 10^{-12}$$

These factors are of great importance in assessing potential environmental hazards and remediation strategies for ecosystems with high levels of natural or anthropogenic chromium.

Cr(VI) Remediation Strategies

Remediation strategies are employed in order to minimize the risk of public exposure to chromium contaminated sites. Several common remediation strategies include the no action option, excavation and removal of contaminated soil, pump and treat strategies, and soil solidification and stabilization. In order to implement the optimal remediation strategy, an understanding of physical and chemical processes affecting the

migration and chemical state of chromium is required. The no action option is adopted if the risk of exposure and potential impact to the environment is marginal. Knowledge of the type of soil reductants present is important for the implementation of this option. Excavation is no longer a very desirable method as it simply moves the contaminated soil from one place to another. Pump and treat is one of the most commonly used methods for aquifer remediation. The two main purposes are to remove contaminants from the subsurface for treatment and to maintain gradient control to prevent contaminants from migrating beyond compliance boundaries. Among the major concerns of employing this method is the residual concentration. The residual concentration is usually much higher than the maximum contaminant level (MCL) level set by EPA. Soil solidification process includes solidification of the contaminated soils by transforming Cr(VI) into an insoluble chemical form that is impermeable to the ground water. Traditional techniques for remediating chromate contaminated water also involve reduction of Cr(VI) to Cr(III) by chemical means (usually with Fe^{2+}) or electrochemical means at pH 5, followed by precipitation and filtration or sedimentation (41). The electrochemical Cr(VI) reduction process uses consumable iron electrodes and electrical current to generate ferrous ions that react with Cr(VI) to Cr(III) is given below. Increased quantity of resultant sludge by this method is one of the drawbacks. This method is often employed in combination with the pump and treat methods.

$$3Fe^{2+} + CrO_4^{2-} + 4H_2O \rightarrow 3Fe^{3+} + Cr^{3+} + 8OH^-$$

These processes can be extremely reagent or energy intensive. Most of these methods take long periods of time to reach the regulatory level for remediating contaminated sites. The cost involved in these chemical enhanced remediation strategies

is very high and this lowers the overall cost-benefit ratio. The discovery of microorganisms that can reduce metals has led to applications in the bioremediation which are potentially more cost effective than traditional methods. One of the major factors that decide the application of the bioremediation strategies is the bioavailability of the preferred electron donor by the indigenous microorganisms that are involved in metal reduction. For bioremediation of Cr(VI), stimulation of the existing microbial populations with bioavailable electron donors may result in increased metal reduction, thereby remediating the contaminated site. Although reduction of Cr(VI) to Cr(III) does not remove chromium from soils, it does limit the mobility and toxicity of chromium in the contaminated soils. Many potential remediation pathways are known for the chromate reduction, but the dominance of one pathway over another has not been established. Furthermore, coupled geochemical and microbiological processes have a potential to dominate the reduction of metals such as Cr(VI).

Finally it must be recognized that there are many factors that effect the microbial reduction of Cr(VI) in soils. Clearly there is a need to understand the various groups of bacteria that reduce hexavalent chromium and the different mechanisms by which Cr(VI) is microbially reduced in soils.

Chromium Resistance in Bacteria

The persistent nature of some metals in environment has led to considerable modifications of the microbial community and their activities. Heavy metals have been shown to inhibit microbial growth and other enzymatic activities by blocking essential functional groups, displacing essential metal ions and modifying the conformations of the biological molecules, (49, 81, 171). In metal-contaminated environments, the responses of the microbial communities depend on the concentrations of the toxic agents they are

exposed to among other factors such as nature of nutrients, chemical form of the toxic agent and so on. The resistance mechanisms proposed for heavy metal resistance in bacteria include exclusion by permeability barrier, exclusion by active transport, intracellular physical sequestration by the binding proteins of the cell, extracellular sequestration and detoxification by chemical modification of the toxic to non-toxic form of the metal.

Microorganisms may adopt several strategies to reduce metal sensitivity to cellular targets: (i) mutations to decrease the sensitivity to the metal (ii) increased production of damaged cell component, (iii) increased efficiency of repair of damaged cell component, (iv) utilization of plasmid-encoded resistance mechanism. These mechanisms may either occur singly or in various combinations. Persistence of metal in environments selects for the resistant strains possessing either the resistant or the reduction capability. Organisms isolated from sediments of Cr(VI)-contaminated metal-processing waste evaporation ponds were found to be more Cr-tolerant compared with those found outside the ponds (85). Plasmid-associated bacterial resistance has been reported in *Streptococcus lactis* (42), *Pseudomonas* sp (148), and *Alcaligenes eutrophus* (27, 112, 119). Studies with *Pseudomonas fluorsencens* LB300 showed the loss of Cr(VI) resistance resulted with the loss of plasmid and transformation of the plasmidless strain done with the purified plasmid DNA resulted in regaining of the Cr(VI) resistant ability of the strain (21).

Increased polysaccharide production has been reported in *Pseudomonas* sp. (1). Further studies with *Pseudomonas ambigua* and its Cr(VI) sensitive mutant S-1 led to the conclusion that the presence of thick membranes around the parent cell decreased the

permeability of Cr(VI) of the cells and increased the resistance of the bacteria (59, 60). *Enterobacter cloacae* strain HO1 and yeast exhibited Cr(VI) resistance by decreased uptake of Cr(VI) (12, 116, 163).

Pathways for Chromium(VI) Reduction

Microorganisms obtain their energy for metabolism by participating in several oxidation-reduction reactions. In environments where the photosynthesis does not occur the transfer of electrons is the driving force that governs all the microbial processes. Depending on the environment the microorganisms have adapted and evolved the ability to be able to mediate various oxidation-reduction couples to conserve energy. Some Cr(VI) resistant bacteria are able to grow by reducing Cr(VI) to Cr(III). Cr(VI) reduction is considered to be a fortuitous reduction process that is employed by some bacteria as a mechanism of defense by detoxification of the environment they have to survive in. Most Cr(VI) reducing bacteria (CRB) reported so far are gram negative bacteria (12,45). Recently the ability to use Cr(VI) as terminal electron acceptor was demonstrated in a sulfate reducing bacterial consortium and *Pantoea agglumerans* (45, 152).

Currently microbial reduction of Cr(VI) can be explained by two prevalent models: (i) direct enzymatic reduction, and (ii) indirect reduction. Distinguishing between these enzymatic and nonenzymatic Cr(VI) reductions is difficult. The direct enzymatic reduction refers to the reduction by the metal reductase system. Indirect mechanism refers to Cr(VI) reduction mainly by conditions provided by bacterial source such as the redox potential, or the bacterial metabolites.

Direct Enzymatic Reduction of Cr(VI)

Although CRB have been studied for many years now, little is known about the biochemistry and mechanism of Cr(VI) reduction. It still remains unclear if Cr(VI) is

taken up by the cell and reduced in the cytoplasm or the periplasm or the electron are transferred to the outside of the cells or both. Direct contact between cells and the metal oxide has shown to be required for the energy conservation process (8). Enzymatic reduction of Cr(VI) has been observed in some CRB (10, 29, 52, 68, 114, 149, 173).The CRB are able to reduce Cr(VI) by either soluble enzyme systems or the membrane-bound system. Membrane-associated chromate reductase activity was first observed in *Enterobacter cloacae* HO1 where the insoluble form of reduced chromate precipitates was seen on the cell surface (164). In the presence of ascorbate reduced phenazine methosulfate (PMS) as electron donor, high chromate reduction was shown by right-side-out membrane vesicles of *E. cloacae* HO1 (164). Membrane-associated constitutive enzyme that mediated the transfer of electrons from NADH to chromate was later elucidated by Bopp et al. (21). In case of *Shewanella putrefaciens* MR-1 chromate reductase activity was associated with the cytoplasmic membrane of anaerobically grown cells (106). Formate and NADH served as electron donors for the reductase. No activity was observed when NADPH or L-lactate were provided as the electron donors. However, in *Pseudomonas putida,* unlike in *Shewanella putrefaciens*, NADPH served as an electron donor for this (117).

Studies conducted by Shen and Wang (141) on *E. coli* suggested the presence of soluble chromate reductase. Cr(VI) reduction in another gram negative bacteria, *Pseudomonas* sp CRB5, was found to be mediated by a soluble enzyme contained in cytoplasm (101). In addition to gram-negative bacteria, soluble chromate reductases have also been observed in gram-positive strains. NADH was the preferred electron donor for the reduction of chromate by the soluble enzyme in *Bacillus coagulans* (122).

Bacterially Mediated Indirect Reduction of Cr(VI)

Redox potential-pH. Changes in pH and redox conditions are known to occur in medium during growth of bacterial cultures due to various biochemical reactions and the metabolites formed. These changes may indirectly affect the reduction of Cr(VI) in the medium. Lower redox and pH has been shown to favor reduction of Cr(VI)(36).

Cr(VI) reduction occurs in a wide range of redox potentials. The optimum redox potential range has not been well established as yet. Reduction of Cr(VI) has been reported in redox conditions as high as +250mV (53). In the same culture, after 48 hours, Cr(VI) reduction was observed even when the redox potential dropped to –500mV. A higher rate of Cr(VI) reduction by *Agrobacterium radiobacter* was observed at –240mV compared with –198mV (82). In contrary, no reduction of Cr(VI) was observed with redox potential of –140mV for the first hour of incubation in cultures of *Escherichia coli.*

Fe(III)-mediated reduction of Cr(VI). Fe(III) is the most abundant electron acceptor for anaerobic respiration in many sedimentary environments due to its ability to act as terminal electron acceptor for many organisms. Microbial reduction of Fe(III) significantly affects Cr(VI) biogeochemistry as reduced iron in sediments is one of the most significant electron donors for the reduction of Cr(VI). Three equivalents of Fe(II) are required for the reduction of one equivalent of Cr(VI).

$$3Fe(II) + Cr(VI) \rightarrow 3Fe(III) + Cr(III).$$

Therefore, Fe(III) reducing bacteria that are unable to support their growth on reduction of Fe(III) can indirectly reduce Cr(VI) via Fe(III) reduction (Figure 1-4). Reduction of chromate by dissimilatory iron-reducing bacteria was reported by Wielinga et al (169). They elucidated the reduction of Cr(VI) to Cr(III) via a closely coupled biotic-abiotic pathway under iron-reducing conditions.

Quinone mediated reduction of Cr(VI). Humic substances are ubiquitous in the environment. They are heterogeneous organic high-molecular-weight macromolecules that are composed of many potentially reactive moieties. Humic substances were considered resistant to microbial degradation until recently, when the ability of humics to serve as electron acceptors and support bacterial growth under anaerobic conditions was reported (90). Humics function as primary electron acceptors for iron-reducing bacteria, and mediate transfer of electrons from humics to Fe(III) oxides, thereby stimulating the reduction of insoluble Fe(III) oxides (Figure 1-5) (90). Quinones serve as the primary electron-accepting moiety in the humic acids when they are reduced to hydroquinones by accepting two electrons, as shown in Figure 1-6. Scott et al. demonstrated the higher free-radical content of humic substances with higher electron accepting capacity with electron spin resonance measurements by showing a proportional increase in semiquinones and electron-accepting capacity of humic substances (137).

To date several humic reducing bacteria have been isolated from a variety of environments (30). All iron-reducing bacteria that have been evaluated to date have shown the ability to transfer electrons to humic substances and other extracellular quinones.(91). Microbially reduced humics are also capable of reducing other metals, including manganese (IV) and technetium (VII) (83). Reduction of Tc(VII) mediated by Fe(III) was enhanced in the presence of anthraquinone di-sulfonate (AQDS), a humic acid analog that behaved as an electron shuttle (90). Although humic-mediated Cr(VI) reduction has not been reported so far thermodynamically, transfer of electron from humics (E = 0.2mV) to Cr(VI) (E = 1.23mV) is plausible.

Factors Affecting Microbial Chromium Reduction

Cell density. Rate of Cr(VI) reduction has been shown to be a affected by cell density under both aerobic and anaerobic conditions. Wang et al. (164) reported increase in the rate of Cr(VI) reduction with increase in cell density under anaerobic conditions. Similar observations were made in both aerobic and anaerobic cultures of *Escherichia coli*. However, the rate of Cr(VI) reduction was not proportional to the increase in the cell density, and the specific rate of Cr(VI) reduction was higher at relatively lower cell densities (142). These observations were also documented in cultures of *Enterobacter cloacae, Agrobacterium radiobacter, Pseudomonas fluorescens* LB300, *Bacillus coagulans,* and *Microbacterium* sp.

Initial Cr concentration. Depending upon the initial concentration of Cr(VI), its complete or incomplete reduction has been observed in *Enterobacter cloacae* HO1, (48, 71). Even though a decrease in cell viability was observed in the culture on addition of Cr(VI) to the growing culture (72, 163), the initial rate of Cr(VI) reduction increased with the increase in the initial rate of Cr(VI) in some cultures of *Enterobacter cloacae* (163), *E. coli* (139) *P. flourescens* (167) and *Bacillus* sp.(167). Similarly, initial specific rate of Cr(VI) reduction by cultures of *E coli* increased with increasing Cr(VI) concentrations. However, an increase in time required for complete reduction was also observed (142).

Effect of other electron acceptors. Presence of oxygen does not completely inhibit Cr(VI) reduction in some bacteria but it represses it as in the case of *Agrobacterium radiobacter* EPS-916, *E. coli* ATCC 33456 and *Pseudomonas stutzeri* CMG463 (9, 71, 82, 139, 141, 165). Microbial reduction of Cr(VI) is completely inhibited in aerobic condition as in the case of *E. cloacae* HO1, even though cell growth was observed (48). Studies with enrichment microcosms showed only 41% reduction of

Cr(VI) under aerobic conditions when compared with the 84% reduction observed in anaerobic conditions (97).

Marsh et al. concluded that lower reducing conditions were required for Cr(VI) reduction because reduction of Cr(VI) was inhibited by oxygen and nitrate (97).

Among other naturally occuring dominant electron acceptors, sulfate and nitrate have little effect on the Cr(VI) reduction upto concentrations of 10 mM and 16mM, respectively. The concentration of sulfate and nitrate to which microbial Cr(VI) reduction is not affected varies with the bacterial species. In the case of Cr(VI) reduction by *Pseudomonas. putida* Cr(VI), reduction was not affected by 1mM of sulfate and 0.2mM of nitrate. Concentrations of sulfate and nitrate, that did not affect Cr(VI) reduction, in case of *Bacillus* sp., were 10 mM and 16mM respectively, in case of *E. coli* were 83 mM sulfate and 129mM nitrate. Sulfate concentration as high as 50 mM did not affect the Cr(VI) reduction by *Desulfovibrio vulgaris* (93). In contrast, the chromate reduction by *Enterobacter cloacae* is inhibited by 32% in the presence of just 25μM of sulfate and 84% in the presence of 5mM $NaNO_3$. Enrichment studies with alternative electron acceptors done by Marsh et al. showed that nitrate reduction preceeded Cr(VI) reduction. However, Fe(III) reduction and sulfate reduction always followed the Cr(VI) reduction. They supported their results by the Gibbs energy obtained by thermodynamic reactions (97).

Temperature and pH effects. Optimum temperature and pH conditions reported for microbial Cr(VI) reduction strongly suggest that the reduction process is related to growth. Cr(VI) reduction was observed in cultures of *Enterobacter cloacae* at pH range of 6.0-8.5, and at pH range of 3.0 -8.0 in cultures of *Escherichia coli* and *Bacillus*

coagulans. However the maximum initial specific rate of Cr(VI) reduction by all three bacteria was at pH 7.0, an optimal pH for most bacterial growth. Even though Cr(VI) reduction by *E. coli* and *Enerobacter. cloacae* occurred at a wide range of temperature of 10°C to 50°C, optimum temperature was found to be 36°C and 30°C respectively. These conditions were found to be optimal for the anaerobic growth of the bacteria. Studies with sediments have shown temperature optima of 22°C and 50°C and a pH optimum of 6.8 (97).

Carbon sources. Studies have been conducted to try and establish the relationship between the electron donors and the rate of Cr(VI) reduction. Enrichment studies with the soils done by Marsh et al showed hydrogen to be an efficient electron donor for the reduction of Cr(VI). Addition of electron donors that increase the bioavailable hydrogen such as glucose, formate, and hydrogen stimulated the Cr(VI) reduction in the soils as compared with acetate and benzoate and lactate. The study also documented the dissolved hydrogen concentration in the Cr(VI)-reducing conditions. Based on the observation that very low hydrogen concentration was present under Cr(VI) reducing conditions similar to that reported under nitrate- and manganese-reducing conditions, and the observation that Cr(VI) reduction occurs before iron or sulfate reduction it was concluded that very highly reducing conditions were not required for Cr(VI) reduction (98).

Rege et al. reported the utilization of sucrose as a carbon source for *Enterobacter* cloacae HO1for reduction Cr(VI) (128).

Outline of Dissertation

The work presented in this dissertation was performed to get more insight into the diversity of electron donors and acceptors utilized by the indigenous chromium reducing bacteria (CRB). Special attention was paid to the kinetics of chromium reduction in the presence of alternative electron acceptors Fe(III) and AQDS. An attempt was made to explain the mechanism of Cr(VI) reduction by fermentative organisms.

Chapter 2 describes the enrichment studies with indigenous CRB capable of utilizing various electron donors and acceptors. Difference in reduction of Cr(VI) is determined by the organisms enriched by the various electron donors chosen to represent the range of electron donors that naturally exist in nature. Effect of electron acceptors viz. Fe (III) oxides and anthraquinone di-sulfonate (AQDS), an humic acid analog on reduction of Cr(VI) is also investigated. Identification of the organisms isolated from the enrichments is also described based on the phylogenetic studies. Chapter 3 describes the isolation of the Cr(VI)-reducing consortium from the glucose enrichments and detailed kinetic studies of Cr(VI)-reduction by this consortium. In chapter 4 the isolation, identification, and detailed characterization of a novel species of chromium-reducing fermentative organism GCAF-1 is described. Chapter 5 includes detailed kinetic studies with fermentative isolate GCAF-1. It describes the possible mechanism adopted by *Clostridium* sp. GCAF1 reduce Cr(VI). Finally, the results presented in this dissertation are summarized and the implications of this research are discussed in Chapter 6.

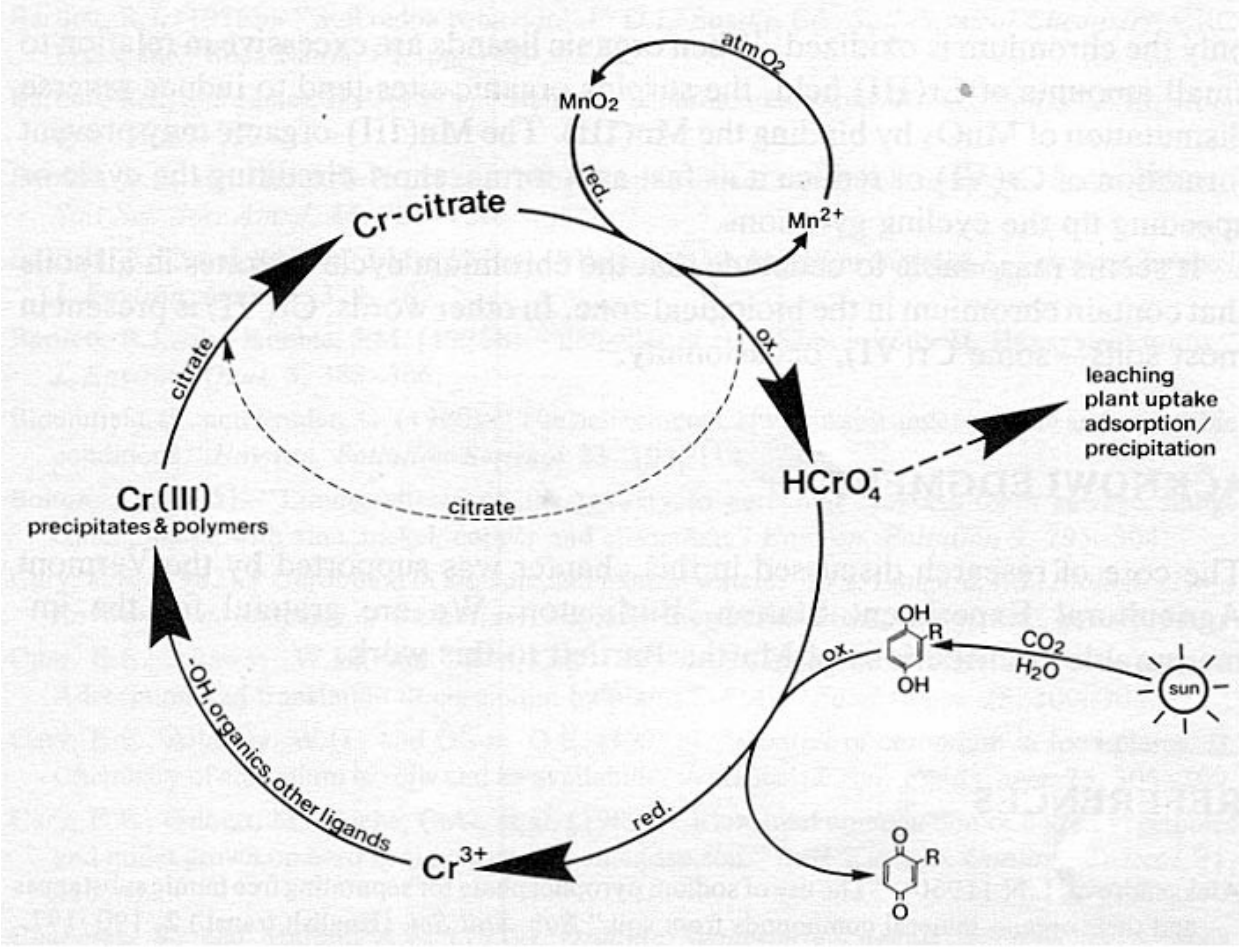


Figure 1-1. Chromium cycle in environment (174)

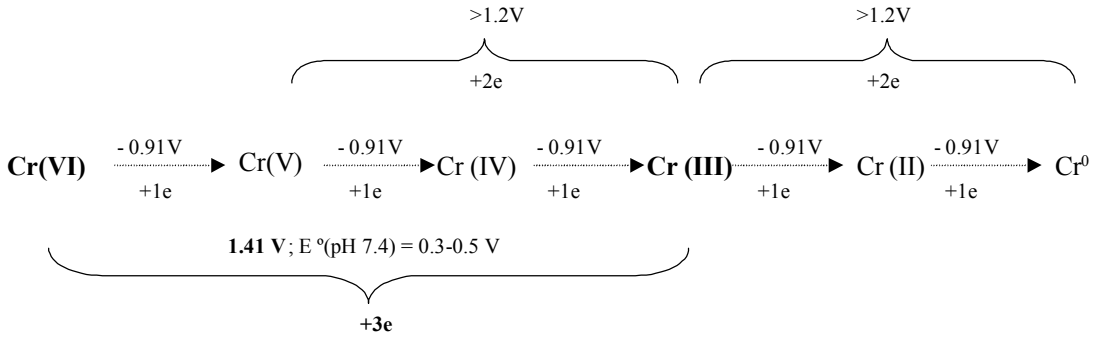


Figure 1-2. Reduction potential diagram for chromium. Positive Eº values favor the reduced form. E ºvalues for Cr(VI) and Cr(V) are dependant on the pH because the protons are involved in the reaction (111).

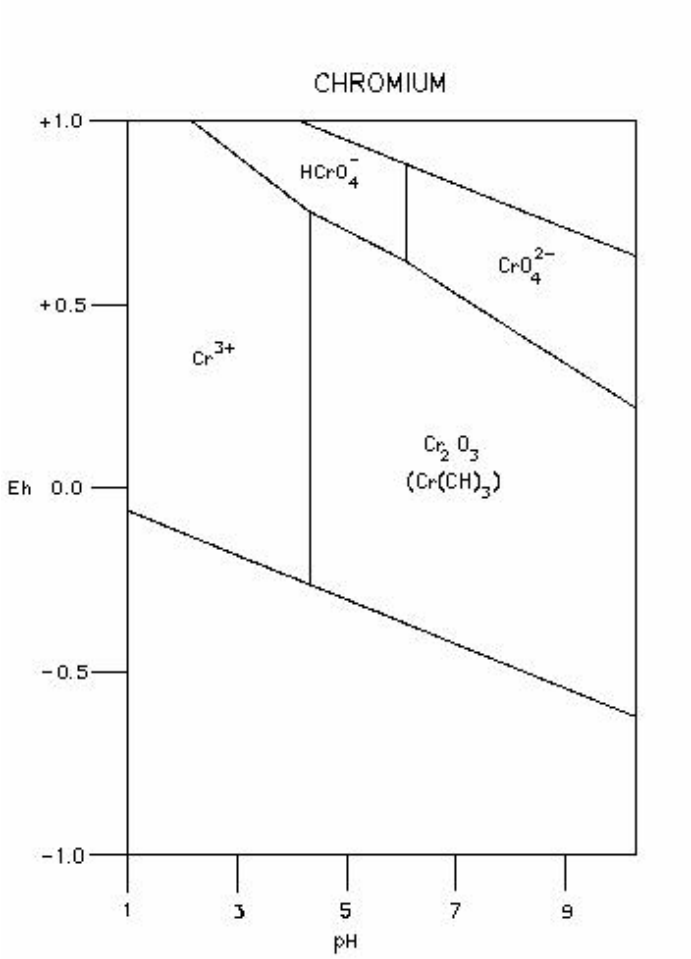


Figure 1-3. Eh-pH diagram for chromium-water system at standard state conditions. Source: Dragun Figure (111)

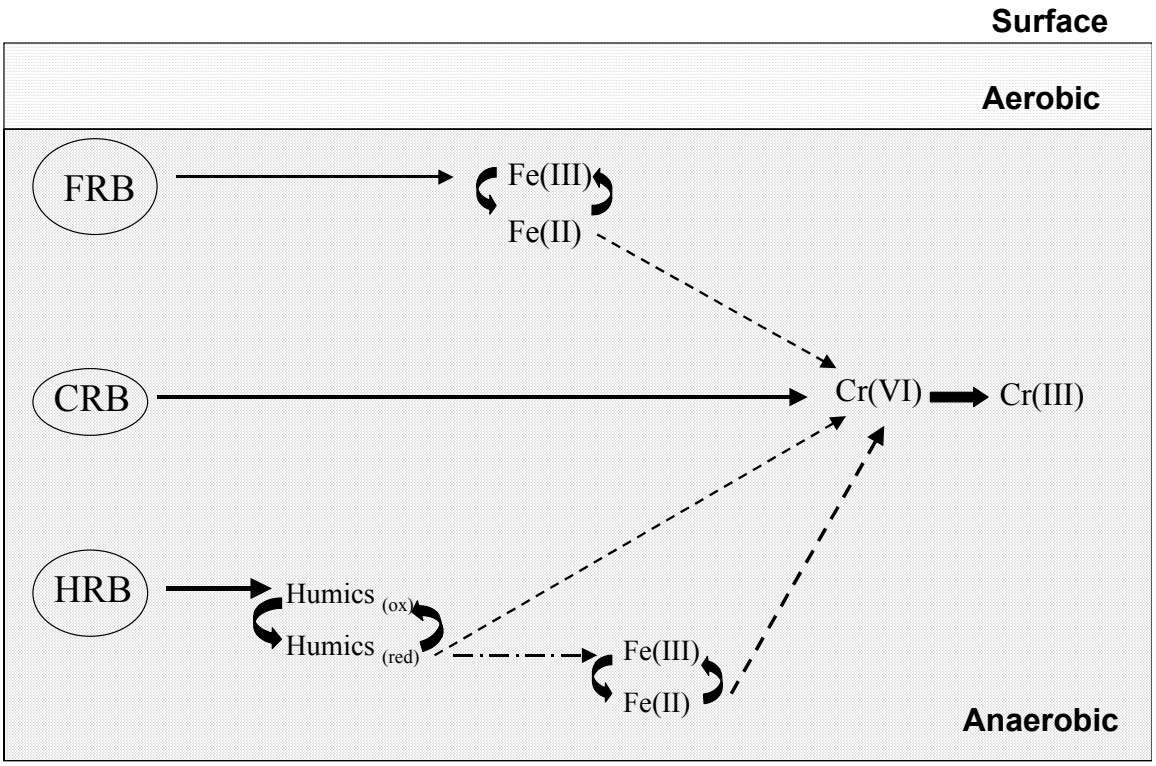


Figure 1-4. Schematic diagram showing the possible pathways for anaerobic Cr(VI) reduction by the three groups of bacteria, Fe(III) reducing bacteria (FRB); Cr(VI) reducing bacteria (CRB); humics reducing bacteria (HRB). Solid lines represent the biotic reduction of Cr(VI) and the abiotic reduction is represented by the dashed lines.

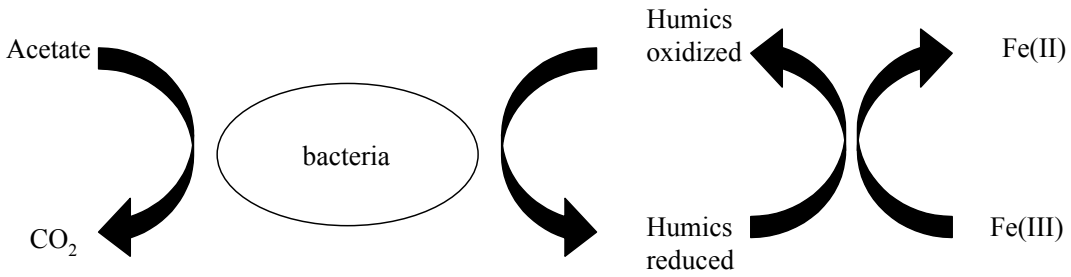


Figure 1-5. Model showing reduction of Fe(III) mediated by humics (90)

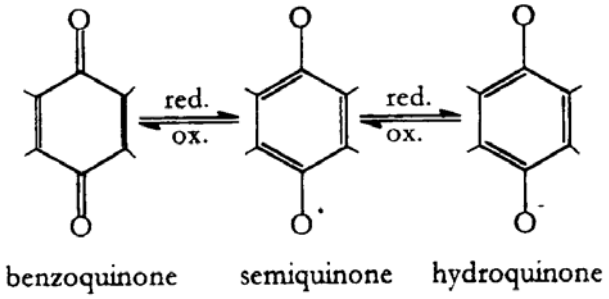


Figure 1-6. Quinone model compound. The semiquinone species contains an unpaired electron (137)

Table 1-1. Cr(VI) reducing bacteria described in literature

Bacteria	Redox potential	Cr(VI) reduced	Gram stain	Reduction conditions	Enzymatic reduction	Carbon source	Reference
P. putida MK1		0.2mM	Gram negative	Anaerobic	ND		(117)
Pseudomonas sp. CRB5		0.1mM	Gram negative	Aerobic and anaerobic	Soluble reductase	Does not require NADH	(101)
Pseudomonas dechromaticans	ND	0.2mM	Gram-negative	Anaerobic	ND	Peptone / glucose	(130)
P. chromatophila	ND	-	Gram-negative	Anaerobic	ND	ribose/ lactate/ acetate/ succinate/ butyrate/ glycerol/ fumarate	(79)
P. fluorescens LB300	ND	0.48mM	Gram-negative	Aerobic and to a lesser extent anaerobic	Membrane associated, NADH dependant	Glucose	(22, 167)
P. ambigua G-1	ND	0.4mM	Gram-negative	Aerobic	NAD(P)H-dependant	Nutrient broth	(59)
P. aeruginosa		-	Gram-negative	Anaerobic	ND	Acetate/ glucose	(56)
P. putida PRS2000		0.038mM	Gram-negative	Aerobi and anaerobic	Soluble protein; NADH or NADPH dependent	Glucose/ lactate	(62)

Table 1-1. continued

Bacteria	Redox potential	Cr(VI) reduced	Gram stain	Reduction conditions	Enzymatic reduction	Carbon source	Reference
E. coli ATCC 33456	ND	0.3 mM	Gram-negative	Anaerobic and aerobic; oxygen repressed Cr(VI) reduction.	Majorly Soluble reductase little activity by membrane associated.	Nutrient broth	(141)
Agrobacterium radiobacter EPS-916 (resting cells)	-200mV	0.5mM under Eh -138mV	Gram-negative			Glucose Fructose lactose glutamate succinate	(82)
Desulfovibrio vulgaris			Gram-negative				(93)
Micrococcus roseus			Gram-positive				(56)
Streptomyces (Actinomycete)			Gram-positive				(35)
Pantoea agglomerans SP1	NA	0.1mM	Gram-negative (facultative anaerobe)	Anaerobic. Cr(VI) used as terminal electron acceptor.	NA	Lactate, acetate, hydrogen	(45)
Achromobacter eurydice	ND	-	Gram-negative	-	-	-	(56)

Table 1-1. continued.

Bacteria	Redox potential	Cr(VI) reduced	Gram stain	Reduction conditions	Enzymatic reduction	Electron donor	Reference
Dienococcus radiodurans R1 (Thermus group)	NA	0.5mM; .	Gram-positive	Anaerobic and to a lesser extent in aerobic conditions	NA	lactate	(47)
Bacillus subtilis	ND	0.1mM to 1mM	Gram-positive	Aerobic	Soluble protein; NADH can act as electron donor		(53)
Enterobacter cloacae HO1		0.5 mM	Gram-negative	Anaerobic	Membrane associated	Acetate/ glycerol/ glucose	(115)
Rhodobacter sphaeroides	ND	0.146µMol h^{-1} (aerobic) 1.6µMol h^{-1}	Gram-negative	Aerobic and Anaerobic	Soluble fraction; NADH required.	Succinic acid	(110)
Desulfotomaculum reducens	ND	Less than 0.20 mM	Gram positive	Anaerobic	ND	Butyrate/ lactate/ propionate/ pyruvate/ glucose	(152)
Thiobacillus ferroxidans	ND	0.289 mM	Gram-negative	Anaerobic	abiotic	sulfur	(125)

ND:not determined
-; no values were found in leterature.

CHAPTER 2
ENRICHMENT, ISOLATION, AND CHARACTERIZATION OF CR(VI)- REDUCING BACTERIA

Introduction

Understanding the microbe-metal interactions in the environment has gained considerable importance in the past decade and a half (67, 94, 160, 161, 170, 175, 177). Among the various aspects of the interactions studied, the role of microorganisms in remediating contaminated water, soils, and sediments is gaining much appreciation (47, 125, 158, 166, 176). Microorganisms can affect the solubility and the toxicity of metals and provide *insitu* remediation of contaminated fields. Field studies, conducted to exploit the ability of microbes to attenuate or remove contaminants from the environment by direct or indirect means, have shown stimulation of indigenous microorganisms to be an effective method of remediation.

Microbial reduction of soluble Cr(VI) to its insoluble Cr(III) form is a cost-effective way to prevent the mobility of Cr(VI) beyond the compliance boundaries and to eliminate the risk of health hazards to humans. Microbial reduction of Cr(VI) is controlled by many factors, including cell density, initial concentration of Cr(VI), pH, and redox potential (97, 98, 176). Among the factors that contribute significantly to microbial reduction of Cr(VI) by influencing the activities of particular groups of soil bacteria are electron donors and acceptors present in the soil. Number and the activity of Cr(VI)-reducing bacteria in soil largely depend on the growth conditions and the organic compounds that serve as electron donors present in soil.

In aerobic environments, oxygen is the most abundant electron acceptor. In the absence of oxygen, other electron acceptors such as NO_3^-, and SO_4^{2-} can be utilized by the microorganisms to conserve energy. Several microorganisms also have the ability to couple the reduction of the metal oxides such as Fe(III) and Mn(IV) to the oxidation of the carbon source. Wide diversity of Fe(III)- and Mn(IV)-reducing has been established to date (84, 152, 177). Although few bacteria with an ability to reduce Cr(VI) have been reported in literature, not many Cr(VI)-respiring bacteria are reported possibly due to two reasons (a) Cr(VI) is a mutagen and is toxic for most organisms, (b) presence of other electron acceptors that can support the growth of the organisms is much higher. Recently, a sulfate reducing-bacteria *Desulfotomaculum* sp and a consortium of sulfate-reducing bacteria with an ability to utilize Cr(VI) were reported (152). Presence of other electron acceptors can influence the reduction of Cr(VI). For instance, it has been shown in the past that Fe(II) abiotically reduces Cr(VI) under reducing conditions, and that soil organic matter with a high content of humics acts as an electron donor in Cr(VI) reduction. However, paucity in data exists regarding the importance of microorganisms in these transformations. The comprehensive study presented here addresses various aspects of microbial Cr(VI) reduction in soil such as the organisms involved in Cr(VI) reduction, and the role of electron donors and acceptors in Cr(VI) reduction.

Comparative analysis of genetic sequences provides insights into the genealogical relationships of prokaryotes. Sequencing studies have used 5S rRNA (58), cytochrome c and ferredoxins (136) among others as possible genetic probes for phylogenetic analysis. However, phylogenetic patterns obtained are not congruent among each other and none of them match the branching patterns of those of 16S rRNA. The sequence of 16S rRNA

genes (rDNA) is considered to be a valuable genetic marker for establishing phylogenetic relationships between organisms. The conserved character of these molecules together with regions of higher variability, their ubiquitous distribution, genetic stability, and functional constancy make them a suitable candidate for this application. The phylogenetic trees cluster organisms based on genetic makeup. Phenotypic characters do not have to be considered. Therefore, 16S rDNA was used in this study for identifying the various CRBs.

Specific objectives of this study were to (i) maximize the diversity of the CRB to be isolated (ii) evaluate the contribution of various electron donors for Cr(VI) reduction, (iii) study the effect of alternative external electron acceptors on Cr(VI) reduction, and (iv) perform phylogenetic analysis of the CRB isolated.

Materials and Methods

Soil was obtained from a highly Cr(VI)-contaminated Superfund site in the Upper Peninsula of Michigan. This site is a wetland receiving Cr(VI) from effluents discharged from an adjacent leather-tanning facility. Soil was collected in sterile containers and immediately shipped, while being maintained below 4°C, to our laboratory. Samples were stored under 4°C until the work began. The concentration of chromium in the soil was determined to be approximately 17 g/ kg of soil. The iron content of these soil samples was determined to be 13 mg/kg.

Enrichment media. Anaerobic enrichments were established with a variety of electron donors and electron acceptors in different combinations. Enrichments were prepared in bicarbonate buffered basal media composed of (per liter) KH_2PO_4 (0.42 g); K_2HPO_4 (0.22 g); NH_4Cl (0.2 g); mineral mix (10ml); vitamin mix (15ml); KCl (0.38 g); NaCl (0.36 g), $CaCl_2$.$2H_2O$ (0.04 g); $MgSO_4.7H_2O$ (0.10 g); $NaHCO_3$ (1.8 g); Na_2CO_3

(0.5 g); 1mM Na_2SeO_4 (1ml). vitamin mix was composed of (per liter) biotin (2.0 mg); folic acid (2.0 mg); pyridoxine HCl (10.0 mg); riboflavin (5.0 mg); thiamine (5.0 mg); nicotinic acid (5.0 mg); pantothenic acid (5.0 mg); vitamin B-12 (0.1 mg); *p*-aminobenzoic acid (5.0 mg); thioctic acid (5.0 mg); mineral mix was composed of (per liter) NTA (1.5g); $MgSO_4$ (3.0g); $MnSO_4.H_2O$ (0.5g); NaCl (1.0g); $FeSO_4.\ 7H_2O$ (0.1g); $CaCl_2\ .2H_2O$ (0.1g); $CoCl_2.6H_2O$ (0.1g); $ZnCl_2$ (0.13g); $CuSO_4.5H_20$ (0.01g); $AlK(SO_4)_2.12H_2O$ (0.01g); H_3BO_3 (0.01g); Na_2MoO_4 (0.025g); $NiCl_2.6H_2O$ (0.024g); $Na_2WO_4.2H_2O$ (0.025g). 90 ml of this medium were dispensed in 117 ml serum bottles under gas ($C0_2$:N_2::20:80) pressure and gassed for 30 min. When required, iron was added to the medium in the form of $Fe(OH)_3$ a from stock solution of 0.5M before autoclaving. After autoclaving, media were anaerobically amended with electron donors and acceptors. Sterile stock solutions of electron donors and acceptors were prepared separately under anaerobic conditions. Final concentrations of supplements in the medium were Cr(VI) (0.4mM); AQDS (0.1mM); and Fe(III) (5mM). Electron donors included 10mM each of acetate, benzoate, citrate, and glucose as required for individual enrichments (Table 2-1).

Enrichments. Four sets of anaerobic enrichments were established with of different electron donors as described above. Each set contained 4 microcosms, each amended with a different combination of electron acceptors (Table2-1). Microcosms contained the following combinations of electron acceptors. Cr(VI) only; Cr(VI) and AQDS; Cr(VI) and Fe(III); Cr(VI), AQDS, and Fe(III). Each set also included one control to monitor abiotic reduction of Cr(VI). Controls were supplemented with all electron acceptors and appropriate donor inoculated with dead (autoclaved) cells.

The inoculum was prepared by adding 10 g of soil to basal medium (10 ml). Soil in media was stirred under continuous flushing with nitrogen gas. After considerable stirring to break all soil aggregates, 10 ml of slurry were used as inoculum for enrichments. The enrichments were incubated at 30ºC in the dark without shaking. The concentration of Cr(VI) remaining in the microcosm was monitored at various times. On depletion of Cr(VI) from the enrichments, transfers were made with 10% inoculum to fresh medium containing appropriate electron acceptors and donor. Rates of Cr(VI) reduction were determined in all enrichments after the third transfer as described below. By the third transfer, soil particles were diluted out, thereby decreasing the likelihood of Cr(VI) reduction by chemicals other those supplemented in the medium. Initial Cr(VI) concentrations were measured in enrichments immediately following inoculation to assess chemical reduction of Cr(VI) by reduced components that carried over during transfers.

Analytical methods. Concentrations of Cr(VI) were determined colorimetrically by UV/Visible spectrophotometer (perker) equipped with 1 cm cuvettes, using the diphenylcarbazide (DPC) assay as previously described (43, 159). 0.25ml solution, prepared with 0.025g of 1,5-diphenylcarbazide in 10 ml of acetone, were added to 10 ml of sample (diluted when necessary). After 15 minutes of incubation at room temperature (25°C), absorbance at 540 nm was determined. This assay has a detection limit of 2 μM All readings were conducted in triplicate.

Strain isolation. Bacteria were isolated by a standard roll tube method, technique used for the isolation of the anaerobic bacteria. Roll tubes are anaerobic, sealed serum tubes that were prepared by rolling tubes containing sterile molten agar in medium with

carbon source. The inoculum was added while the agar was still in molten form. The tubes were rolled till the agar layer was set along the walls of the tube. Isolated colonies appeared either embedded or on the surface of the agar layer. Each culture was passed through roll tubes several times until colonies with uniform morphology were obtained. Colonies selected and picked by sterile long stemmed pasteur pipettes were immediately transferred liquid basal media while maintaining the electron donors and acceptors. This set up was continuously maintained under nitrogen gas flow to keep it anaerobic. Isolates were obtained from enrichment cultures in which the fastest reduction of Cr(VI) was observed. Isolated colonies were tested for their ability to reduce Cr(VI). Isolates were tested for facultative and obligate anaerobic growth. Strains prefixed as GCAF were isolated from Glucose-Cr(VI) enrichment supplemented with Fe(III) and AQDS; Strains designated as GCF were from the glucose-Cr(VI) enrichment with Fe(III); GCA isolates were obtained from the glucose-Cr(VI) and AQDS enrichment. GC isolates were from the Glucose-Cr(VI) enrichment with no external electron acceptors.

DNA isolation and amplification of 16S rDNA. Genomic DNA was extracted from each of the 12 isolates (1ml culture of pure isolates). Cells were harvested and lysed by boiling in 500 µl of sterile water. 16S rRNA gene was amplified using universal bacterial primers 27f and 1492r (76)with Perkin Elmer thermocycler Model 240 (Norwalk, CT). Conditions for amplifications were as follows: 95^{o}C for 15 min, followed by 35 cycles of 94^{o}C for 30 seconds, 58^{o}C for 1 min and 72^{o}C for 30 sec. The final extension step was 7 minutes at 72^{o}C. The amplified product was purified using a commercially available kit (Qiagen, Inc.) and sequenced by the DNA Interdisciplinary Center for Biotechnology Research sequencing facility at the University of Florida.

Phylogenetic analyses of the isolates 16S rDNA sequences were screened using the BLAST (ref.) program to identify organisms of highest similarity with 16S rDNA sequences of the various isolates obtained. Sequence alignment were either performed manually with sequences obtained from Ribosomal Database Project (96)or by using PILEUP function of GCG (Genetics Computer Group version). CLUSTALX was used to view the alignment and finer adjustments were made manually using McCLADE version 3.0 (95) Phylogenetic trees were constructed using maximum parsimony analyses of the aligned sequences by PAUP 4.0b8 (150). Bootstrap values were assigned on 100 replicates after reweighing the characters by heuristic search strategy to assess the confidence level of various clades. The GenBank accession numbers for the sequences shown in Figure 2-2.

Results and Discussion

Effect of Electron Donors and Acceptors on Cr(VI) Reduction

The results strongly suggested that the rate of Cr(VI) reduction by indigenous soil microorganisms was affected by the available electron donor and acceptors. Even though reduction of Cr(VI) was observed in all sets of enrichments, there was a difference in rate of Cr(VI) reduction. Concentrations of Cr(VI) remained constant in treatments that were not inoculated with cells, and insignificant amount of reduction was observed in treatments inoculated with heat killed cells (Figure 2-1). Cr(VI) was rapidly reduced in enrichments with glucose and citrate as electron donors. Cr(VI) concentrations fell below detection levels in less than a week in glucose enrichments. Higher turbidity was also observed in these enrichments. Enrichments with citrate as electron donor reduced Cr(VI) within 10 days. Bacterial enrichments amended with acetate and benzoate also showed loss of Cr(VI) although at a much lower rate. Cr(VI) in these enrichment cultures was

completely reduced after three weeks. The pH of cultures was monitored and remained constant at 7.4.

Enrichments with different electron donors were established in order to maximize the diversity of the CRB enriched. Acetate was chosen as an electron donor as it is abundant in nature and most metal reducing organisms have the ability to couple acetate oxidation to metal reduction (51, 109, 147). No loss of Cr(VI) and no bacterial growth was observed in enrichments with Cr(VI) as sole electron acceptor. There are three possible explanations; (i) toxicity of high concentrations of Cr(VI) (ii) absence of acetate utilizing bacteria that couple their growth to Cr(VI) reduction (iii) bacterial communities being among the 99.9% uncultivable bacteria. Benzoate and lactate were expected to support the growth of organisms that conserve energy by oxidizing aromatic compounds and fermentation products respectively. Bacteria with the ability to oxidize benzoate with Cr(VI) reduction has been reported (140). Citrate was one of the chosen electron donors for this study as it is a part of citric acid cycle that has major biosynthetic as well as energetic functions and many organisms have the ability to utilize it an electron donor and carbon source. Reduction of Cr(VI) in enrichment sets with the electron donors was observed only in the presence of other electron acceptors. Variation in rate of Cr(VI) reduction observed in different donor sets suggested diversity in organisms being enriched and possibly different mechanisms of Cr(VI) reduction.

Slight turbidity was observed in glucose enrichment with Cr(VI) as sole electron acceptor but there was no reduction of Cr(VI) indicating growth of Cr(VI) resistant fermentative organisms.

Reduction of Cr(VI) was observed in all enrichment set when the media were supplemented with additional electron acceptor AQDS or Fe(III). In presence of both Fe(III) and AQDS, an accelerated rate of Cr(VI) reduction was observed. These results can be explained by efficient channeling of electrons towards the reduction of Cr(VI) due to Fe(III) and AQDS acting as electron shuttles and the amount of electrons shuttled in these systems are much higher and therefore Cr(VI) is rapidly lost from the system. Microbially reduced form of AQDS can act as a shuttle by transferring electrons to insoluble Fe(III) and increasing the rate of Fe(III) reduction. Fe(II), in turn, can reduce Cr(VI) to Cr(III). In the absence of either of the electron acceptors, effective electron shuttle trains are broken, thereby lowering the rate of Cr(VI) reduction. Increased reduction of Cr(VI) is also indicative of the combined contribution of the AQDS respiring bacteria, Fe(III) reducing bacteria, and Cr(VI) reducing bacteria (Figure 1-4). The results also suggested that the presence of Fe(III) and AQDS in combination alleviate the toxicity of Cr(VI) to the bacteria more than when they are present alone with Cr(VI).

Fastest rate of Cr(VI) was observed in enrichment culture with glucose as electron donor. In addition higher turbidity was also observed. Preference of glucose by the enriched indigenous CRB, over other electron donors used in the study was clearly evident. Anaerobic Cr(VI)-enrichment with glucose as electron donor was expected to enrich fermentative bacteria in addition to other CRB. In the environment fermentative organisms form the primary level where they oxidize more complex electron donors and form simpler metabolites that are used up by the secondary level microorganisms. Therefore the fermentative organisms that are resistant to Cr(VI) were expected to enrich

first along with the other Cr(VI) resistant, non fermentative CRB. The fermentation metabolites formed could then be used by other non fermentative CRB that are unable to utilize glucose as carbon source. However, results from the phylogenetic study revealed that the enrichment culture that showed the fastest reduction of Cr(VI) was dominated by the fermentative gram positive bacteria. One of the possible explanation of the dominance of these bacteria in the enrichment culture is that the major indigenous CRB in the Cr(VI)-contaminated soil were fermentative bacteria, mainly *Clostridium* sp. and *Celullomonas* sp. At this point, it was not clear if *Clostridium* sp. reduces Cr(VI) directly or indirectly via Fe(III) and AQDS. The confirmation of this hypothesis and the elucidation of the mechanism of Cr(VI) reduction by these organism was the objective of the next study.

Phylogenetic Analysis of Cr(VI) Reducing Bacteria

Phylogenetic analysis of 16S rDNA sequences of the isolates obtained from enrichments with glucose as electron donor yielded organisms that belonged mostly to the high G+C gram positive *Cellulomonas* group and low G+C gram positive *Clostridium* group of bacteria. Several isolates belonging to *Micrococcus*, *Bacillus*, and *Staphylococcus* genera were also obtained. With exception of *Bacillus* and *Micrococcus,* attempts to grow these isolates aerobically were unsuccessful. Previously described CRB are capable of reducing Cr(VI) to Cr(III) and are phylogenetically diverse. Most CRB are gram-negative and facultative anaerobes. Few gram positive bacteria capable of reducing Cr(VI) have been reported to date. Reduction of Fe(III) by *Clostridium* sp has been documented previously, but no reports of Cr(VI) reduction by *Cellulomonas* sp or *Clostridium* sp have been published so far. Results from this study strongly suggest

significant role played by the fermentative organisms in the reduction of Cr(VI) and perhaps other heavy metals.

As more chromium reducing bacteria will be isolated from various environments it is likely that the diversity will continue to increase. It still remains to be seen if the capability to reduce Cr(VI) evolved independently and specifically in some organisms or if there are some organisms that can support their growth on the reduction of Cr(VI). For example, numerous studies suggest that NADH dependant-reductase enzyme was invariably involved in reduction of Cr(VI) to Cr(III) in *E. coli* and *Pseudomonas* sp. the finding that many other bacteria can reduce Cr(VI) without any enzyme indicates that there is more than one pathway for the reduction of Cr(VI). These studies emphasize that much study is required before the microorganisms in various environments will be known and before the mechanisms for Cr(VI) reduction will be understood.

Table 2-1. Combination of electron acceptors and donors supplemented in the media for anaerobic enrichment studies

Electron donors	Electron acceptors	Code
Acetate	Cr(VI)	AC
	Cr(VI) and AQDS	ACA
	Cr(VI) and Fe(III)	ACF
	Cr(VI), AQDS and Fe(III)	ACAF
Benzoate	Cr(VI)	BC
	Cr(VI) and AQDS	BCA
	Cr(VI) and Fe(III)	BCF
	Cr(VI), AQDS and Fe(III)	BCAF
Citrate	Cr(VI)	CC
	Cr(VI) and AQDS	CCA
	Cr(VI) and Fe(III)	CCF
	Cr(VI), AQDS and Fe(III)	CCAF
Lactate	Cr(VI)	LC
	Cr(VI) and AQDS	LCA
	Cr(VI) and Fe(III)	LCF
	Cr(VI), AQDS and Fe(III)	LCAF
Glucose	Cr(VI)	GC
	Cr(VI) and AQDS	GCA
	Cr(VI) and Fe(III)	GCF
	Cr(VI), AQDS and Fe(III)	GCAF

Table 2-2. Accession numbers for 16S rDNA sequences used in this study

Bacterial species	**Acession numbers***
Clostridium cellasea	X83804
Cellulomonas flavigena	AF140036
Cellulomonas persica	AF064701
*Cellulomonas sp.*strain 1533	Y09658
Cellulomonas hominis	X82598
Oerskovia turbata	X79454
Clostridium acetobutylicum	X81021
Clostridium beijerinckii	X68179
Clostridium roseum strain DSM 51	Y18171
Clostridium sp. (*C.corinoforum*)	X76742
Clostridium sp. (*C.favososporum*)	X76749
Clostridium .puniceum	X71857
Clostridium butyricum	X68176
Clostridium paraputrificum strain M-21	AB032556
Staphylococcus sp. strain LMG-19417	AJ276810
Bacillus megaterium	D16273
Bacillus macroides	X70312
Bacillus macroides	AF157696
Bacillus subtilis N5	AF270793
Desulfotomaculum acetoxidans	Y11566
Pseudomonas putida	AF307869

*Gen Bank accession numbers except for those species sequenced used in this study

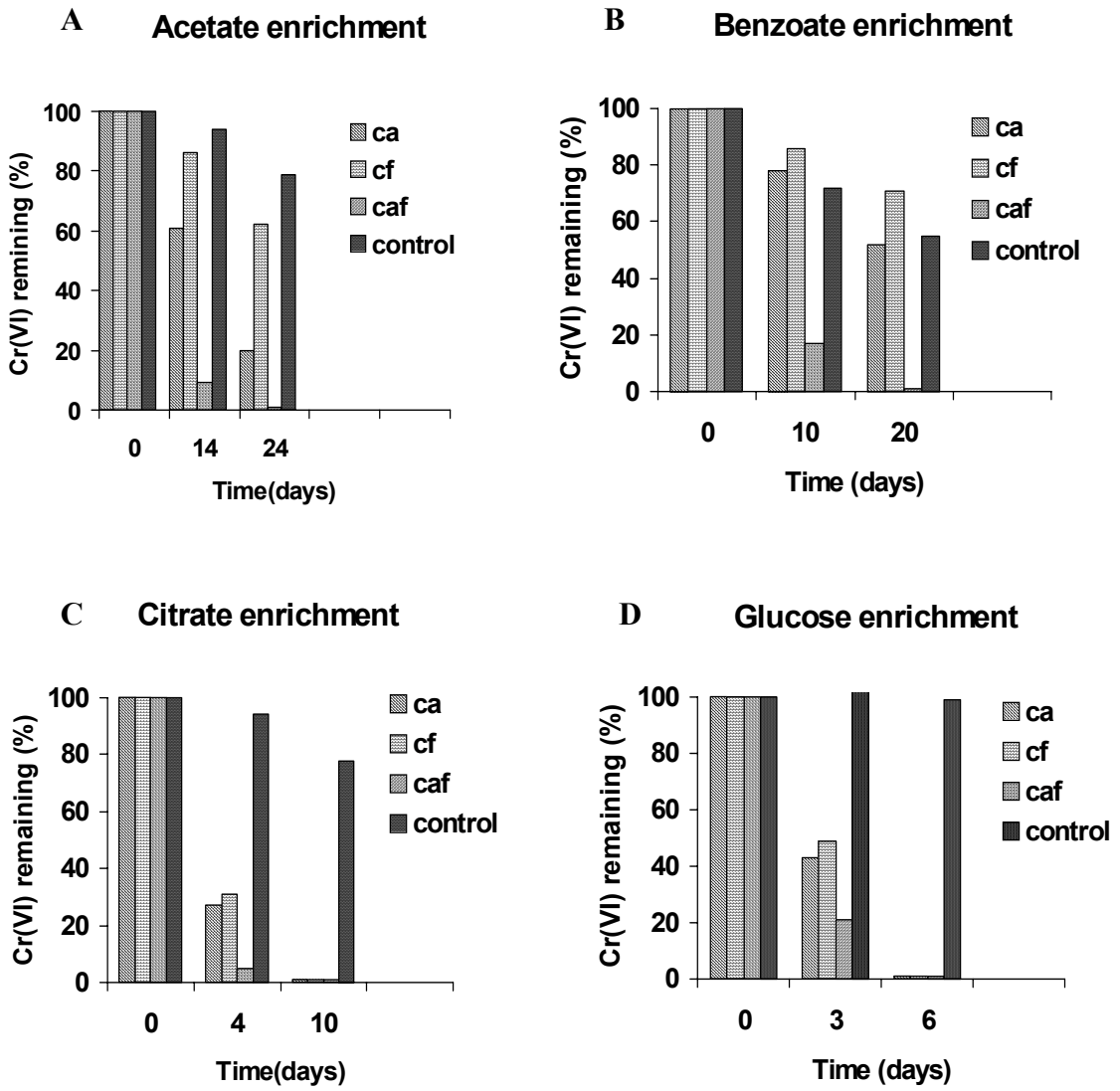


Figure 2-1. Rate of reduction of Cr(VI) in enrichment cultures amended with different electron donors and electron acceptors**.** A: acetate amended enrichments. B: benzoate amended enrichments; C: citrate amended enrichments, D: glucose amended enrichments. Each of the enrichment sets was supplemented with adiitional electron acceptors. **ca :**Cr(VI) and AQDS, **cf**: Cr(VI) and Fe(III); **caf**: Cr(VI),AQDS and Fe(III); control was set up with dead (autoclaved)cells amended with Cr(VI),AQDS and Fe(III) as electron acceptors. No cell control showed very insignificant reduction of Cr(VI).

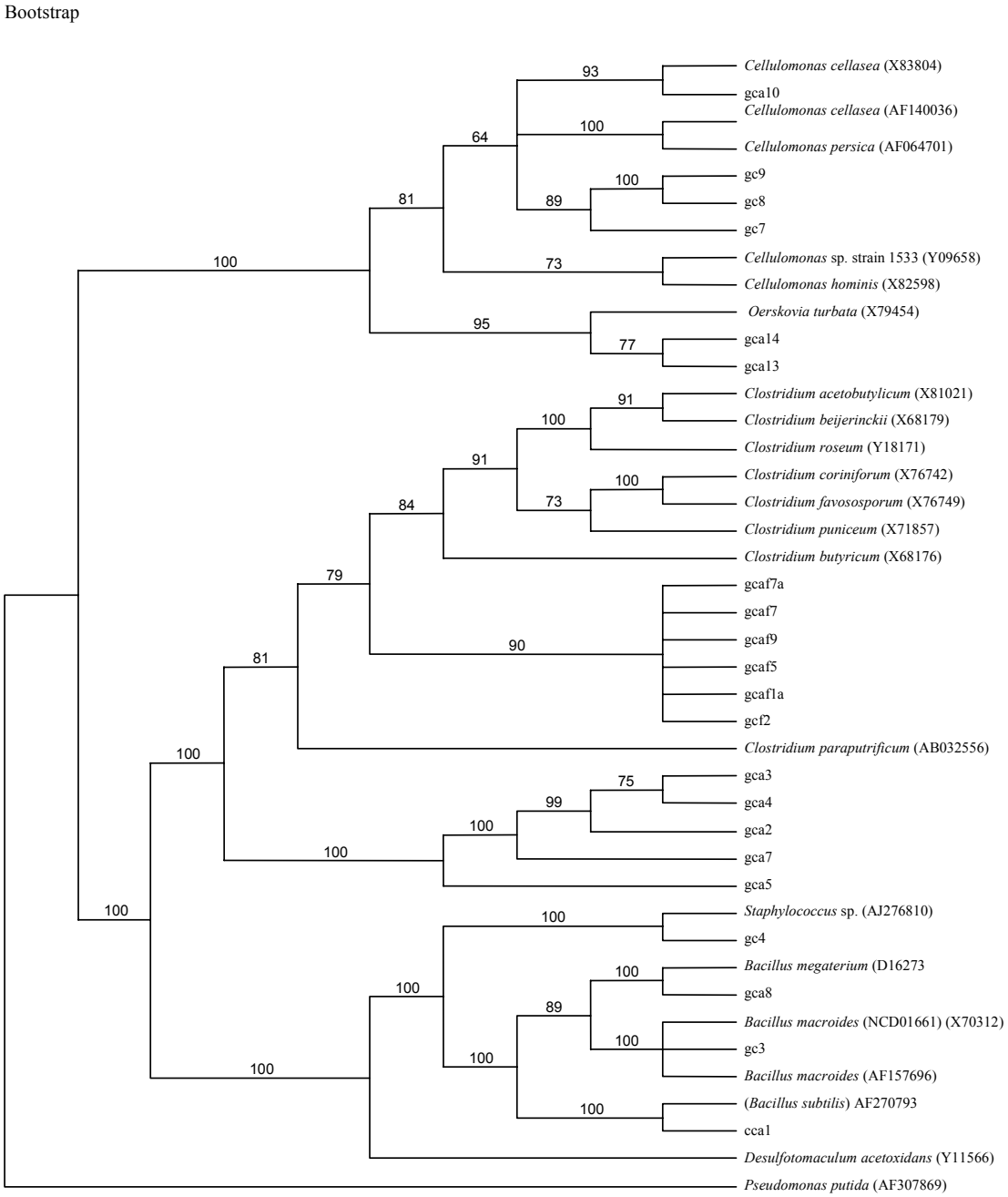


Figure 2-2. Phylogenetic tree constructed using maximum parsimony (PAUP version 4.0b8). Numbers above the branches represent the bootstrap values (100 replicates). Codes for isolates are provided in Table 2-1.

CHAPTER 3
CR(VI) REDUCTION BY A CONSORTIUM OF GRAM POSITIVE FERMENTATIVE BACTERIA

Introduction

Cr(VI) is mainly generated as an effluent by many industries. Due to its soluble nature it has been found in groundwater not only at the point source but also away from its source. Mutagenic and carcinogenic nature of Cr(VI) makes its contamination a matter of intense concern. Chemical processes that reduce Cr(VI) before it is discharged into the environment include chemical reduction, ion exchange and electrodepositing. The use of microbial reduction offers an inexpensive and long term alternative strategy to treat the Cr(VI)-contaminated soils and ground water. In order to implement the bioremediation strategy, it is important to understand microorganisms involved in Cr(VI) reduction and the mechanisms by which Cr(VI) is reduced. Data from enrichment studies (chapter 2) indicated that among the various carbon sources tested glucose was the preferred electron donor by the organisms to carry out the reduction of Cr(VI). Enrichments showing the fastest reduction of Cr(VI) had enriched gram positive bacteria belonging predominantly to *Clostridium* sp and *Cellulomonas* sp. Presence of fermentative bacteria like *Clostridium* sp. in metal contaminated sites has been described in the past, but no detailed study has been done to elucidate their role in metal reduction. Their major role in the metal contaminated environments has been described as being the provider for the carbon sources for other metal reducing bacteria. The metabolic products of fermentative

bacteria act as electron donors for other microorganisms that are involved in metal reduction.

Studies with consortium are significant as they provides a closer picture of what happens in the field where the microorganisms do not live in pure cultures. Pure culture studies are important to study the particular microbial activity and the consortium studies explain how the activity may be influenced by the presence of other organisms. Considering the above mentioned reasons the following study was done to understand the kinetics of Cr(VI) reduction by the cosortium GCAF isolated from glucose enrichment. The objectives of this study were to (i) monitor the kinetics of Cr(VI) reduction by the consortium GCAF, in the presence of Fe(III) and AQDS (ii) to elucidate the mechanism of Cr(VI) reduction by the consortium, (iii) to study the effect of Cr(VI) on the fermentation

Materials and Methods

Culture conditions for bacterial consortium. Fermentative consortium GCAF-1 was obtained during the previously conducted enrichment study (chapter 2). Glucose (10mM) was supplied as the electron donor. Cr(VI) (0.4 mM), Fe(III) (5 mM) and AQDS (0.1mM) were provided as electron acceptors. For inoculum purposes, the consortium was grown in the absence of any electron acceptor. All manipulations were made under an atmosphere of N_2-CO_2 (80:20).

Metal reduction experiments. Late log phase cultures were inoculated in fresh anaerobic medium with glucose as electron donor and incubated under 30ºC till early log phase was reached. The optical density of the inoculum was 0.6 (OD at 550nm). Media supplemented with appropriate electron donor and acceptors was inoculated with 1 % inoculum. Experimental set up consisted of eight different treatments.The treatments

consisted of basal medium supplemented with (i) glucose and all three electron acceptors, (ii) glucose, Cr(VI) and Fe(III), (iii) glucose and Cr(VI), (iv) glucose, Fe(III) and AQDS, (vi) glucose and no electron acceptors, (vii) all three electron donors and no glucose and, (viii) glucose and all three electron acceptors and no bacterial inoculation. The concentration of glucose, Cr(VI), Fe(III) and AQDS were maintained as in the original consortium. All the addition of electron acceptors and donors was done separately from sterile stock solutions. After inoculation the experimental cultures were incubated at 30ºC throughout the study. Samples were taken with sterile syringe and needles that were flushed with N_2 to avoid any contamination of the cultures with oxygen at appropriate time intervals. Each test was performed in triplicates.

Determination of cell numbers. Number of cells in the cultures were determined by direct count using acridine orange stain and the fluorescence microscope. Dilutions of the cultures were made wherever necessary to keep the cell count within the range of 50-100 per field area. 25% gluteraldehyde was used to fix the cells for counting. Cells were suspended in oxalate solution prior to counting to dissolve any insoluble Fe-oxides in the solution.

Chemical analyses. Chromium analysis was performed by colorimetric method using UV/Vis spectrophotometer (Shimadzu) as mentioned previously (chapter 2). Glucose analyses was done by the colorimetric method using UV/Vis spectrophotometer at 490nm. Sample was filtered through 0.2µm filter. 200µl of 5% phenol was added to the equal amount 200µl of sample. Immediately 1ml of conc. H_2SO_4 was added to the mixture and gentally shaken. The reaction was kept stationary for 30 minutes to allow the solution to cool down. The solution was gently shaken before taking the reading at

490nm. Precipitate analysis was done by EDX. Organis acids formed as he fermentation products were measured by High pressure liquid chromatography (Waters Co.) equipped with a UV detector (Waters, Co). Aminex HP 87H column was used as the separating column (300 X 7.5 mm). Sulfuric acid (5mM) was used as an eluent at the flow rate of 0.6 ml/ minute.

Electron microscopy. Precipitates formed in the consortium was obtained by using the microcentrifuge (12000X g), rinsed twice with distilled water, and air dried on carbon coated mounts prior to viewing it via scanning electron microscopy.

Results and Discussion

Composition of Fermentative Consortium GCAF

Active Cr(VI) reducing consortium isolated from the glucose enrichment was dominated by high G+C and low G+C gram positive bacteria. Fermentative consortium GCAF was unable to grow or reduce Cr(VI) when Cr(VI) was added as the sole electron acceptor to the medium. Growth of cells was inhibited by the toxicity of Cr(VI). However, in the presence of Fe(III) and AQDS, reduction of Cr(VI) was observed suggesting the possibiliy of Fe(III) alleviating the toxicity of the Cr(VI) to the cells of Consortium GCAF (Figure 3-1).

Biotic versus Abiotic Reduction of Cr(VI)

Cr(VI) reduction was a biotic process that did not occur in medium that was not inoculated with cells. No reduction of Cr(VI) was observed in the medium in the absence of the electron donor suggesting that the biotic reduction of Cr(VI) required the metabolically active cells.

Kinetics of Cr(VI) Reduction

Fermentative consortium GCAF was able to reduce Cr(VI) under anaerobic conditions. Presence of other electron acceptors had an effect on the amount of Cr(VI) reduced, the rate at which Cr(VI) was reduced, and the rate at which glucose was oxidized by the consortium GCAF. Media that were supplemented with other electron acceptors showed high turbidity indicative of bacterial growth. This was also confirmed by observing the sample under the microscope. Cr(VI) reduction was also observed in the active cultures. Complete reduction of Cr(VI) was observed in medium with AQDS and Fe(III) as additional electron acceptors. However, in the absence of AQDS only 66% of Cr(VI) was reduced. The rate of reduction of Cr(VI) was slower in the absence of AQDS. Similar trends were observed in the oxidation of glucose. In the absence of AQDS complete glucose (10mM) was not utilized and the rate of oxidation was much slower. These results suggested that the electrons being generated by oxidation of glucose, by the consortium were being transferred to Cr(VI) via Fe(III) and AQDS. In the absence of AQDS, the electrons were shuttled from the cells to the insoluble Fe(III). Soluble Fe(II) was then behaving as an electron shuttle and transferring the electrons to Cr(VI). Due to the requirement of contact between bacterium and insoluble Fe(III) to transfer electrons, the process was slow. This affected the overall reduction of Cr(VI). In presence of AQDS there is a higher turnover of the electrons and faster reduction of Fe(III). This in turn increases the rate of Cr(VI) reduction. AQDS is soluble unlike Fe(III) and it alleviates the need for contact between the cells and the metal. It accepts two electrons unlike Fe(III) that can accept only one electron at a time. Therefore higher reduction of Fe(III) occurs in the presence of AQDS which in turn augments the rate of Cr(VI) reduction. The higher rate of Cr(VI) reduction corresponds well to the high rate of glucose consumption in the

presence of AQDS. In the absence of Cr(VI) the rate of glucose consumption was much higher suggesting that Cr(VI) effects the metabolic machinery of the cells.

The initial color of the medium was yellow but with the reduction of Cr(VI) it became colorless. Insoluble amorphous Fe(III) was red in color that reduced to white precipitate that had disc shaped crystals when seen under a SEM microscope(Figure3-6). The X-ray diffraction analysis indicated this precipitate to be vivianite

Effect of Electron Donor on Cr(VI) Reduction

Concentration of electron donor in the medium affected the amount of Cr(VI) reduced (Table 3-2). However the rate of reduction of Cr(VI) was not depedant on the concentration of the electron donor. These results have implications in the field of bioremediation. Limitation of electron donors in the environment can impede the microbial reduction of Cr(VI) by the fermentative organisms.

Effect of Cr(VI) Reduction on Cell Growth in Consortium GCAF

The final cell concentration was lower by 47% in the medium in the presence of Cr(VI). Although presence of AQDS in the medium with Fe(III) increased the rate of Cr(VI) reduction, there was no significant difference in the cell numbers observed when compared with those in the absence of AQDS (Table 3-2).

Effect of Cr(VI) on Metabolites

Since consortium GCAF comprised mainly of fermentative bacteria effect of Cr(VI) on the fermentative products was determined. The variability in the rate of glucose oxidation during Cr(VI) reduction in presence of different electron acceptors suggested varied rates of product formation. Furthermore, reduction of external electron acceptors by the fermentative bacteria suggested some change in the fermentative metabolic products. Therefore the production of fermentation products of the consortium

were monitored with time. The results observed showed that formation of products corresponded well with the time when oxidation of glucose started and the production carried on till glucose was consumed (Figure 3-3, 3-4, 3-5).

The major fermentation products that were generated by the consortium GCAF when grown in presence of glucose were acetate, butyrate and lactate. No change in the proportions of products was observed when Fe(III) and AQDS was added to the medium. However, there was a shift in fermentation product pattern observed when Cr(VI) was added to the medium containing Fe(III) and AQDS as other external electron acceptors. Oxidation of glucose by the cells in consortium resulted in significantly less amount of butyrate, and more amount of acetate in the presence than in the absence of Cr(VI) (Table 3-3). These results suggested that the reduction of Cr(VI) effects the cells to shift the fermentation products to more oxidative forms. During the glucose fermentation by the fermentative cells, glucose is oxidized to pyruvate and generates two molecules of NADH (four reducing equivalents). The oxidative decarboxylation of pyruvate generates one molecule of formate and one molecule of acetyl CoA. Acetyl CoA has two alternative fates: it either forms acetate with a generation of ATP or can sacrifice the generation of energy by forming a more reduced form ethanol. The other possibilities for disposing reducing equivalents is by the formation of lactate. Lactate results in the reoxidation of one NADH. Butyrate formation is at the expense of 4 reducing equivalents (or 2 NADH). In the presence of Cr(VI) the drop in butyrate and lactate formation indicated that less of NADH were being reoxidized by those pathways. Increase in acetate could perhaps be the result of excess pyruvate being converted to acetate and formate. The energy generation step that is liked to acetate would prove beneficial to the

cell as it has to survive in the toxic environment of Cr(VI) and reduce it. The excess NADH that were not reoxidized by lactate and butyrate were then channeled towards the reduction of Cr(VI).Whether reduction of Cr(VI) occurs inside the cell membrane or on the cell surface was not clear at this point. No difference in the pattern of the products formed in the medium in the presence of Fe(III) and AQDS and in their absence indicated that mechanism for reduction of Fe(III) and AQDS was different from the way Cr(VI) was reduced by the cells in consortium GCAF.

So far there is no evidence that energy conservation by fermenting bacteria during reduction of Cr(VI). Although, reduction of Cr(VI) results in increased formation of acetate which is energetically favorable for fermenting bacteria.

Mechanism of Cr(VI) reduction by a pure strain isolated from this consortium GCAF has been explained in chapter 5. Identification and characterization studies of the isolate have been conducted in order to explain the phylogenetic importance of the strain. GCAF may be a good candidate for the bioremediation of heavy metal laden waters and sediments.

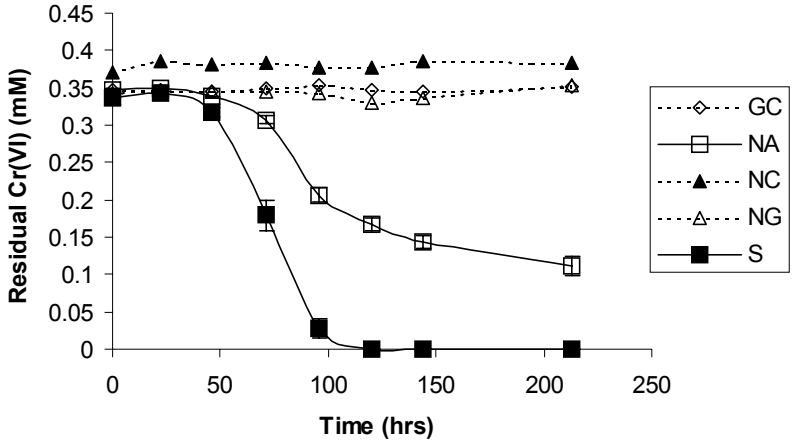


Figure 3-1. Cr(VI) reduction and removal from the solution as an insoluble precipitate. Glucose (10mM) was supplied as an electron donor. Fe(III) and AQDS were supplied as extra electron acceptors. Reduced insoluble Cr(VI) was removed from the solution by centrifugation prior to analysis. GC: glucose, Cr(VI); NA. Glucose, Cr(VI), Fe(III); NC. Glucose, Cr(VI), Fe(III), AQDS with no inoculation of cells; NG: Cr(VI), Fe(III), AQDS; S: glucose, Cr(VI), Fe(III) and AQDS.

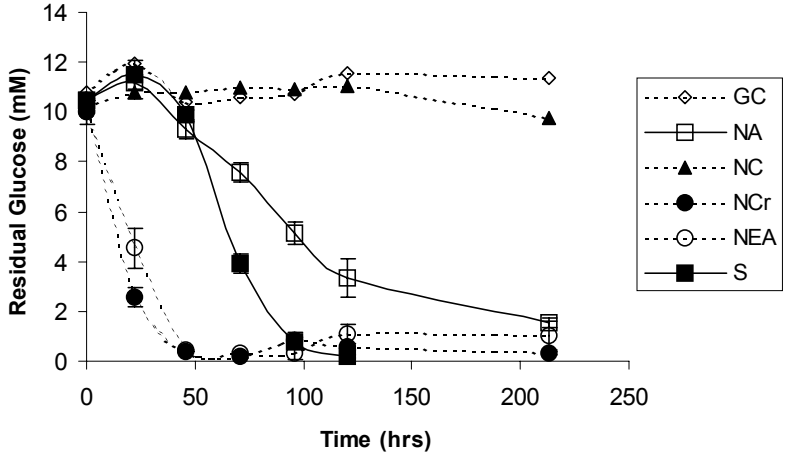
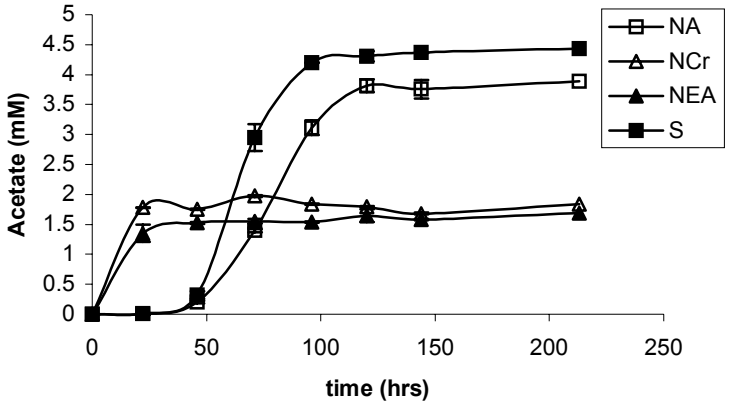


Figure 3-2. Glucose consumption by consortium GCAF-1 during reduction of Cr(VI) (0.4mM) in the presence of Fe(III) (5mM) and AQDS.(0.1 mM). GC: glucose, Cr(VI); NA. Glucose, Cr(VI), Fe(III); NC. Glucose, Cr(VI), Fe(III), AQDS with no inoculation of cells; NCr; glucose, Fe(III) , AQDS; NEA: glucose; S: glucose, Cr(VI), Fe(III) and AQDS.

A



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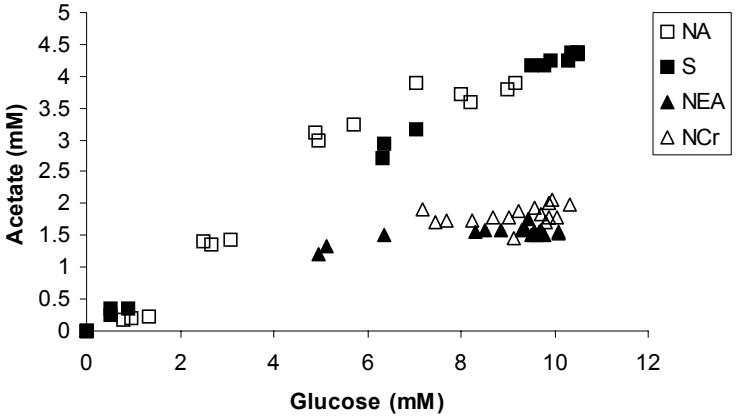

Figure 3-3. Acetate produced by oxidation of glucose by consortium GCAF.during the reduction of Cr(VI) in presence of added electron acceptors. (A) Acetate produced with respect to time. All treatments were set up in triplicates with eeror bars representing the standard error. (B) Acetate produced per mole of glucose consumed.NA. Glucose, Cr(VI), Fe(III); AQDS with no inoculation of cells; NCr; glucose, Fe(III) , AQDS; NEA: glucose; S: glucose, Cr(VI), Fe(III) and AQDS.

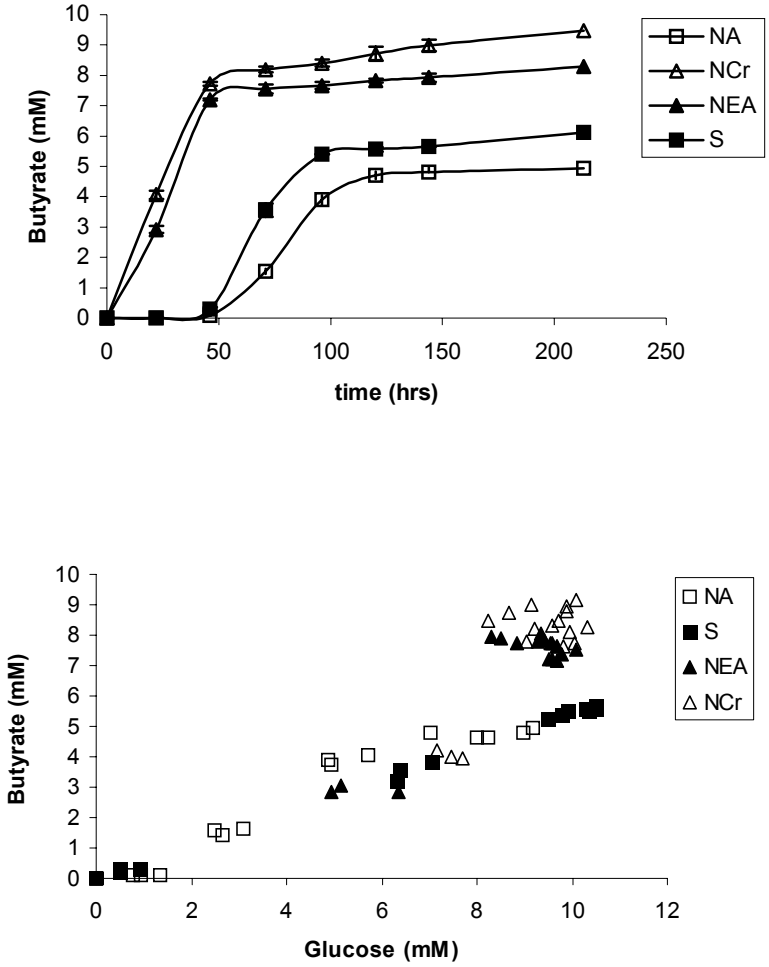

Figure 3-4. Butyrate produced by oxidation of glucose by consortium GCAF during the reduction of Cr(VI) in presence of added electron acceptors. (A) Butyrate produced with respect to time. All treatments were set up in triplicates with eeror bars representing the standard error. (B) Butyrate produced per mole of glucose consumed.NA. Glucose, Cr(VI), Fe(III); AQDS with no inoculation of cells; NCr; glucose, Fe(III) , AQDS; NEA: glucose; S: glucose, Cr(VI), Fe(III) and AQDS .

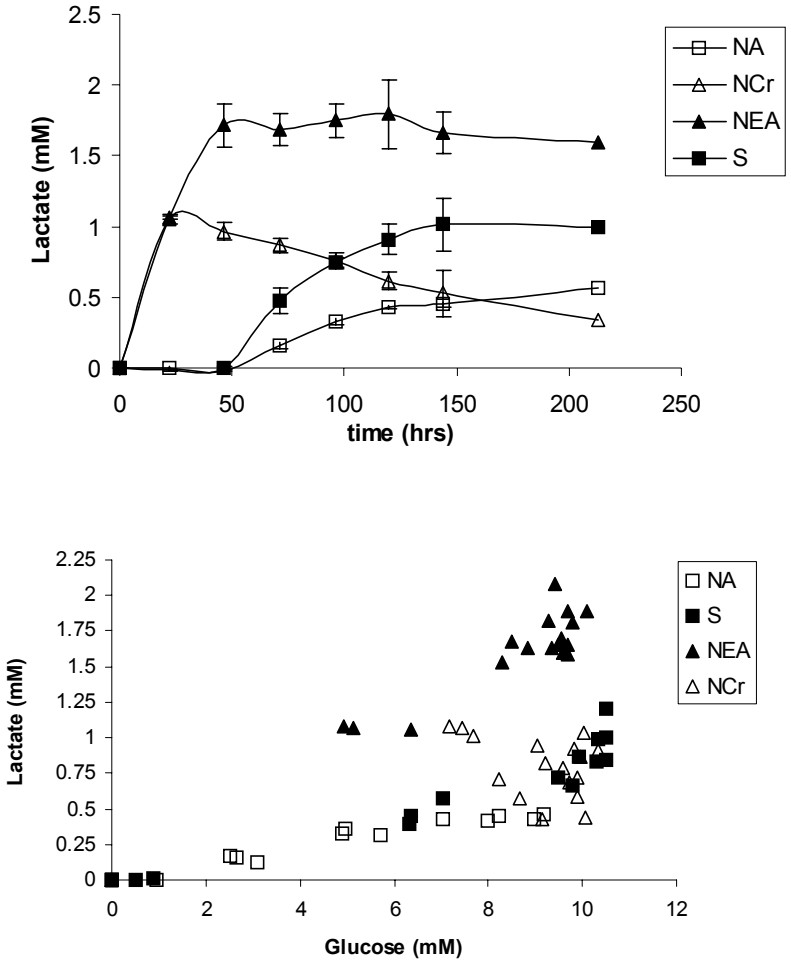

Figure 3-5. Lactate produced by oxidation of glucose by consortium GCAF during reduction of Cr(VI) in presence of added electron acceptors. (A) Lactate produced with respect to time. All treatments were set up in triplicates with eeror bars representing the standard error. (B) Lactate produced per mole of glucose consumed. NA. Glucose, Cr(VI), Fe(III); AQDS with no inoculation of cells; NCr; glucose, Fe(III) , AQDS; NEA: glucose; S: glucose, Cr(VI), Fe(III) and AQDS.

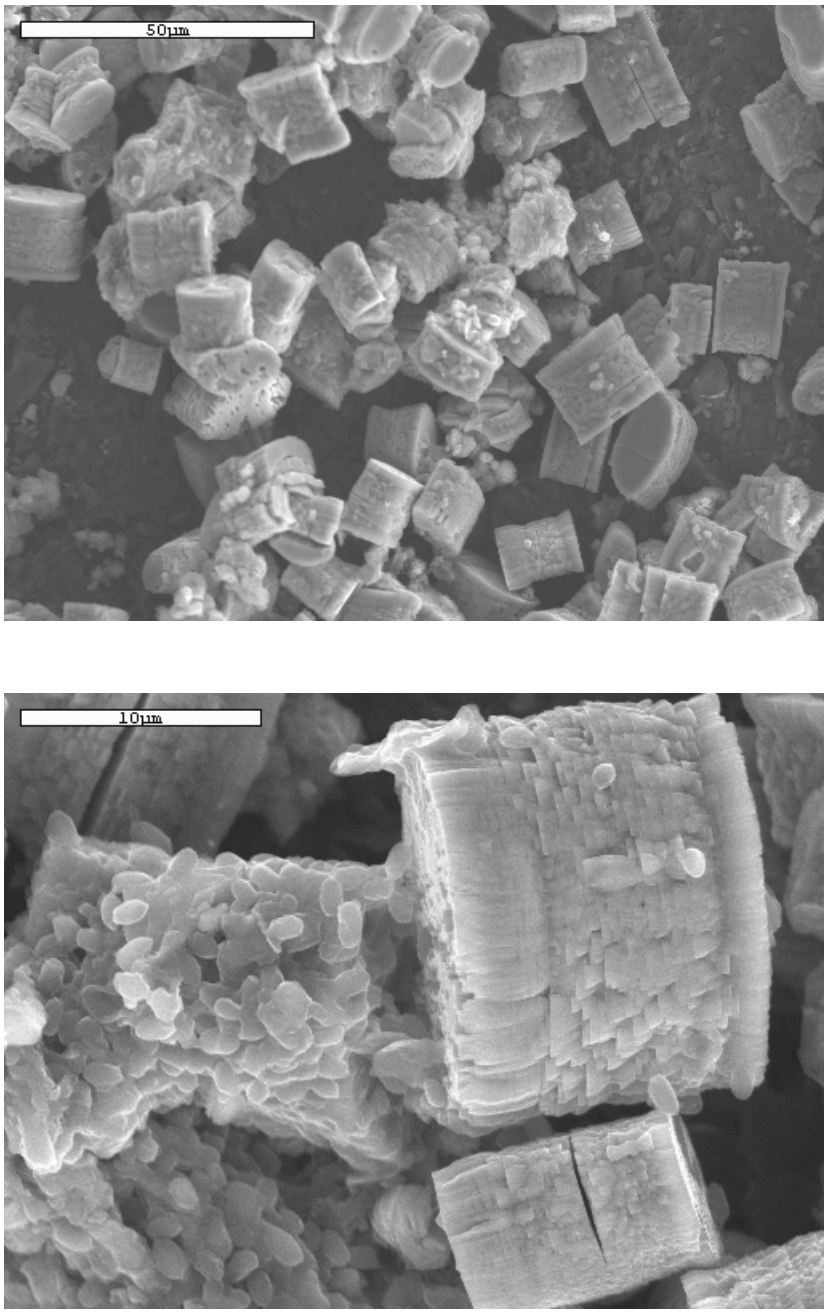

Figure 3-6. SEMs showing the insoluble precipitates formed by consortium GCAF-1 during the reduction of Cr(VI) via Fe(II)- and AQDS-mediated mechanisms

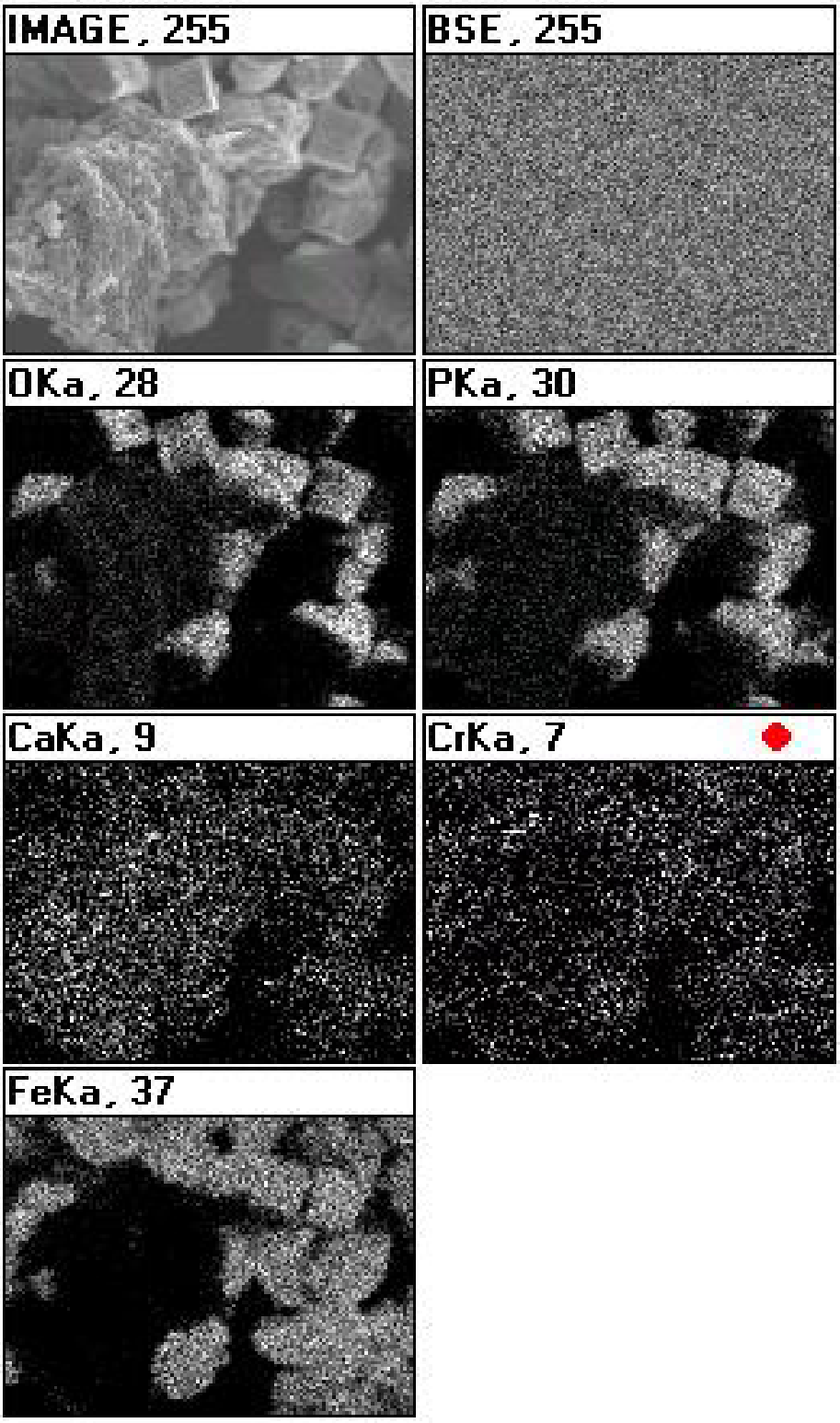

Figure 3-7. EDX of precipitate formed by consortium GCAF-1 showing the distribution of Cr in the precipitate formed during Cr(VI) reduction.

Table 3-1. Cell growth in the presence of different electron acceptors. Cells were grown in Basal Media supplemented with glucose as electron donor and Fe(III) as external electron acceptor. Cr(VI) and AQDS were added as described in the table. Cell counts were estimated by Acridine Orange Direct Count method using a fluorescence microscope. All treatments were set up in triplicates.

Treatment	Fe(III)	AQDS	Cr(VI)	Cell numbers/ ml (x 10^7)	Reduction in Cell number (%)
I	+	+	-	80.6 ± 4.6^a	-
II	+	+	+	50.3 ± 5.3^b	37.61
III	+	-	+	43.4 ± 1.6^b	47.18

Table 3-2. Cr(VI) reduction by consortium GCAF in presence of varying concentrations of electron donor.

Glucose Initial *(mM)*	Glucose utilized *(mM)*	Rate of Cr(VI) reduction *(mM/day)*	Amount of Cr(VI) reduced *(mM)*
5.73	5.62	0.066	0.19
10.92	9.05	0.066	0.34
17.96	8.42	0.066	0.37

Table 3-3. Effect of Cr(VI) on the on the pattern of products of glucose fermentation by consortium GCAF.

	AQDS	Fe(III)	Cr(VI)	Concentration of substrate consumed (*mM*)	Products formed (*mM*)		
					Acetate	Butyrate	Lactate
A	_	_	_	10	1.7	7.9	1.9
B	+	+	_	10	1.9	8.4	1.1
C	+	+	+	10	4.1	5.5	0.9

CHAPTER 4
IDENTIFICATION AND CHARACTERIZATION OF THE CHROMIUM REDUCING ISOLATE *CLOSTRIDIUM* SP. GCAF1

Introduction

In the last few decades, microbial metal reduction has been identified as an important process for mineralization of organic compounds (92) and for detoxification and remediation of soils contaminated with toxic metals (6, 88, 89). Microbial Cr(VI) reduction was first demonstrated by Romanenko (129), following which a wide diversity of CRB have been isolated. Cr(VI) reduction by organisms belonging to the genera *Bacillus, Escherichia*, *Pseudomonas*, and *Pantoea*, among others.. This dissertation describes the involvement of *Clostridium* and *Cellulomonas* species (Chapter 2) in Cr(VI) reduction. The presence of fermentative bacteria in Cr(VI)-contaminated soils and sediments have been reported previously, no detailed studies on the direct involvement of these bacteria in Cr(VI) reduction have been documented to date.

The genus *Clostridium* forms one of the largest gram-positive taxa and has significance in several fields. Many toxin producing pathogens (*Clostridium perfringens, C. botulinum, C. tetani and C. difficile*) and industrially important solvent producing fermentors (*C. acetobutylicum, C. butyricum, C. aceticum*) belong to this group (69, 145, 146, 172).

This study reports the discovery of a new spore forming bacterium, *Clostridium* sp. GCAF-1. This strain has an ability to reduce Cr(VI) directly and indirectly via Fe(III-reduction). Detailed results of extensive analysis of 16S rRNA gene sequences, DNA-

DNA homology and G+C content analyses are described here. Additional studies supporting the designation of GCAF-1 as a novel species of genus *Clostridium* are also reported.

Material and Methods

Source of sample and organism. Soil used as inocula for the enrichments originated from the Cr(VI) contaminated wetlands in Michigan. Tranfers were made several times into the fresh media to dissolve the soil particles out. transferred to Strain GCAF-1 was isolated from Cr(VI)-reducing anaerobic enrichment that was provided with glucose as electron donor and Fe(III) and AQDS as additional electron acceptors. The enrichments were amended with Fe(III) and AQDS as additional electron acceptors.

Media and growth conditions. Standard anaerobic culture techniques were used during the preparation of the medium (11). A bicarbonate buffered mineral medium, as described in chapter 2, was used for the growth of the strain GCAF-1. The final pH was adjusted with HCl ca 7.0 and N_2/CO_2 was bubbled through it to remove oxygen. The medium was dispensed into anaerobic pressure tubes or serum bottles under N_2/CO_2, sealed with thick butyl rubber stoppers and then sterilized by autoclaving. Electron donor, glucose (10mM) and appropriate electron acceptor [Cr(VI) (400µM); $Fe(OH)_3$ (5mM); AQDS (100µM)] was added later from sterile anaerobic stocks. All incubations were done in the dark under 30ºC without any shaking.

Isolation of strain GCAF-1. Strain GCAF-1 was isolated from the consortium enriched under glucose-Cr-Fe(III) and –AQDS conditions. All procedures were carried out under the anoxic conditions in a glove box equipped with charcoal filter and with $N_2/CO_2/H_2$ (v/v) as gas phase. Traces of oxygen were removed by circulating the gases over the palladium catalyst and desiccant.

Biochemical analyses. The biochemical features of the strain GCAF-1 were determined to establish its species status. Production of riboflavin (yellow pigment) by GCAF-1, under anaerobic conditions, was tested by inoculating sterile milk under anaerobic conditions and incubating at 30C for 24 hours (70). Sensitivity to antibiotic rifampicin was tested and turbidity was monitored to determine the resistant and sensitive nature of the strain GCAF-1(70).

Fatty acid analysis. Methyl esters of cellular fatty acids (FAME) were prepared according Metcalfe et al (105) and were analyzed using Microbial Identification System (MIDI, Inc., Newark, DE, USA). Fatty Acids were identified by comparison of retention times with those of commercial standards (Sigma Co., USA).

Determination of G+C content of the DNA. The base composition of the DNA was determined by Deutsche Sammlung von Mikrooganismen und Zellkulturen (DSMZ, Germany). DNA was isolated by cell disruption with French pressure cell. DNA was purified on hydroxylapatite columns (26). The DNA was further hydrolyzed with P1 nuclease and nucleotides were dephosphorlized with bovine alkaline phosphatase (104). The resulting deoxiribonucleosides were analyzed by HPLC using column SelectaPore 90M, C18, 5 µ m (250 x 4.6mm) equipped with guard column 201gd54H (Vydac, Hesperia, CA 92345, USA). Chromatography conditions were as follows: temperature 45ºC, 10 µl sample, solvent used was 0.3 M (NH_4) H_2PO_4 / acetonitrile, 40:1 (v/v), pH 4.4, flow rate 1.3 ml / min (adapted from Tamaoka and Komagata) (151). Non-methylated lambda DNA (sigma) with GC content of 49.85 mol% (104) was taken as standard. GC ratio calculated from the ratio of deoxyguanosine (dG) and thymidine(dT) according to Mesbah (1989) (104).

16S rRNA gene sequencing and phylogenetic analysis..Cells from actively growing culture of GCAF-1 were harvested by centrifuging at 3000xg. The pellet was washed three times and resuspended with a phosphate buffer saline. Suspended culture was lysed by boiling for fifteen minutes. The 16S rRNA gene was amplified by using the universal bacterial primers 27F (5`-AGAGTTTGATCCTGGCTCAG-3`) and 1492 R (5`-TACGGTTACCTTGTTACGACTT- 3`) (76). Purified PCR products were cloned using TOPO TA Cloning kit (Invitrogen, Life Technologies). 14 clones forming white colonies were chosen for further analysis. Insert DNA was amplified using M13 forward and reverse primers to under conditions described in appendix B. The insert was digested with *Hae III* and *Alu I* separately. Digestion reaction was set at 37ºC for 14 hours. The digestion pattern of the clones was compared on a 2% agar electrophoresis gel. Based on the results obtained, 16S rDNA amplification products of two clones were sent for sequencing. Sequencing was done at ICBR core sequencing facility at University of Florida with an automated sequencer.

Sequences obtained were compared with those available from public databases using BLAST search and were aligned with type strains showing 98% or greater similarity using GCG. Phylogenetic tree was constructed based on 1400 bp 16S rRNA sequences using PAUP version 4.0.

DNA-DNA hybridization. These studies were performed at the DSMZ with strains showing 98% (or higher 16S rDNA similarity) by thermal denaturation method described by DeLey et al (ref) with Gilford System 2600 spectrophotometer equipped with a Gilford 2527-R thermoprogrammer. DNA was isolated by chromatography on hydoxylapatite as described by Cashion et al. (26).

Results and Discussion

Cell morphology. GCAF-1 is a spore forming obligate anaerobe. Cells of strain GCAF-1 were rod shaped (4-7μ long and 1-1.5 μ in diameter), occurring singly or in chains of 4-7cells (Figure 4-1). Terminal and subterminal endospores were observed under scanning electron microscope (SEM). Spores were oval in shape (1-1.5 μ by 0.5 μ) (Figure 4-2). Spores constitute the dormant form of the bacterial cell that are produced during the advent of starvation. The spores are resistant to adverse conditions including high temperatures and organic solvents. Gram staining of GCAF-1 resulted in faint blue color that was regarded as positive. Cells were motile and motility could be demonstrated under electron microscopy when a freshly withdrawn sample was studied immediately Negative staining of GCAF-1 revealed the presence of peritrichous flagella and pili. Flagella represent the locomotory organelles of the cell. They are embedded in the cell membrane and extend through the cell envelope and project as a long strand. Flagella consist of many proteins including flagellin. Pili (synonym fimbriae) are hair like projections of the cell that may be involved in the sexual conjugation or may allow adhesion to the surfaces. Electron micrograph of the ultra thin section of the Cr(VI) reducing Clostridium sp GCAF-1 showed the presence of the S-layer. S-layers are the outer most component of the cell wall of several bacteria and archaea. They confer stability to the cell structure and protects cell from lytic enzymes.

Biochemical characteristics. The Cr(VI) reducing strain GCAF-1 reduced higher concentrations of Cr(VI) (400μM) completely in the presence of AQDS and Fe(III). In cultures with Cr(VI) as sole electron acceptor, complete reduction of Cr(VI) was observed only when Cr(VI) was present in low concentrations such as 20 μM. Reduction of Cr(VI) occurred simultaneously with the growth of the organism. The generation time

of this culture in the absence any electron acceptor was 2.04 Hrs. In the presence of 15μM of Cr(VI) the time increased to 2.5 hrs. However, when the concentration of Cr(VI) was further increased to 35μ and 50 μM the generation time also increased to 4.0 and 4.5 hrs respectively (Figure 4-3). Other substrates that were tested for the chromium reduction but were not utilized were lactate, acetate, butyrate, formate and citrate. GCAF-1 was found to be resistant to rifampicin. It did not curdle the milk in 24 hrs and yellow pigment, riboflavin formation was not observed. This strain seemed closer to the *C. beijerinckii* than to *C. acetobultylicum* based on the few physiological properties tested.

Chemotaxonomic Data.

Fatty acid analysis. Cellular fatty acids profile of the isolate GCAF-1 is given in Table 4-1. The most prevalent fatty acids were 16 and 18 carbon atoms. Hexadecanoic acid was most abundant. Dimethylacetals (DMA) were found in the esterified preparation. DMA`s are the esterification products of plasmalogens, unique lipids found in anaerobes (118).

DNA base composition. The G+C content of DNA from strain GCAF-1 was 30.7 mol %. The characteristics determined for strain GCAF-1 are summarized in Table 4-2

Phylogeny of Strain GCAF-1

Analyses of the 16S rRNA gene sequence. Comparative sequence searches of EMBL and Genbank databases revealed that 16S rDNA sequence of strain GCAF-1, was related to those of low G+C genus *Clostridium* of the gram-positive bacteria. Strain GCAF-1 clusters with group I organisms of genus *Clostridium*. Within this subphylum the highest sequence identity (98%) was obtained with 16S rRNA gene sequences of type strains of*, C. beijerinckii, C. saccharybutylicum, C. saccharoperbutylacetonicum, C. butyricum, C. roseum* (Table 4-3) (Figure 4-4).

***Clostridium* sp. GCAF-1 contains at least two 16S rRNA genes**. Restriction patterns obtained with *HaeIII* were identical for all DNA fragments obtained from 14 clones selected. However, two kinds of restriction patterns obtained with *Alu I* indicated the presence of more than one kind of 16S rRNA gene operon. Sequencing of the selected clones revealed the difference of 5 base pairs, one being at the site of Alu I.

The results obtained from the cloned 16S rDNA of isolate GCAF-1 suggests the possibility of at least two types of 16S r RNA gene operons, but may be greater. A difference of 5 bases (0.36%) can result from PCR- introduced errors (Figure 4-6). Alternatively there is a possibility of sequence heterogeneity between 16S rRNA gene operons as previously described in *E. coli* and *Clostridium paradoxum* (25, 107). Presence of multiple 16S rRNA genes with heterogeneous intervening sequences has been described in *Clostridium paradoxum* (127).

Such results may have an implication on the microbial ecology studies, wherein the group of highly related environmental 16S rDNA clone sequences obtained from many environments may represent not a group of separate, phylogenetically highly related strain but rather the sequence heterogeneity of the 16S rDNA contained within one strain.

DNA -DNA hybridization. As described previously**,** the 16S rRNA gene of 5 Clostridial species showed similarity of greater than 97% with that of GCAF-1. DNA-DNA hybridization was carried out as with these 5 type strains of genus *Clostridium* (Table 4-3). Determination of genomic similarities revealed that strain GCAF-1 had only 23% reassociation values with *Clostridium beijerinckii.*

Based on the general conclusion that strains with more than 97% 16S rRNA gene-sequence similarities that do not exhibit DNA-DNA homologies of 70% of more are accepted as being representatives of a single species (66, 168). GCAF-1 is a novel species in the cluster I of *Clostridium.* The 16S rDNA sequence is being submitted tot he Genbank.

In summary, isolate GCAF-1, obtained from a glucose-oxidizing Cr(VI) reducing enrichment was identified as a novel species belonging to genus *Clostridium*. The name *Clostridium chromoreductans* sp. nov is proposed. Other biochemical and chemotaxonomic data further describes the isolate GCAF-1. The Cr(VI) reducing ability of the organism is investigated in chapter 5 in detail. This strain can be used as a model organism to provide an insight to the role of fermentative bacteria in metal reduction

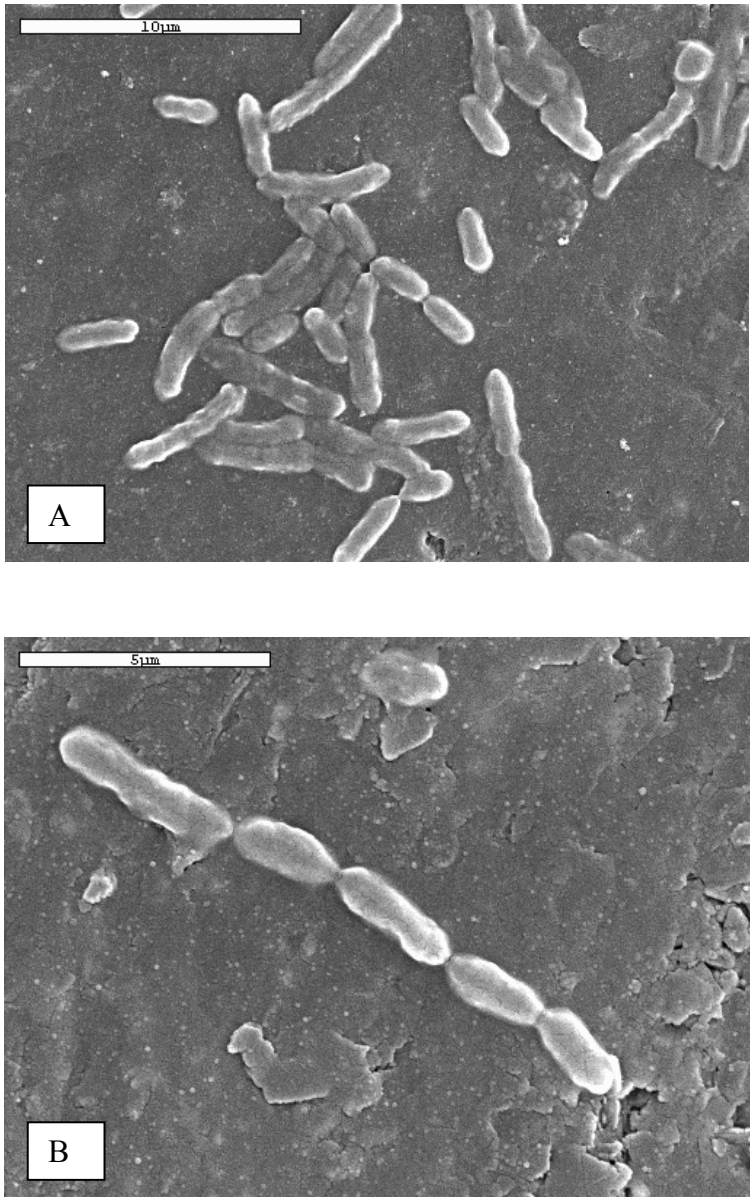

Figure 4-1. Scanning electron micrograph of isolate GCAF-1.A. Rod shaped cells. Scale bar, 10µM. B. Cells occur in chains. Scale bar, 5Mm.

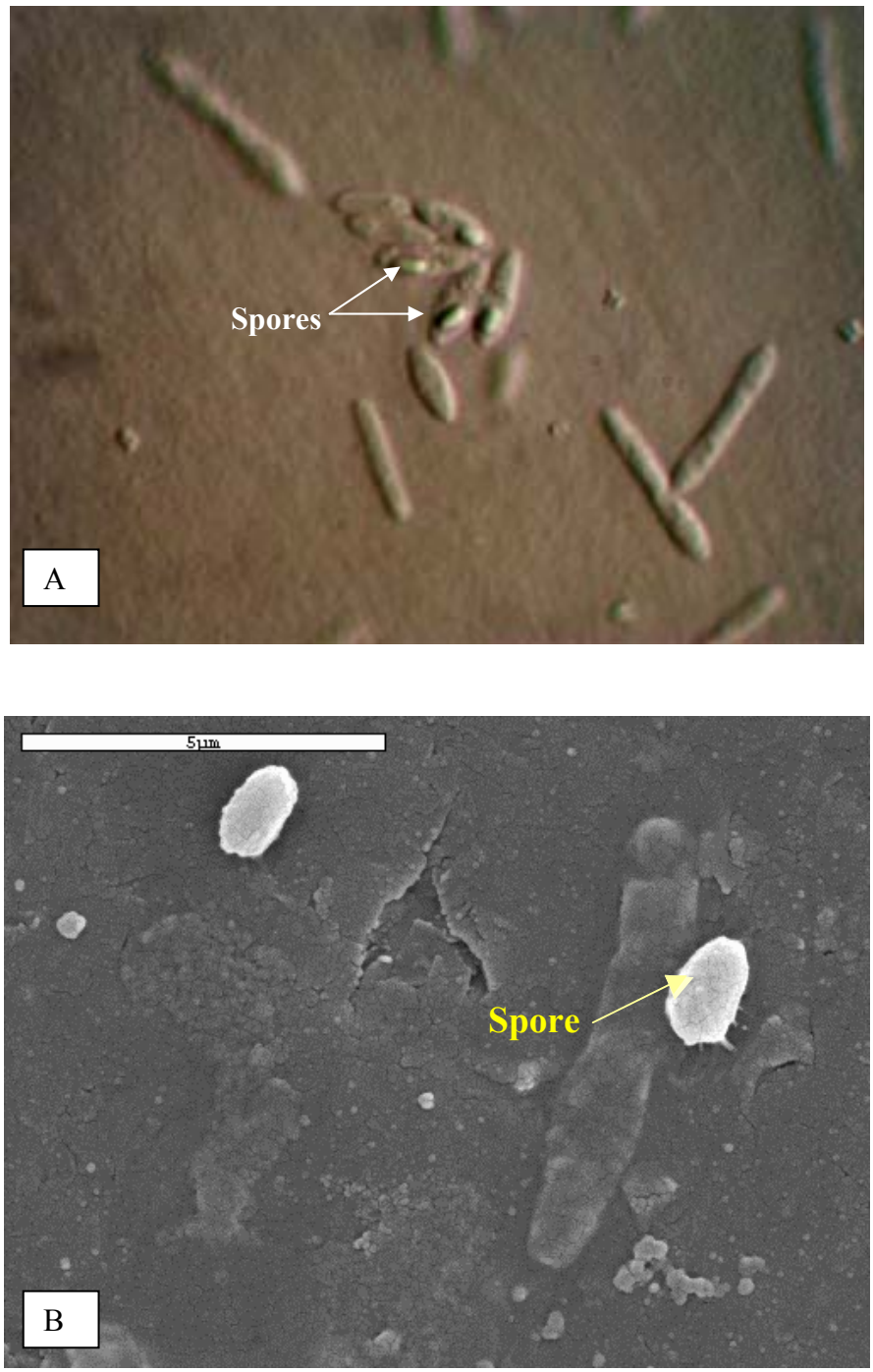

Figure 4-2. Micrograph of spores of isolate GCAF-1. A. Differential Interference image of sporulating cells with subterminal spores. B. Scanning electron microscopy of GCAF-1 spores. Scale bar 5µm.

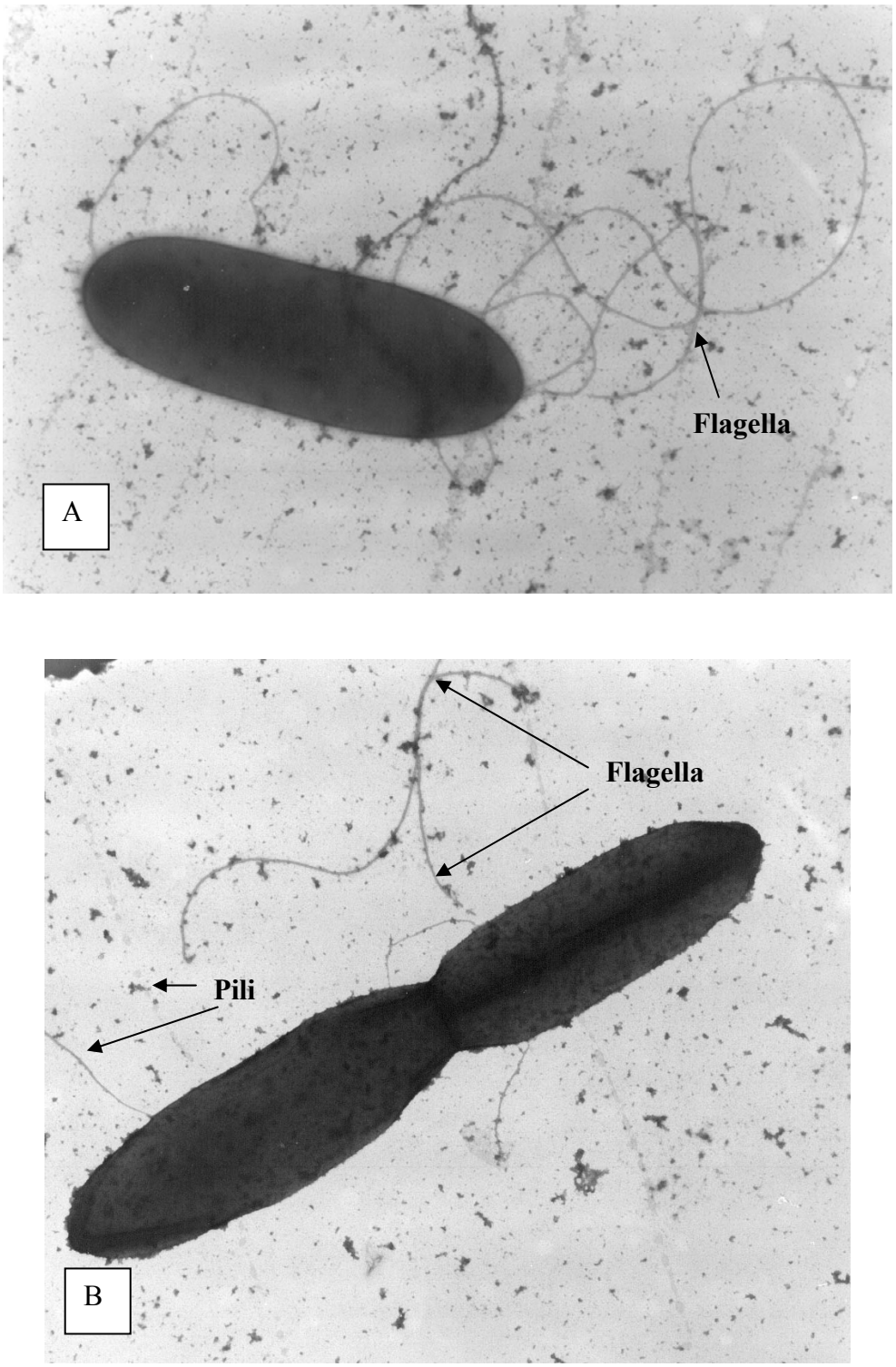

Figure 4-3. Negatively stained preparations of Cr(VI) reducing *Clostridium* sp. GCAF-1 showing peritrichous flagella. A. Bacterium showing peritrichous flagella. B. Dividing cells still maintain the flagella.

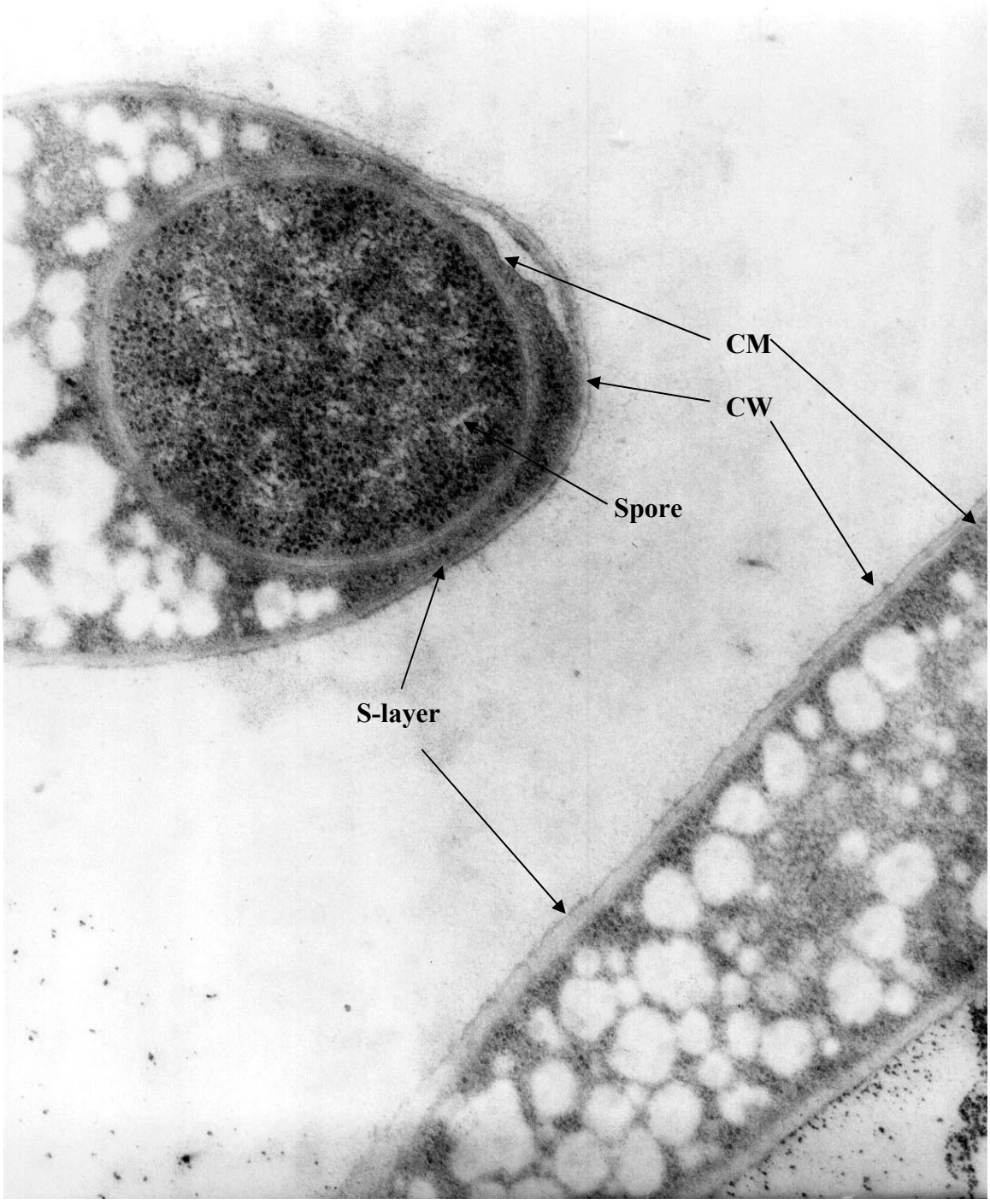

Figure 4-4. Electron micrograph of an ultra thin section of *Clostridium* sp. GCAF-1 showing the S-layer. Due to slight plasmolysis, the protoplast has drawn away from the cell wall. CM, cell membrane; CW, cell wall.

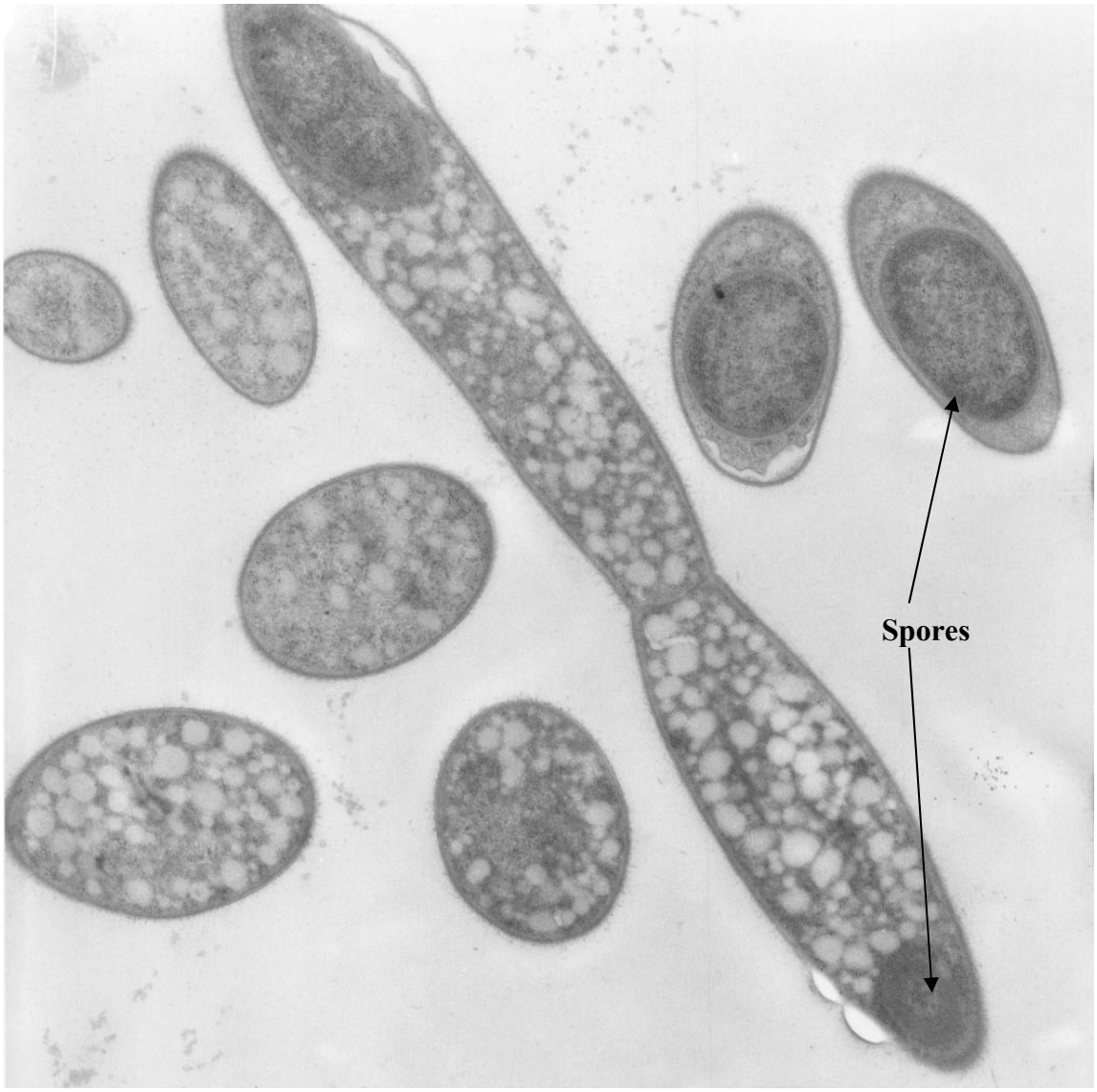

Figure 4-5. Electron micrographs of Cr(VI) reducing *Clostridium* sp. GCAF-1 showing the dividing cells containing terminal spores and glycogen inclusions in the cells.

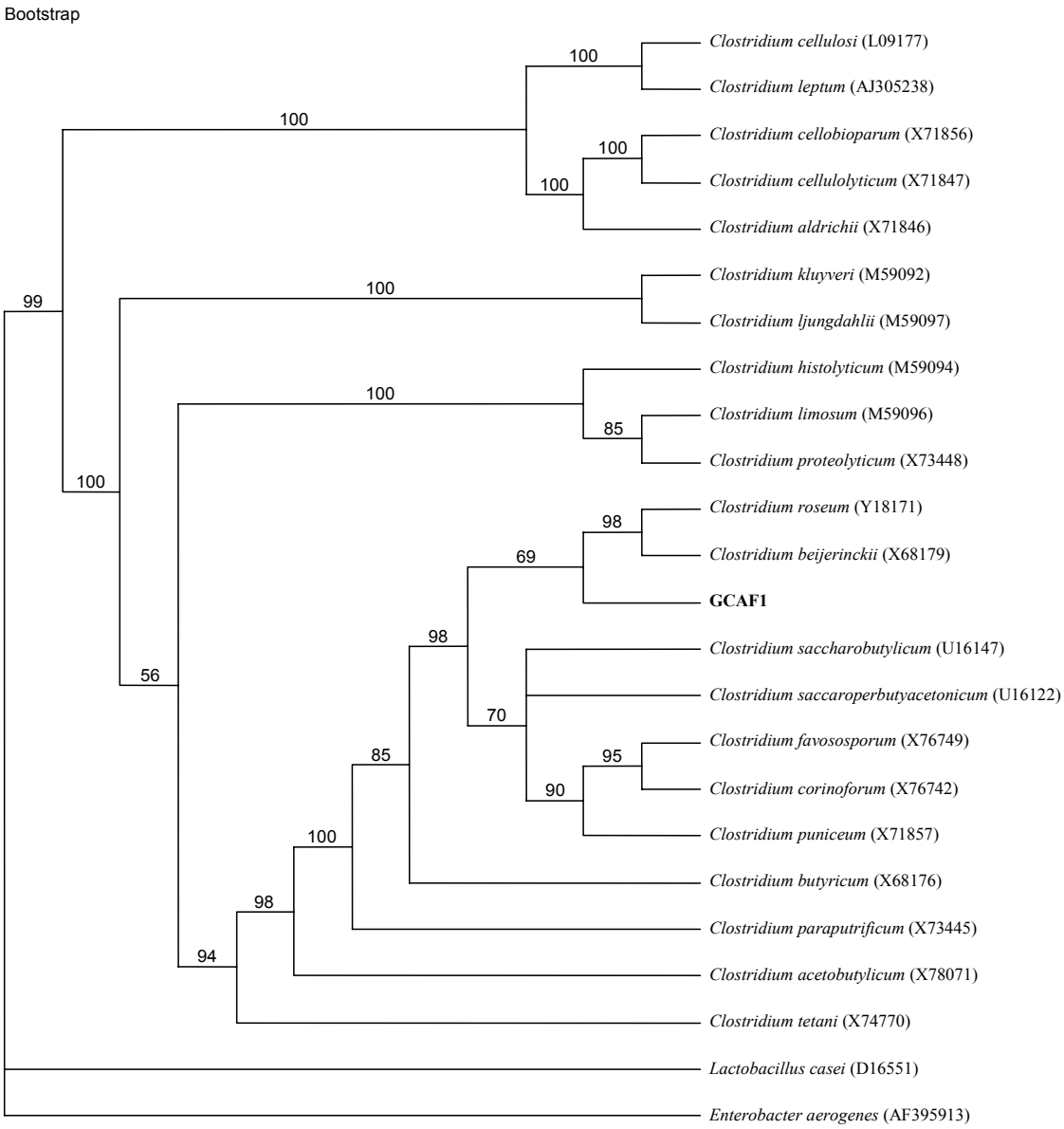

Figure 4-6. Phylogenetic tree based on 16S rDNA comparisons showing the relative position of strain GCAF-1 among other representative species of genus *Clostridium*. The branching pattern was generated by the neighbour joining method and the bootstrap values, shown at the nodes were calculated from 100 replicates. Bootstrap values less than 50% are omitted from the figure. Bar, 0.01 substitutions per nucleotide position. The gen bank acession numbers for the 16S r RNA sequences are given after the strain names.

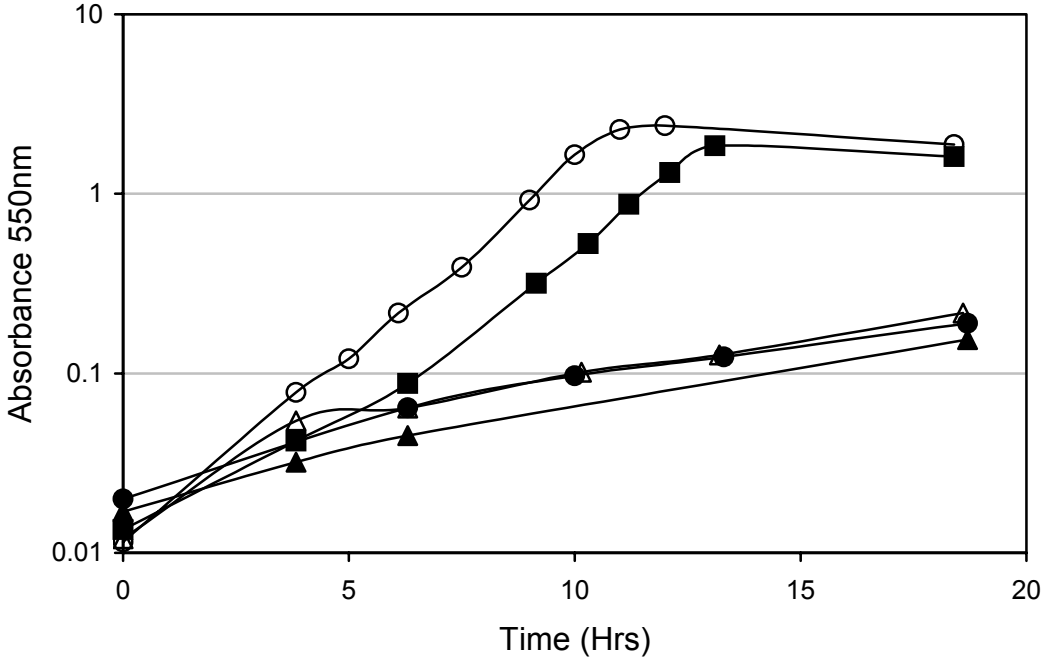

Figure 4-7. Anaerobic growth curve of GCAF-1 in under various Cr(VI) concentrations. ○--in absence of Cr(VI); --■-- in presence of 0.015 mM Cr(VI); --Δ-- 0.032 mM Cr(VI);---●--0.050 mM Cr(VI) ; ---▲-- 0.070 mM Cr(VI). [a]Values are represented by the average of the duplicates. [b]Growth rate was determined in cultures kept at 30ºC in dark without shaking.

Figure 4-8. Comparison of two 16S rRNA gene sequences from *Clostridium* sp. GCAF-1. Two sequences are represented by CLS-1 and CLS-2. □ highlight the different bases.

Identities = 1378/1383 (99%)
Strand = Plus / Plus

```
CLS-1: 13   gttccttcgggaacggattagcggcggacgggtgagtaacacgtgggtaacctgcctcat 72
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CLS-2: 1    gttccttcgggaacggattagcggcggacgggtgagtaacacgtgggtaacctgcctcat 60

CLS-1: 73   agaggggaatagcctttcgaaaggaagattaataccgcataagattgtagtttcgcatga 132
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CLS-2: 61   agaggggaatagcctttcgaaaggaagattaataccgcataagattgtagtttcgcatga 120

CLS-1: 133  aacagcaattaaaggagtaatccgctatgagatggacccgcgtcgcattagctagttggt 192
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CLS-2: 121  aacagcaattaaaggagtaatccgctatgagatggacccgcgtcgcattagctagttggt 180

CLS-1: 193  gaggtaacggctcaccaaggcgacgatgcgtagccgacctgagagggtgatcggccacat 252
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CLS-2: 181  gaggtaacggctcaccaaggcgacgatgcgtagccgacctgagagggtgatcggccacat 240

CLS-1: 253  tgggactgagacacggcccagactcctacgggaggcagcagtggggaatattgcacaatg 312
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CLS-2: 241  tgggactgagacacggcccagactcctacgggaggcagcagtggggaatattgcacaatg 300

CLS-1: 313  ggggaaaccctgatgcagcaacgccgcgtgagtgatgacggtcttcggattgtaaaactc 372
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CLS-2: 301  ggggaaaccctgatgcagcaacgccgcgtgagtgatgacggtcttcggattgtaaaactc 360

CLS-1: 373  tgtctttggggacgataatgacggtacccaaggaggaagccacggctaactacgtgccag 432
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CLS-2: 361  tgtctttggggacgataatgacggtacccaaggaggaagccacggctaactacgtgccag 420

CLS-1: 433  cagccgcggtaatacgtaggtagcaagcgttgtccggatttactgggcgtaaagggagcg 492
            |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
CLS-2: 421  cagccgcggtaatacgtaggtggcaagcgttgtccggatttactgggcgtaaagggagcg 480

CLS-1: 493  taggtggatatttaagtgggatgtgaaatactcgggcttaacctgagtgctgcattccaa 552
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CLS-2: 481  taggtggatatttaagtgggatgtgaaatactcgggcttaacctgagtgctgcattccaa 540

CLS-1: 553  actggatatctagagtgcaggagaggaaagtagaattcctagtgtagcggtgaaatgcgt 612
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CLS-2: 541  actggatatctagagtgcaggagaggaaagtagaattcctagtgtagcggtgaaatgcgt 600

CLS-1: 613  agagattaggaagaataccagtggcgaaggcgactttctggactgtaactgacactgagg 672
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CLS-2: 601  agagattaggaagaataccagtggcgaaggcgactttctggactgtaactgacactgagg 660

CLS-1: 673  ctcgaaagcgtggggagcaaacaggattagataccctggtagtccacgccgtaaacgatg 732
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CLS-2: 661  ctcgaaagcgtggggagcaaacaggattagataccctggtagtccacgccgtaaacgatg 720
```

```
CLS-1: 733  aatactaggtgtaggggttgtcatgacctctgtgccgccgctaacgcattaagtattccg 792
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CLS-2: 721  aatactaggtgtaggggttgtcatgacctctgtgccgccgctaacgcattaagtattccg 780

CLS-1: 793  cctggggagtacggtcgcaagattaaaactcaaaggaattgacgggggcccgcacaagca 852
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CLS-2: 781  cctggggagtacggtcgcaagattaaaactcaaaggaattgacgggggcccgcacaagca 840

CLS-1: 853  gcggagcatgtggtttaattcgaagcaacgcgaagaaccttacctagacttgacatctcc 912
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CLS-2: 841  gcggagcatgtggtttaattcgaagcaacgcgaagaaccttacctagacttgacatctcc 900

CLS-1: 913  tgaattacccttaatcggggaagcccttcggggcaggaagacaggtggtgcatggttgtc 972
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CLS-2: 901  tgaattacccttaatcggggaagcccttcggggcaggaagacaggtggtgcatggttgtc 960

CLS-1: 973  gtcagctcgtgtcgtgagatgttgggttaagtcccgcaacgagcgcaacccttattgtta 1032
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CLS-2: 961  gtcagctcgtgtcgtgagatgttgggttaagtcccgcaacgagcgcaacccttattgtta 1020

CLS-1: 1033 gttgctaccatttagttgagcactctagcgagactgcccgggttaaccgggaggaaggtg 1092
            ||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CLS-2: 1021 gtcgctaccatttagttgagcactctagcgagactgcccgggttaaccgggaggaaggtg 1080

CLS-1: 1093 gggatgacgtcaaatcatcatgccccttatgtctagggctacacacgtgctacaatggct 1152
            |||||||||||||||||||||||||||||||||||||||||  ||||||||||||||||||||
CLS-2: 1081 gggatgacgtcaaatcatcatgccccttatgtctagggccacacacgtgctacaatggct 1140

CLS-1: 1153 ggtacagagagatgctaaaccgcgaggtggagccaaacttcaaaaccagtctcagttcgg 1212
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CLS-2: 1141 ggtacagagagatgctaaaccgcgaggtggagccaaacttcaaaaccagtctcagttcgg 1200

CLS-1: 1213 attgtaggctgaaactcgcctacatgaagctggagttgctagtaatcgcgaatcagaatg 1272
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CLS-2: 1201 attgtaggctgaaactcgcctacatgaagctggagttgctagtaatcgcgaatcagaatg 1260

CLS-1: 1273 tcgcggtgaatacgttcccgggccttgtacacaccgcccgtcacaccatgagagttggca 1332
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CLS-2: 1261 tcgcggtgaatacgttcccgggccttgtacacaccgcccgtcacaccatgagagttggca 1320

CLS-1: 1333 atacccaaagttcgtgagctaaccgcaaggaggcagcgacctaaggtagggtcagcgatt 1392
            |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
CLS-2: 1321 atacccaaagttcgtgagctaaccgtaaggaggcagcgacctaaggtagggtcagcgatt 1380

CLS-1: 1393 ggg 1395
            |||
CLS-2: 1381 ggg 1383
```

Table 4-1. Cellular fatty acid composition of GCAF-1 grown with 10mM glucose.

Component		Fatty acids in profile (area%)
14:0	FAME	5.44
16:0	ALDE	1.14
16:1	CIS 7 FAME	4.25
16:1	CIS 9 FAME	6.73
16:0	FAME	29.46
16:1	CIS 9 DMA	2.37
16:0	DMA	5.44
18:1	CIS 9 FAME	22.58
18:1	CIS 13 FAME	0.57
18:0	FAME	2.97
18:1	CIS 9 DMA	3.42
18:1	CIS 11 DMA	1.73
20:1	CIS 11 FAME	0.52
ECL	Unknown 14:762 (15:2 FAME, 15:1 CIS 7)	1.61
15:0	ANTEI 3 OH FAME, 16:1 CIS 7 DMA	2.08
ECL	Unknown 16:760 (17:2 FAME , 17:1 CIS 8 FAME)	0.96
ECL	Unknown 16:801 (17:1 CIS 9 FAME, 17:2 FAME)	0.56
ECL	Unknown 17:834 (18:1 c11/t9/t6/ FAME)	5.84
ECL	Unknown 18:622 (19:0 ISO FAME)	0.63

The cellular fatty acid compositions were analyzed by Microbial ID, Inc.
In fatty acid designation the first and second numbers indicate the number of carbon atoms, and the number of double bonds, respectively.
Peaks lower than 0.5% in area are not represented.
[a]FAME, fatty acid methyl ester;
[b]DMA, dimethyl acetyl;
[c]ISO, iso;
[d]CIS, cis form double bond;
[e]c, cis form;
[f]ECL, Equivalent chain length;
[g]ALDE, aldehyde;
[h]ANTEI, anteiso;
[i]xOH indicates the position of hydoxylation;
[j]t, trans from double bond.

Table 4-2. Characteristics of GCAF-1

Organism	**GCAF-1**
Genus	Clostridium , cluster I
G+C Mol content	30:7%
Carbon sources not utilized	Acetate, Lactate, butyrate, Lactate
Resistance to Rifampicin	+
Riboflavin formation in milk	-
Facultative growth	-
Spore forming	+
Spore size	1-1.5 µ by 0.5µ
Length of rods	4.0-7.0 µ y 1.5µ
MIC of Cr(VI)	> 0.1 mM
Major fermentation products	Acetate, butyrate, lactate[a]
Motility	motile

a Fermentation products as determined by HPLC equipped by UV /vis detector:

Table 4-3. Sequence similarity between 16S rRNA gene of isolate GCAF-1 and type strains of the genus *Clostridium* showing closest similarity.

Species	Strain	Sequence similarity of 16S rDNA to isolate (%)	DNA DNA hybridization with the isolate (%)	Reference
C. *acetobutylicum*	[a]ATCC 824, [b]DSM 792, [c]NCIB, 8052	91	[d]ND	Mc Coy Emend Keis
C. butyricum	ATCC 19398 DSM 10702, DSM 552	94	ND	Prazmowski
C. beijerinckii	ATCC 25752, DSM, 791, NCIB 9362	98	23.5	Donker Emend Keis
C. paraputrificum	ATCC 25780, DSM 2630, NCIB 10671	96	ND	Bienstocksnyder
C. puniceum	DSM 2619, NCIB 11596	98	32.9	Lund
C. roseum	ATCC 17797, DSM 2619, NCIB 11596	98	33.6	Mc Coy Cato
C. saccharobutylicum	ATCC BAA-117, DSM 13864	98	[e]ND	Keis
C. sacchaoperbutylicum	ATCC 27021, DSM 14923	98	36.3	keis

Similarity higher than 97% is indicative of sequences belonging to same genus.
[a]ATCC American Type Culture Collection, Manassas, VA, USA
[b]DSM: Cultures from Deutsche Sammlung von Mikroorganismen und Zelkulturen, Braunschweig, Germany
[c]NCIMB: National Collections of Industrial, Food and Marine Bacteria, Abeerdeen, UK.
[d]ND: Not determined
[e]ND: Not determined. Biochemical test conducted.

CHAPTER 5
ELECTRON SHUTTLE-MEDIATED CHROMIUM REDUCTION BY *CLOSTRIDIUM* sp GCAF-1

Introduction

Strictly anaerobic fermentative organisms, although ubiquitous in the environment, have not been studied extensively in terms of heavy metal remediation. Direct metal reduction by fermentative organisms is not generally considered to be significant, however, there are a few reports documenting the ability of these obligate anaerobes to reduce metals such as iron and selenium (39, 118). The currently accepted hypothesis regarding the role of fermentative bacteria in metal reduction is that complete oxidation of fermentable compounds to carbon dioxide is coupled to Fe(III) reduction by the cooperative activity of fermentative and Fe(III) respiring bacteria (92) that utilize the fermentative products as electron donors. This does not consider that fermentative organisms may play a significant role in direct reduction of metals. Thereby it disregards any ecologically significant role of fermentative bacteria in direct reduction of metals.

Many bacterial strains have been shown to mediate reduction of Cr(VI) to Cr(III) both aerobically (22, 59, 75) and anaerobically (28, 93, 152, 163). Most of these organisms belong to the gram-negative group of bacteria. Cr(VI) reducing gram-positive bacteria reported to date, belong to the genera *Bacillus* (122), *Staphylococcus* (134) and *Micrococcus* (9). Although studies based on the preliminary screening of organisms isolated from Cr(VI) contaminated soil have reported the presence of gram-positive

fermentative bacteria in metal contaminated sites, there is no report documenting the active participation of fermentative organisms in Cr(VI) reduction.

Results from our enrichment studies (Chapter 2) show that the most rapid rate of Cr(VI) reduction was observed in enrichments amended with glucose as electron donor. Even though acetate is considered to be one of the most preferred electron donors for most metal reducing bacteria, our data indicated efficient chromium reducers to be among glucose utilizers. Isolation and identification studies revealed that the predominant enriched microorganisms belonged to the genus *Clostridium*. Cr(VI) reducing isolate GCAF-1 was obtained from this enriched culture. In order to understand the mechanisms adopted by GCAF-1 to reduce Cr(VI), detailed kinetic studies were undertaken. In doing so, direct and Fe-dependant indirect pathways for Cr(VI) reduction were identified.

These results suggest that the selective advantage of strain GCAF-1 in Cr(VI) contaminated environments, due to its ability to grow in the presence of typically toxic concentrations of Cr(VI) and to reduce high concentrations of Cr(VI) in presence of Fe(III) and humics, is of potential environmental relevance. This study not only adds to the growing list of organisms involved in the Cr(VI) reduction, but also suggests the significant role played by fermentative organisms in the reduction of Cr(VI).

Materials and Methods

Source of organism and isolation. Strain GCAF-1 was isolated from anaerobic enrichment cultures that were initiated with the soil sample from a Cr(VI) contaminated wetland in Michigan. Enrichment cultures were supplemented with glucose as electron donor and Cr(VI), AQDS and Fe(III) as electron acceptors. Strain GCAF-1 was isolated by using standard anaerobic technique of roll tube.

Cultivation of strain GCAF-1. Strict anaerobic techniques were used throughout the course of study as described previously (87). All incubations were in dark at 30ºC unless specified. Medium was prepared in serum bottles and bubbled with (N_2/ CO_2 :: 80: 20) to remove the dissoled oxygen. These bottles were then capped with blue butyl rubber stoppers and aluminum crimp seals under (N_2/ CO_2 :: 80: 20). Media was sterilized by autoclaving it for 30 minutes. Appropriate electron donor and acceptors were added from sterile stock solutions. For routine maintenance of cultures, 10 ml of medium were dispensed into anaerobic pressure tubes (Bellco glass, Inc., Vineland, N.J) and sparged with appropriate gas mixture for 10 minutes before sterilizing the medium by autoclaving.

Cell growth and kinetics study. Growth of cells was monitored during the kinetic study by cell counts with acridine orange direct count. For the kinetics study, 48 ml of basal medium was dispensed into 120 ml serum bottles and sparged for 30 minutes with appropriate gas mixture to remove traces of oxygen from the medium. Medium was sterilized by autoclaving it for 30 minutes. Glucose was added as electron donor for all treatments except in controls that were set up with no electron donor. To determine the effect of Fe(III) and AQDS on Cr(VI) reduction, several treatments were set up with different combinations of electron acceptors: (i) Cr(VI) with Fe(III)and AQDS (ii) Cr(VI) with Fe(III), (iii) Cr(VI) with AQDS, and (iv) Cr(VI). In order to account for the effect of Fe(III) and AQDS on the metabolism of strain GCAF-1 another treatment with Fe(III) and AQDS in the absence of Cr(VI) was set up as a control. $Fe(OH)_3$ was synthesized by titrating a solution of $FeCl_3.6H_2O$ with 10% NaOH to pH of 9.0. Cr(VI) was provided in the form of $K_2Cr_2O_7$. Anthraquinone di-sulfonate (AQDS), humic acid analog (Sigma)

was used to mimic the effect of humic acids on Cr(VI) reduction. Appropriate addition of electron donor and acceptors were made to the bottles to get a final concentration of glucose (10 mM), Cr(VI) (0.4 mM), Fe(III) (5 mM), and AQDS (0.1mM) in the media. Samples were taken from serum bottles with a syringe.

Analytical techniques. Cell enumeration was done by staining the cells with acridine orange and counting the cells under epifluorescence microscope as previously described (87). 0.1 ml of cell culture was taken from the serum bottle and fixed with 0.1 ml of 25% gluteraldehyde for 10 minutes. 0.8ml of PBS was added to make up the volume to 1 ml. Samples were diluted in oxalate solution in order to dissolve the particulate forms of iron. Sample was passed through 0.2 µm millipore filter and stained with the acridine orange (5 drops of 0.1 M). After washing off the excess stain with filtersterlized distilled water, the filter was placed on the glass slide and viewed under the microscope. Cr(VI) concentrations were measured by colorimetric methods using UV spectrophotometer as described in chapter 2 (159). Glucose concentrations were determined spectrophotometrically at 450nm. Fermentation products were analyzed with high pressure liquid chromatography (HPLC, Waters) equipped with an Aminex HPX-87H column (7.8 by 300 mm column) (Biorad) and UV detector (Waters) at 210 nm. Sulfuric acid (5mM) was used as the eluent buffer. Flow rate was maintained at 0.6 ml min^{-1}.

Results and Discussion

Strain GCAF-1 was isolated from a Cr(VI) reducing enrichment culture that was supplemented with glucose as electron donor and Fe(III) and AQDS as additional electron acceptors. Earlier studies with Cr(VI) reduction have reported Fe(II) to be one of its ecologically significant reductant in soils (13, 24, 54, 138). Also the ability of

Clostridium beijerinckii and *C. butyricum* (118) to reduce Fe(III) to Fe(II) made Fe(III) a potential candidate to study the effect of Fe(III) on Cr(VI) reduction.

The Biphasic Mechanisms for Cr(VI) Reduction by Clostridium sp GCAF-1.

Strain GCAF1 was capable of direct and Fe-dependant indirect reduction of Cr(VI). It reduced 400 μM of Cr(VI) in the presence of Fe(III) and AQDS. In the absence of these additional electron acceptors strain GCAF-1 was able to reduce up to 100μM of Cr(VI). Cell growth in GCAF-1 cultures supplemented with Cr(VI) as sole electron acceptors, with concentrations higher than 100μM, was negligible. Toxicity of the heavy metal at higher concentrations may have prevented the cell proliferation. Growth rate of the cells of strain GCAF-1 decreased as the initial Cr(VI) concentration in the medium increased (Figure4-?). The fermentative growth rate of GCAF-1 was similar to that when 16μM of Cr(VI) was present in the medium (Figure 5-1). However, slight increase in lag phase is observed indicating that cells need to get acclimatized to Cr(VI) before they enter the logarithmic growth phase. Cr(VI) was completely reduced during growth. No growth was observed in medium with Cr(VI) concentration of 200μM. GCAF-1 was a fermentative spore-forming organism and formation of spores was observed in the fermentative culture in 24 hours. In presence of Cr(VI) delayed sporulation was observed and cells exhibited altered morphology. The cells appeared longer and much thinner than those grown in absence of Cr(VI) suggesting the effect of Cr(VI) on cell division of the cells as documented by Theodotou et al. (153). Negligible Cr(VI) reduction was noted in the absence of electron donor or in the absence of cells. These results indicated that reduction of Cr(VI) was biotic and was carried out by metabolically active cells.

In the presence of Fe(III) and AQDS, strain GCAF-1 was able to reduce higher concentrations of Cr(VI). An interesting observation was that reduction of Cr(VI) in medium containing both AQDS and Fe(III) (GCAF medium) was faster than the Cr(VI) reduction in the medium containing only Fe(III) (GCF medium). These results suggested that Fe(III) acted as an electron shuttle for the reduction of Cr(VI). In the presence of Fe(III), AQDS also formed a part of the train of shuttles transferring electrons to Cr(VI). Besides acting as electron shuttle, Fe(III) also played an important role in alleviating the Cr(VI) toxicity to the cells of GCAF-1. This was evident by the absence of cell growth and negligible reduction of Cr(VI) in culture medium supplemented with AQDS only. It must be noted that since strain GCAF-1 is fermentative it is capable of growing in the absence of electron acceptor and the therefore, the absence of Fe(III) should not have been the limiting factor for its growth. Toxicity of Cr(VI) concentration used in this study however, has been established earlier. Reduction of Cr(VI) corresponded well to the oxidation of glucose by strain GCAF-1 (Figure 5-2). There was negligible change in the glucose concentrations in the absence of cells. No oxidation of glucose was observed in cultures that showed no reduction of Cr(VI). Glucose oxidation by strain GCAF-1 was observed during its fermentative growth in the absence of any external electron acceptor. When the rate of glucose oxidation in GCF and GCAF media was compared there was no significant difference observed. The cell numbers in GCAF cultures were slightly higher when compared with those in GCF cultures (Figure 5-3). Cell growth in both the cultures ceased with the complete oxidation of glucose. However, complete reduction of Cr(VI) was only observed in GCAF medium. Incomplete and slower rate of Cr(VI) reduction was noted in the GCF medium. This indicated that electrons generated during glucose

oxidation by GCAF-1 are channeled more efficiently towards Cr(VI) reduction in the presence AQDS and Fe(II). This can be explained as follows. Reduction of Cr(VI) in the presence of Fe(III) depended on the reduction of Fe(III) by the cells. Fe(III) in this case acted as an electron shuttle. Under the prevailing pH conditions Fe(III) oxide is insoluble and the microbial reduction of Fe(III) requires the cells to be in contact with the metal oxide as described previously (ref). Presence of AQDS in the medium stimulates the reduction of Fe(III) by alleviating the need for the contact by the cells. AQDS is soluble and is capable of shuttling electrons from the cells to Fe(III) oxide. Reduced AQDS is oxidized once the electrons are transferred to metal oxide and it is ready to accept electrons again from the cells. Fe(II) generated directly or indirectly by microbial activity then reduces Cr(VI). Therefore the rate of Cr(VI) reduction was dependant on the reduction of Fe(III). Cr(VI) reduction by strain GCAF-1 was possible by four different pathways: (i) by Fe(II) that is directly generated by the cells, (ii) by Fe(II) that is reduced by reduced AQDS (iii) by reduced form of AQDS (iv) directly by the cells.

Effect of Cr(VI) on Production of Metabolic Products of Strain GCAF-1

In order to determine the effect of Cr(VI) on the metabolism of strain GCAF-1, the metabolites in different media were compared. Formation of metabolic products (Figure 5-4, 5-5, 5-6) corresponded well with the oxidation of glucose (Figure 5-2) and the reduction of Cr(VI) (Figure 5-1). Metabolites were detected in the spent growth medium of cells. As expected, absence of fermentation products was observed in media that did not show any oxidation of glucose. Oxidation of glucose by strain GCAF-1 in the absence of any external electron acceptor yielded acetate, butyrate and lactate as the three major fermentative products (Table 5-1). In the absence of Cr(VI), no change in the fermentation products was observed in growth media supplemented with Fe(III) and

AQDS. However, the effect of Cr(VI) on the metabolic products was apparent by the change in concentrations of acetate, butyrate, and lactate formed per 10 mM of glucose in the presence of Cr(VI) in the medium (Table 5-1). There was an increase in the acetate concentrations noted along with a decrease in the butyrate concentrations (Figure 5-4, 5-5) in GCAF in GCF cultures. This indicated the possibility that (i) Cr(VI) was directly reduced by strain GCAF-1, and (ii) Fe(III) and AQDS were reduced by strain GCAF-1 by a pathway that was different from that by which Cr(VI) was reduced.

Proposed Mechanism of Cr(VI) Reduction by Strain GCAF-1.

Based on the results presented above a hypothesis elucidating the pathway for Cr(VI) reduction by fermentative strain GCAF-1 is proposed. Generally, in glycolytic pathways the oxidation of one mole of glucose by Embden-Meyerhof-Parnas pathway yields two moles of pyruvate. This pathway also produces two NADH, plus two ATP molecules. The pyruvate is further decarboxylated to acetyl CoA, CO2, and h2 using pyruvate-ferredoxin oxidoreductase and hydrogenase. The acetyl CoA has two fates. Some of it is condensed to form acetoacetyl-CoA, which is reduced to β-hydroxybutyryl-CoA using one of the two NADHs. This product is reduced to butyryl-CoA using the second NADH. CoASH is displaced by inorganic phosphate and butyryl phosphate donates phosphoryl group to ADP and forms ATP and butyrate. Some aetylCoA is also converted to acetate via acetyl -P in a reaction that yields an additional ATP. Twice as much ATP is generated per acetate produced as opposed to butyrate. Mass balance of fermentation products formed (within 10% error) by *Clostridium* sp. GCAF-1 during oxidation of 10mM of glucose in the absence of Cr(VI) showed that 20 NADH produced by the oxidation of 10 mM of glucose, were used as follows: 15 NADH for butyrate, 1 NADH for lactate and 2 NADH used for NADH:ferredoxin oxidoreductase. In presence

of Cr(VI), 11 NADH were used for the formation of butyrate, 1 NADH was utilized for lactate formation and 1.2 for Cr(VI) reduction. 5.2 NADH was utilized for the reduction of Fe(III) and AQDS. In presence of Cr(VI), decrease in concentration of butyrate would result due to the channeling of electrons from NADH towards reduction of Cr(VI) and more ATP formation per acetyl CoA. Reduction of Cr(VI) was perhaps one of the defense mechanism adopted by the cell to detoxify the environment for its survival. Therefore, NADH was perhaps acting as electron source for the reduction of Cr(VI).

This speculation is further supported by several reports of microbial NADH dependant enzymatic reduction of Cr(VI) (10, 44, 52, 117, 149). The electrons from NADH are transferred through the electron transport chain and transported via a cytochrome to Cr(VI). Presence of cytochromes has been reported in some members of *Clostridium* sp. Decrease in formation of butyrate content was coupled with the increase in acetate concentrations. Increase in acetate concentrations are advantageous for the cell as it requires to make more energy to compensate for the energy expended for activating its defense mechanisms against the toxic Cr(VI) molecules. Whether Cr(VI) is used as an electron acceptor by the strain GCAF-1 is currently unclear. Location of Cr(VI) reduction by the cell is also not clear

Environmental Relevance of Cr(VI) Reduction by Gram Positive Spore-forming Fermentative Species

Presence of fermentative organisms in soil is ubiquitous. These organisms generally form the primary group of bacteria that oxidize the complex carbon sources into simple carbon forms that are utilized as electron donors by the known metal reducing bacteria. In anaerobic Cr(VI)- contaminated environments like the wetlands fermentative bacteria such as *Clostridium* sp GCAF-1 are active participants in reduction of Cr(VI) as

they can reduce directly reduce Cr(VI) in addition to providing carbon sources for most metal reducers. The spore forming ability of the organism is advantageous in the advent of nonconducive conditions for its survival. In environments with low concentrations of Fe(III) and humics, Cr(VI) can still be reduced by strain GCAF-1. These results suggest that microbial Cr(VI) reduction by *Clostridium* sp. GCAF-1 via Fe(II) is of potential environmental relevance.

Further investigation of Cr(VI) reduction by fermentative organisms is warranted so as to determine the environmental significance of this group of bacteria in field of metal reduction.

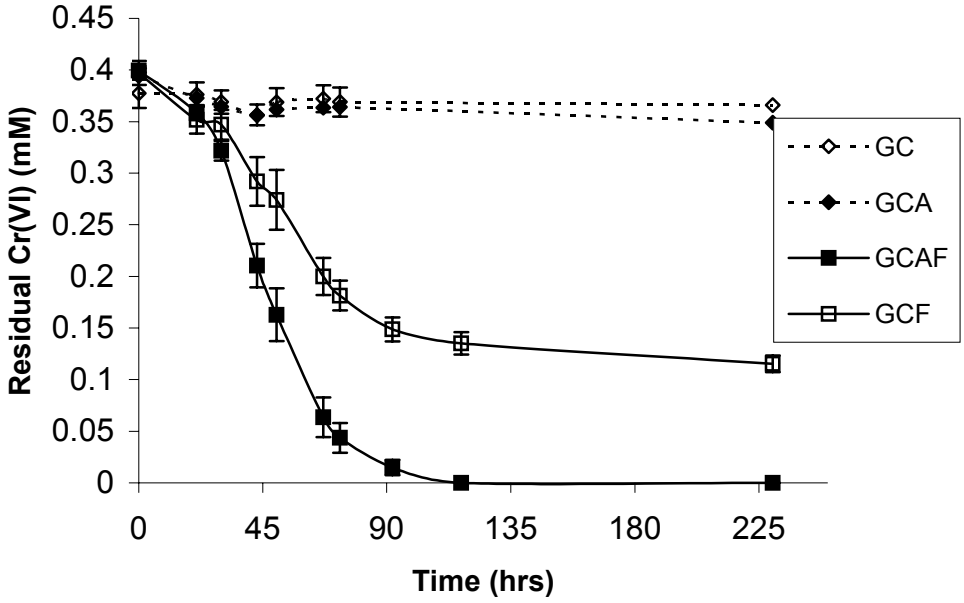

Figure 5-1. Cr(VI) reduction by strain GCAF-1. G, glucose (10mM) was provided as electron donor. Electron acceptors included A, AQDS (0.1mM); C, Cr(VI) (0.4mM); F, $Fe(OH)_3$ (5mM). All treatments were set up in triplicates with error bars representing the standard error

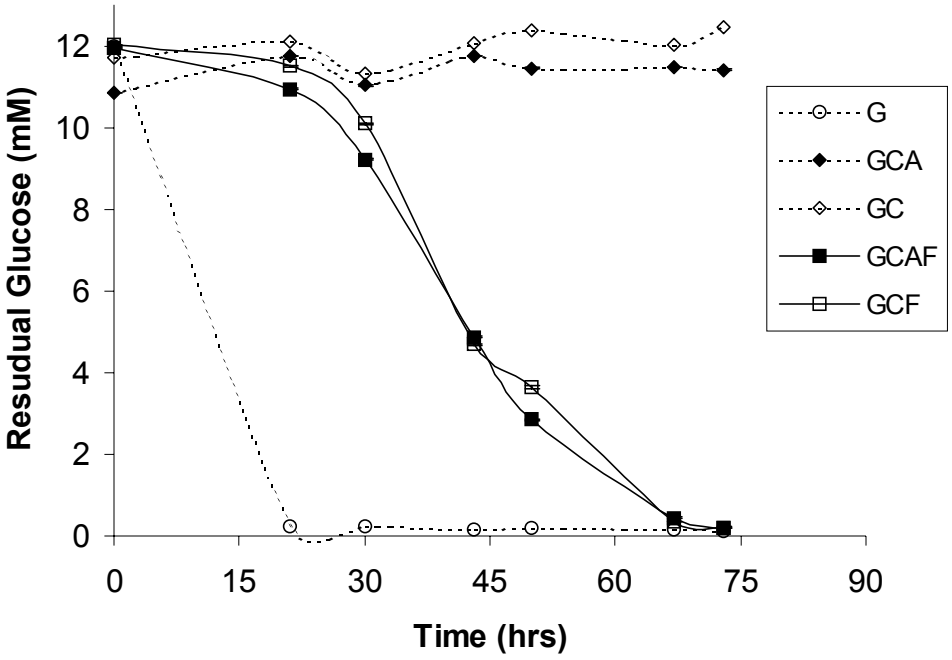

Figure 5-2. Glucose consumption during Cr(VI) reduction by strain GCAF-1. G, glucose (10mM) was provided as electron donor. Electron acceptors included A, AQDS (0.1mM); C, Cr(VI) (0.4mM); F, $Fe(OH)_3$ (5mM). All treatments were set up in triplicates with error bars representing the standard error.

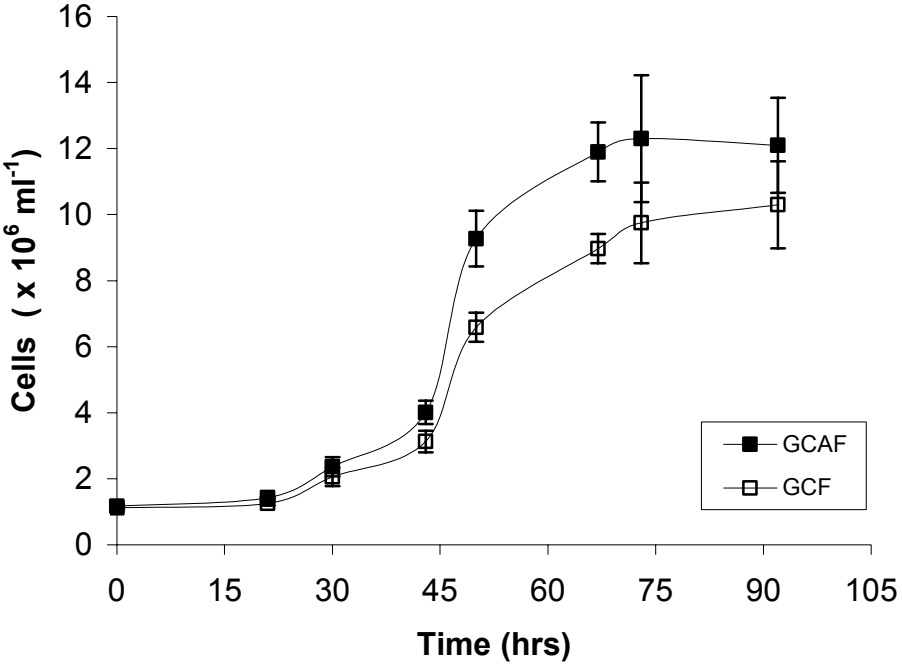

Figure 5-3. Cell growth during Cr(VI) reduction by strain GCAF-1. G, glucose (10mM) was provided as electron donor. Electron acceptors included A, AQDS (0.1mM); C, Cr(VI) (0.4mM); F, $Fe(OH)_3$ (5mM). All treatments were set up in triplicates with error bars representing the standard error.

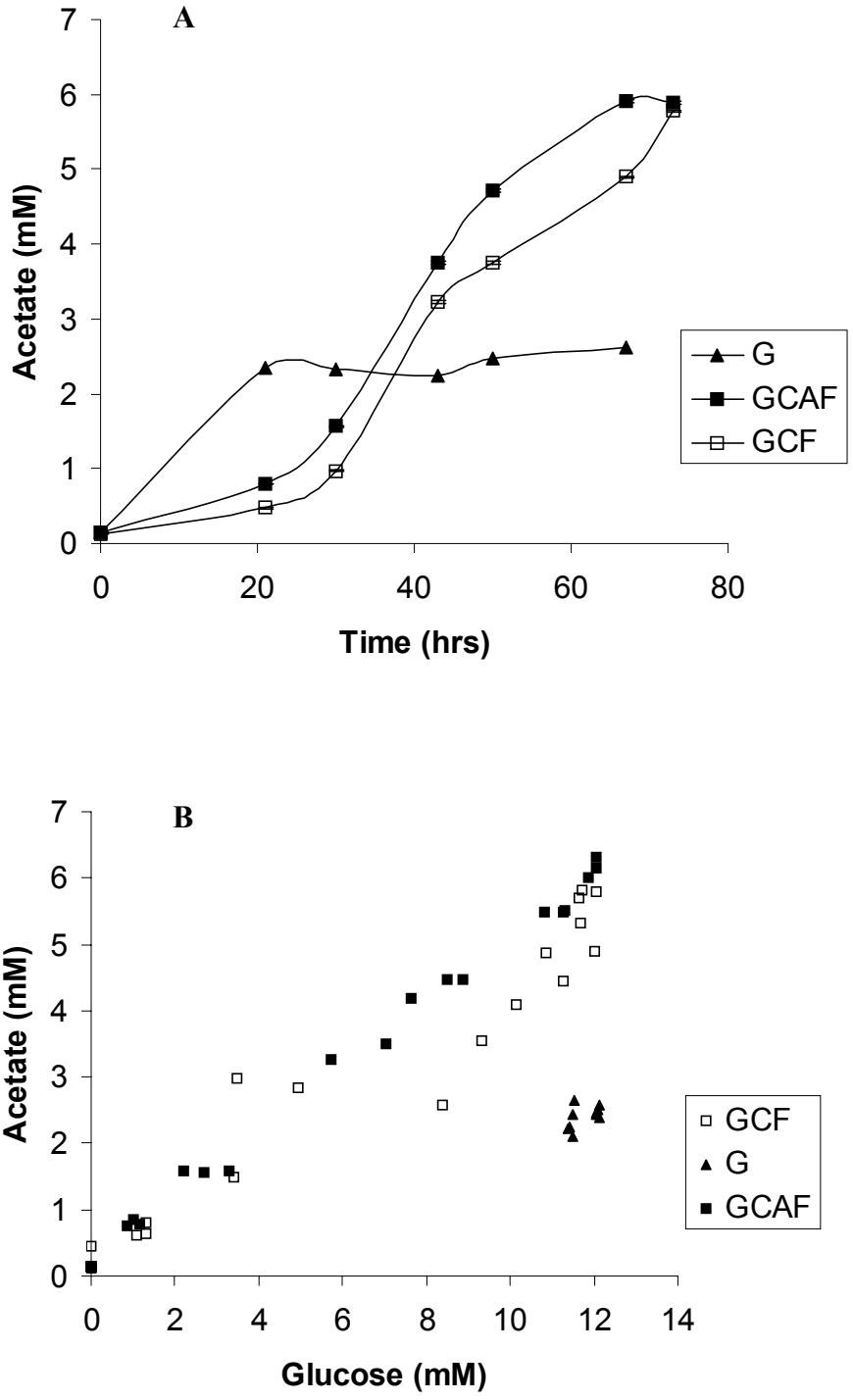

Figure 5-3. Acetate production during oxidation of glucose by GCAF-1. G, glucose (10mM) was provided as electron donor. Electron acceptors included A, AQDS (0.1mM); C, Cr(VI) (0.4mM); F, $Fe(OH)_3$ (5mM).

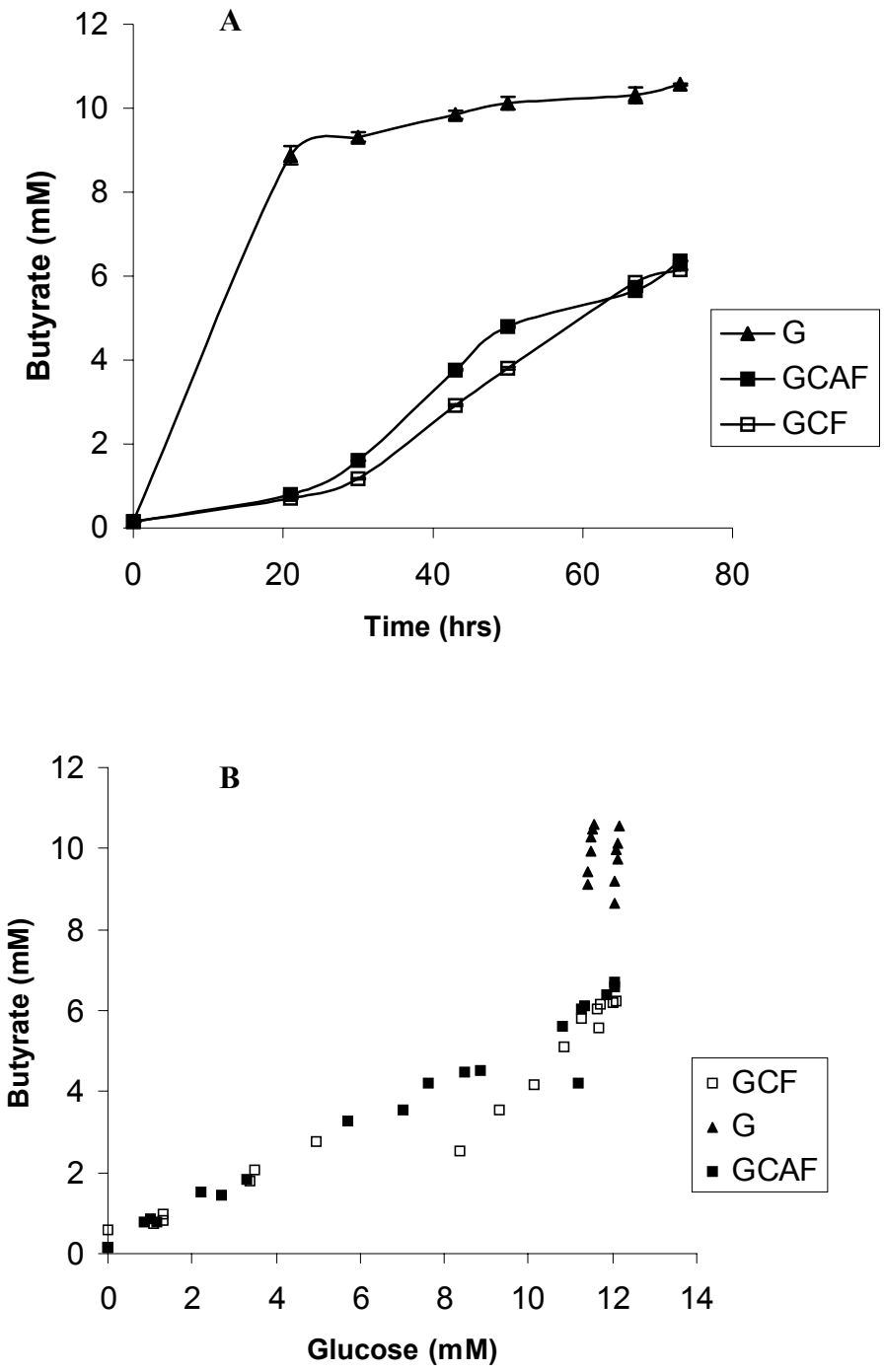

Figure 5-4. Butyrate production during oxidation of glucose by GCAF-1. G, glucose (10mM) was provided as electron donor. Electron acceptors included A, AQDS (0.1mM); C, Cr(VI) (0.4mM); F, $Fe(OH)_3$ (5mM).

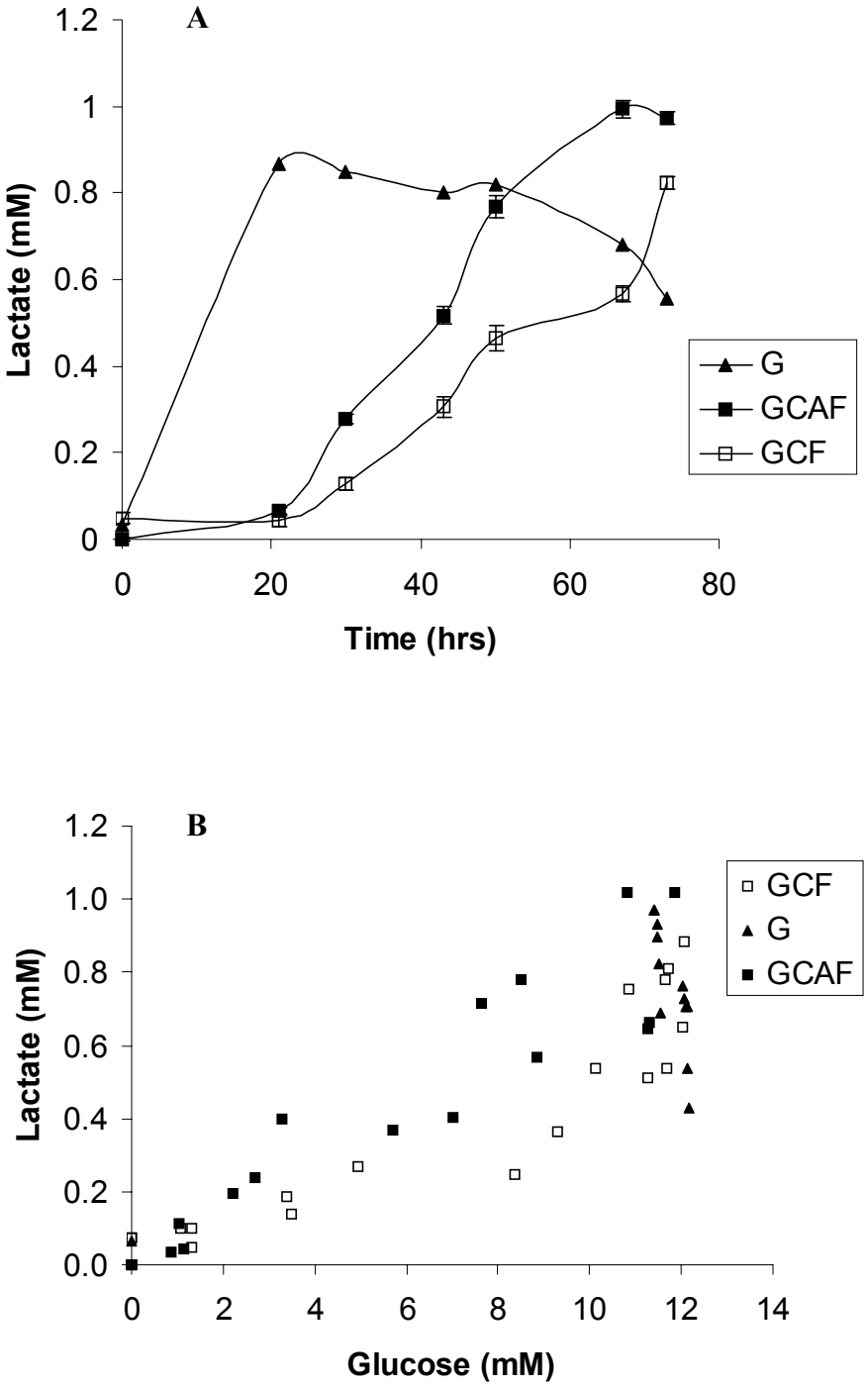

Figure 5-5. Lactate production during oxidation of glucose by GCAF-1. G, glucose (10mM) was provided as electron donor. Electron acceptors included A, AQDS (0.1mM); C, Cr(VI) (0.4mM); F, $Fe(OH)_3$ (5mM).

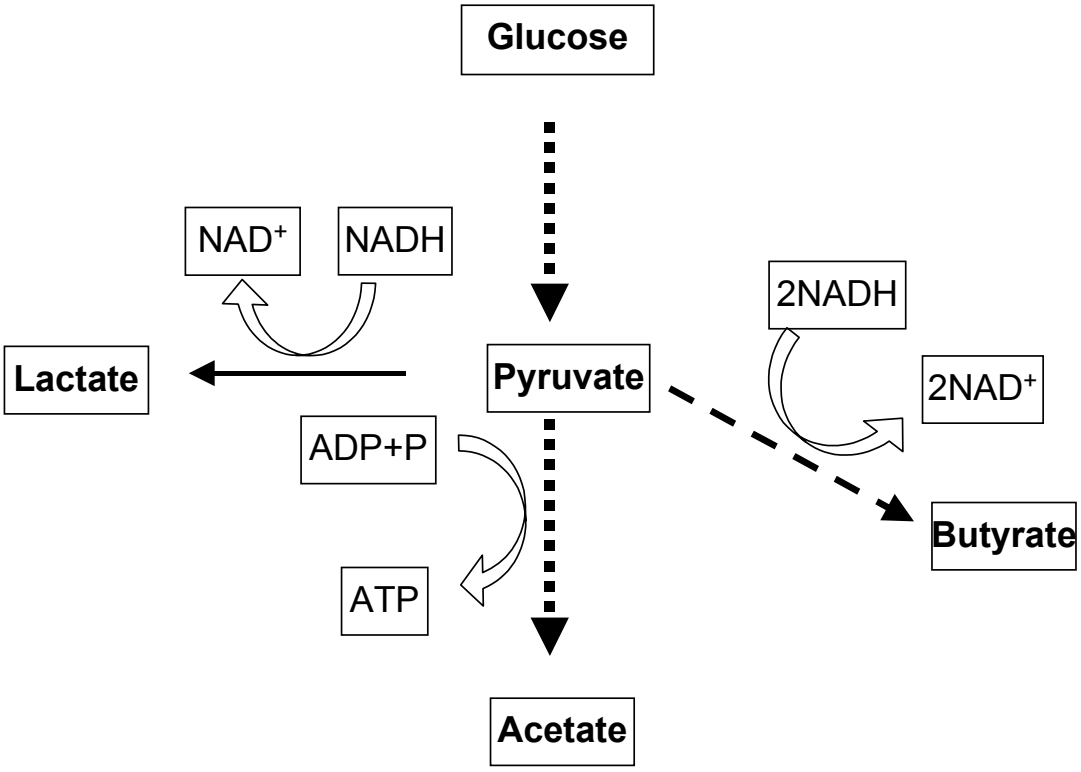

Figure 5-6. Schematic diagram of proposed metabolic pathway of formation of fermentative products by *Clostridium* sp. GCAF-1. Presence of Cr(VI) causes the cell to form lower concentrations of butyrate as NADH serves as an electron donor for the reduction of Cr(VI). Increase in acetate concentrations is coupled with the energy forming step.

Table 5-1. Effect of Cr(VI) on metabolites formed by strain GCAF-1. Glucose oxidation by strain GCAF-1 in medium containing glucose as electron donor and Fe(III) and AQDS as additional electron acceptors formed the following fermentation products in the presence and absence of Cr(VI).

Fermentation products	**Without Cr(VI)**	**With Cr(VI)**
	mM / 10mM of glucose	
Acetate	2.01	5.01
Lactate	0.74	0.83
Butyrate	7.57	5.41
Ratios		
Acetate: Lactate	2.72	6.06
Acetate: Butyrate	0.27	0.93

CHAPTER 6
SUMMARY AND CONCLUSIONS

Cr(VI) contamination in soils is not uncommon, especially near industries involved in glass work, chrome plating, and leather tanning. The mutagenic and carcinogenic properties of Cr(VI) necessitate effective remedial processes. Difficulties associated with chemical and physical techniques to remediate a Cr(VI)-contaminated site to EPA recommended levels (50ppb), in addition to the higher costs involved, assert the need for bioremedial measures. Implementation of these techniques requires knowledge of the following factors: the organisms involved; the factors that govern the optimum reductive ability of these organisms; and the mechanisms involved in Cr(VI) reduction . This dissertation addresses the above mentioned factors and investigates the Cr(VI)-reducing ability of a previously undescribed fermentative strain, thereby adding a representative of the genus *Clostridium* to the growing list of Cr(VI)-reducing bacterial groups. This work provides a greater understanding of processes by which microorganisms reduce Cr(VI) in nature.

Soils contaminated with Cr(VI) for over 5 decades were used in this study to enrich a diversity of indigenous Cr(VI)-reducing microorganisms. To maximize the diversity of the bacteria enriched and to ascertain the preferred electron donor by these bacteria, enrichments were amended with different electron donors and electron acceptors. The donors were chosen to represent those found in anaerobic soils. Among the various electron donors tested, enrichment cultures supplemented with glucose showed the fastest microbial Cr(VI) reduction, suggesting: (i) many rapidly growing Cr-

reducing bacteria preferred glucose as electron donor; and (ii) organisms enriched by glucose were more efficient at reducing Cr(VI) than those enriched by other electron donors in our systems.

Enrichments with Cr(VI) as sole electron acceptor showed insignificant microbial growth and Cr(VI) reduction. The effect of additional electron acceptors was apparent when a higher rate of Cr(VI) reduction was observed in the presence of Fe(III) or anthraquinone di sulfonate (AQDS), a humic acid analog. In the presence of both AQDS and Fe(III), the rate of Cr(VI) reduction was further augmented. These results indicated the following: (i) microbially-mediated reduction of Cr(VI) by the cooperative activity of Fe(III)-reducing, AQDS-reducing and Cr(VI) reducing bacteria (Figure 1-4), (ii) the toxicity of Cr(VI) is alleviated in the presence of Fe(III) or AQDS.

Characterization of cultivable members of the glucose-oxidizing consortium revealed the dominance of the genera *Cellulomonas* and *Clostridium*. Few isolates belonging to *Staphylococcus*, *Micrococcus* and *Bacillus* were also obtained. The presence of fermentative bacteria was not surprising owing to their ability to ferment glucose; however, their dominance in the Cr(VI) amended culture suggested their significant role in Cr(VI) reduction.

Detailed kinetic studies were conducted with the enriched Cr(VI)-reducing consortium that was dominated by organisms belonging to *Clostridium* sp. Reduction of Cr(VI) was observed only in the presence of an additional electron acceptor (Fe(III) or AQDS). The rate of Cr(VI) reduction observed was higher when both Fe(III) and AQDS were present in the medium, compared to when only Fe(III) was present. The presence of AQDS, a humic acid analog, has been shown to act as an electron shuttle and enhance the

Fe(III) reduction (90). Therefore, the lower rate of Cr(VI) reduction in system with no AQDS may be attributed to the slow rate of Fe(III) reduction. We speculate that microbially generated Fe(II) acts as an electron shuttle for the reduction of Cr(VI). In the presence of AQDS, the rate of Cr(VI) reduction is faster due to the stimulatory effect of AQDS on Fe(III) reduction. These results supported the role of AQDS as electron shuttle to enhance the Fe(III) reduction. Another possibility could be absence of AQDS-reducing organisms in the culture in absence of AQDS.

Similar studies with a pure isolate were undertaken to further elucidate the mechanism of Cr(VI) reduction by the *Clostridium sp* and to compare the rate of Cr(VI) reduction by the consortium and a pure isolate. An isolate, GCAF-1, obtained from this consortium was used for further studies. Studies with both consortium and the pure isolate showed comparable rate of Cr(VI) reduction in the presence of Fe(III) and AQDS. Results suggested that the Cr(VI) reducing ability of the organism is maintained at the same level when in isolation or when in a consortium with other organisms. Studies further demonstrated the requirement of Fe(III) by *Clostridium sp*. GCAF-1 to reduce Cr(VI) concentrations as high as 20 ppm. It was also observed that the amount of Cr(VI) reduced per unit of glucose in Fe(III) systems was lower than when AQDS was present along with Fe(III) in the medium.

This study also demonstrated the ability of GCAF-1 to directly reduce Cr(VI) in addition to Fe(III)-mediated Cr(VI) reduction as described above. GCAF-1 was capable of reducing 5 ppm Cr(VI) directly. Whether Cr(VI) was being used an electron acceptor with growth by *Clostridium* sp. GCAF-1 is not clear. At higher Cr(VI) concentrations in the absence of other electron acceptors, no bacterial growth and no reduction of Cr(VI)

were observed. Reduction of Cr(VI) by GCAF-1 was also found to be dependant on the presence of glucose. In the absence of glucose no reduction of Cr(VI) was observed. The concentration of glucose, however, was not a rate limiting factor for the reduction of Cr(VI) under the experimental conditions. These results may have great implications in the environments with much lower electron donor concentrations. Under glucose-limiting conditions, reduction of Cr(VI) may be effected negatively.

Analysis of the metabolic products of the consortium and GCAF-1 revealed that acetate, lactate and butyrate were the major fermentation products formed during glucose oxidation. No difference was observed in the ratios of metabolic products of cultures grown in the absence of an external electron acceptor and those grown in the presence of Fe(III) and AQDS. However, cultures grown with Fe(III), AQDS and Cr(VI) showed an increase in the acetate: butyrate and acetate: lactate ratios. These changes in cell metabolism may serve two purposes: (i) Energy generation by adenosine tri-phosphate (ATP) formation; and/or (ii) reduction of Cr(VI) to detoxify the immediate environment. Formation of acetate in glycolytic pathway is coupled with ATP production, and an increase in acetate concentration is perhaps a strategy adopted by strain GCAF-1 to generate more energy to survive in toxic Cr(VI) environments. A decrease in butyrate and lactate concentrations was accompanied by reduction of Cr(VI), suggesting that NADH, electron donor for the reduction of pyruvate to butyrate and lactate, was channeling its electrons towards the reduction of Cr(VI) via the electron transport chain.

Identification and phylogenetic characterization of isolate GCAF-1 revealed this strain to belong to the genus *Clostridium*. DNA-DNA hybridization with all type strains showing 98% similarity in 16S rDNA indicated the strain to be a novel *Clostridium*

species. GCAF-1 also exhibited the presence of at least two 16S rRNA copies. Other detailed biochemical analyses of GCAF-1 are also documented in this study. In view of the ability of this strain GCAF-1 to reduce Cr(VI), the species name *Clostridium chromoreductans* sp. nov. is proposed.

Studies presented in this dissertation provide significant implications for bioremediation of soils and water contaminated with toxic metals such as Cr(VI). In order to implement bioremediation in soils use of bacterial strain with minimal growth limiting requirements can decrease costs and be more beneficial for the working. GCAF-1 is one such easy maintenance organism. In anoxic soils, GCAF-1 can directly reduce Cr(VI) in soils thereby limiting its mobility. GCAF-1 is a fermentative organism that can grow in the absence of an external electron acceptor, unlike most other known Cr(VI) reducing bacteria, *Enterobacter cloacae*, *Pseudomonas putida, Desulfovibrio, Desufotomaculum sp. Pantoea agglumorens.* Environments that are rich in Fe(III) and humic acids may be dominated by FRB. Microbial reduction of Cr(VI) in these environments may predominantly be due to FRB. Under Fe(III)- and humics- limiting conditions, Cr(VI) reduction by FRM and HRM is insignificant. In such conditions organisms such as GCAF-1 play a significant role in reducing Cr(VI). As demonstrated in this study, GCAF-1 can reduce Cr(VI) significantly by using Fe(III) and AQDS as electron shuttles. The spore-forming ability of GCAF-1 offers another advantage over non-spore forming organisms. This ability enables these organisms to persist in the environment under unfavorable conditions. Among the advantages offered by the use of this strain is the source of carbon for the growth of this organism. Wastes such as molasses from sugar

industries may be provided as the carbon source, thereby making the implementation of bioremediation by these organisms economical.

Future studies with *Clostridium* sp. GCAF-1 to test its ability to reduce other toxic metals may reveal the full potential of this organism. Field studies of the application of these concepts may provide a more accurate picture of the potential importance of *Clostridium* GCAF-1 in the environment.

APPENDIX
MEDIA USED FOR ENRICHMENT STUDIES

NB BASAL MEDIA

Chemicals	**L^{-1}**
Q H_2O	900ml
KH_2PO_4	0.42 g
K_2HPO_4	0.22 g
NH_4Cl	0.2 g
NB Mineral elixir	10ml
Vitamin mix(DL)	15ml
KCl	0.38 g
NaCl	0.36 g
$MgSO_4 \bullet 7H_2O$	0.10 g
$NaHCO_3$	1.8 g
Na_2CO_3	0.5 g
1mM Na_2SeO_4	1ml

- Bring to final volume to 1L and bubble out media with 80/20 N2/CO2,
- Final pH ca. 7.0-7.2
- Donors and acceptors should be added anaerobically and aseptically to the media after autoclaving. Adjust all donors and acceptors to pH 7.0.

VITAMIN MIX

	mg/L
biotin	2.0
folic acid	2.0
pyridoxine HCl	10.0
riboflavin	5.0
thiamine	5.0
nicotinic acid	5.0
pantothenic acid	5.0
B-12	0.1
p-aminobenzoic acid	5.0
thioctic acid	5.0

MINERAL MIX

	g/L
NTA	1.5
$MgSO_4$	3.0
$MnSO_4 \bullet H_2O$	0.5
NaCl	1.0
$FeSO_4 \bullet 7H_2O$	0.1
$CaCl_2 \bullet 2H_2O$	0.1
$CoCl_2 \bullet 6H_2O$	0.1
$ZnCl_2$	0.13
$CuSO_4 \bullet 5H_20$	0.01
$AlK(SO_4)_2 \bullet 12H_2O$	0.01
H_3BO_3	0.01
Na_2MoO_4	0.025
$NiCl_2 \bullet 6H_2O$	0.024
$Na_2WO_4 \bullet 2H_2O$	0.025

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BIOGRAPHICAL SKETCH

Kanika Sharma was born in Sagar, Madhya Pradesh, India, on 23rd April 1974, to Nishchint and Amarnath Sharma. She had an excellent opportunity to visit a lot of places and make a lot of friends owing to her father`s job as defense personnel. In 1994, she graduated from Miranda House, the University of Delhi, with a first class and received a Bachelor of Science degree in botany (honors). She obtained a master`s degree in biochemical technology in 1995. Kanika went back to graduate school to get another master's degree in agrochemical and pest management from the University of Delhi, India, in 1997.

In January 1998, Kanika joined the University of Florida to pursue doctoral studies in environmental microbiology. Her research was supported by a part of the Department of Energy (DOE) research grant awarded to Dr. Andrew Ogram. Amidst the friendly and easygoing members of the Ogram lab and the department, it did not take long for her to make several everlasting friendships. In future, Kanika hopes to pursue an academic career in science full time and to hone the scientific skills acquired during the course of her graduate studies.