Evaluation of Enrichment Strategies for Cr(III)-Oxidizing Bacteria

Miniproject by

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Center for Biofilm Engineering, Montana State University

Microbial Diversity 2001, Marine Biological Laboratory, Woods Hole, MA

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Introduction

Chromium(VI) compounds have been used extensively in the manufacture of alloys, the electroplating industry, the manufacture of dyes and pigments, the preservation of wood, in the leather tanning industry, and as corrosion inhibitors in conventional and nuclear power plants (Hayes, 1997; Langard, 1980). Due to leakage, poor storage, and improper disposal practices, Cr(VI) has become one of the most frequently detected contaminants at hazardous waste sites (National Research Council, 1994; Watts, 1997). The spills are often extensive and the frequent presence of co-contaminants such as chlorinated aliphatics, radionuclides, metals, and organic solvents makes Cr(VI) remediation an environmental challenge (James, 1994; Watts, 1997; Pagilla and Canter, 1999).

Cr(VI) is highly soluble and therefore easily transported with the ground water (Bartlett and James, 1988). It is toxic and mutagenic to humans and other organisms (Committee on Biologic Effects of Atmospheric Pollutants, 1974; Gibb, 1999; Keith and Telliard, 1979; Watts, 1997). In its reduced trivalent state [Cr(III)], chromium forms insoluble hydroxides (Hug and Laubscher, 1997; Richard and Bourg, 1991) and remains immobile under most environmentally-relevant pH and redox conditions (Blowes et al., 1997; James, 1996; Lantz, 1992; Pohland et al., 1993; Richard and Bourg, 1991). Cr(III) is less toxic than Cr(VI) and is neither readily taken up by organisms or plants nor transferred through the food chain (Hayes, 1997; National Research Council, 1994).

Many remediation strategies are aiming on a chemical or microbiological reduction of Cr(VI) to produce immobile Cr(III)hydroxides. These processes however do not remove chromium from the aquifer but immobilize it by precipitation. Although Fe(III)Cr(III)hydroxides and pure Cr(III)hydroxide have very low solubilities and are unlikely to result in chromium concentrations above the drinking water limit for chromium, the reoxidation of Cr(III) compounds is a concern. While manganese oxides have been found to quickly oxidize Cr(III) in soils (Rai and Zachara, 1988, Bartlett and James, 1979), the microbially mediated reoxidation of Cr(III) is usually considered negligible in systems containing natural soil material (James, 1994, Bader et al., 1999).

To the best of the author's knowledge, pure cultures of Cr(III)-oxidizing bacteria have not yet been described in the literature. This project was designed to develop strategies on how

the enrichment and isolation of Cr(III) oxidizing bacteria could be accomplished. The experimental design was based on a miniproject of a previous Microbial Diversity Course (Colleen Hansel, Microbial Diversity 2000 "The Search for Cr(III) Oxidizers"). The methods were modified in an attempt to improve the described enrichment strategies and a number of control treatments were established.

Theoretical Background: Much like Fe(II) oxidizing bacteria, Cr(III)-oxidizing bacteria are expected to be chemolithoautotrophic bacteria capable of gaining energy from the oxidation of Cr(III) to Cr(VI) and producing biomass through the fixation of CO_2. Thermodynamic calculations suggest that the microbial oxidation of Cr(III) by aerobic or denitrifying organisms could provide sufficient energy for microbial growth.

$$4\ Cr(OH)_{3\,(s)} + 3\ O_{2\,(g)} \rightarrow 4\ HCrO_4^- + 2\ H_2O + 4\ H^+ \qquad \Delta G^{0'} = -\ 143\ kJ\ mol^{-1}$$

$$\Delta E^{0'} = 123\ mV$$

$$5\ Cr(OH)_{3\,(s)} + 3\ NO_3^- \rightarrow 5\ HCrO_4^- + 1.5\ N_2 + 4\ H_2O + 2\ H^+ \qquad \Delta G^{0'} = -\ 81\ kJ\ mol^{-1}$$

$$\Delta E^{0'} = 56\ mV$$

Colleen Hansel's work suggests that Cr(III) oxidizers are microaerophilic. Therefore enrichments were attempted in oxic and anoxic gradient tubes and in liquid cultures with $Cr(OH)_{3(s)}$ as sole electron donor.

Materials and Methods

Enrichment Strategies: Batch microcosms and gradient tubes were established providing oxygen or nitrate as electron acceptor. Batch microcosms consisted of 120 mL and 160 mL serum bottles sealed with blue butyl rubber stoppers; gradient tubes were established using Balch tubes.

Batch Microcosms contained 50 mL of a 30 mmol L^{-1} bicarbonate buffered basal salt medium at pH 7.1 – 7.3 supplemented with vitamins and trace elements (see appendix 1 for exact composition). Ammonium was excluded from the basal salt medium in order to discourage the growth of ammonium oxidizing bacteria. Thus, the basal salt medium contained nitrate (0.4 mmol L^{-1}) as the sole source of nitrogen. Chromium(III) was added from a $CrCl_3$ x 6 H_2O stock solution to a final concentration of 5 mmol/L. The addition of $CrCl_3$ to the medium resulted in the formation of a blueish precipitate which was assumed to be amorphous $Cr(OH)_3$. The addition of nitrilotriacetic acid (NTA, 1 mmol L^{-1}) was designed to potentially increase the solubility of the Cr(III) precipitate.

Aerobic enrichments were established using oxygenated medium and headspace oxygen concentrations of 20%, 10%, 5%, and 1%. The different headspace oxygen concentrations were

achieved by purging the headspace with a nitrogen-carbon dioxide gas mix (90:10) and subsequently injecting the appropriate amount of air via a syringe through a 0.2 um sterile filter and a sterile needle. Nitrate reducing enrichments were established by minimizing the amount of oxygen in the culture medium. This was achieved by preparing and dispensing the medium under an atmosphere of nitrogen and carbon dioxide (90:10) using a Widdel flask. The concentration of nitrate was increased to 4 mmol L^{-1} in order to provide sufficient nitrate for the complete oxidation of Cr(III). The microcosms were incubated statically at 30°C. A total of 15 aerobic and six nitrate reducing treatments along with six uninoculated controls was established.

Eleven days after the initial inoculation, a number of transfers were performed. During these transfers, a number of yeast extract amended treatments (final concentration 10 mg L^{-1}) were established (see below for details) in order to provide carbon for accelerated cell growth.

Gradient Tubes: Gradient tubes were established by adding 3 mL of a 0.4 % agarose plug containing 5 mmol of $CrCl_3$ x 6 H_2O to Balch tubes. After the plugs solidified, approximately 20 mL of a 0.2 % agarose overlay was added (see appendix 2 for media composition). Resazurin was added to serve as an indicator for redox conditions. Nitrate enrichments were prepared under a nitrogen/carbon dioxide atmosphere and nitrate was added to a final concentration of 4 mmol L^{-1}. The inoculated gradient tubes were incubated at 30°C.

Inoculum: Each batch microcosm was inoculated with approximately 0.5 g of soil obtained from a brownish-reddish layer of a soil which was found in the School Street Marsh area in Woods Hole, MA. The gradient tubes were inoculated with approximately 0.1 mL of a soil suspension in mineral medium.

Analytical: Enrichments were monitored using phase contrast microscopy. The enrichments were also chemically analyzed for Cr(VI), the potential product of Cr(III) oxidation, using a spectrophotometric method using diphenylcarbazide (Urone 1955). In brief, 0.25 mL of sample filtered through a 0.45 um syringe filter were added to 5 mL of 0.2 N H_2SO_4 mixed, and 0.25 mL of 0.25 % diphenylcarbazide (DPC) in 100% acetone were added. After mixing, the absorbance was read at 540 nm. In order to decrease the detection limit to approximately 10 ug L^{-1}, the assay was subsequently modified as following. A larger amount of filtered sample (i.e. 0.75 mL) was added to 0.25 mL of 0.8 N H_2SO_4, mixed, and 0.1 mL of DPC solution was added before the absorbance was read at 540 nm.

Results and Discussion

Batch Microcosms: Periodically, the microcosms were visually and microscopically examined. No increase in turbidity was detected. The fine blueish-gray precipitate that formed after the addition of the $CrCl_3$ solution (presumably $Cr(OH)_3$) was easily resuspended and masked

any possible increase in turbidity that might have been due to bacterial growth. Microscopic analysis revealed the presence of predominantly small motile rods (approximately 1-3 um in length, 1 um in width) in all treatments. Non motile, larger rods were also observed. It should be noted that the presence of the Cr-precipitate complicated the microscopic observation of bacterial cells. Some of the $Cr(OH)_3$ particles were not easily distinguished from non motile rods or cocci. Generally, a higher abundance of cells was initially observed in the oxygen-free (i.e. nitrate reducing) enrichments and the treatments with low headspace oxygen concentrations (i.e. 1% and 5%). In addition, higher cell numbers were observed in NTA-amended setups compared to setups in which NTA was omitted. Uninoculated controls did not show any evidence of biological activity.

Five days after inoculation, four treatments and their appropriate controls were chosen for Cr(VI) analysis. Cr(VI) was not detected (detection limit approximately 500 ug L^{-1}) in any of the treatments analyzed (0.4 mM NO_3^- plus NTA plus Cr(III), 4 mM NO_3^- plus NTA plus Cr(III), uninoculated nitrate amended controls +/- NTA, 20% O_2 +/- NTA, and uninoculated 20% O_2 +/- NTA).

Since no evidence of Cr(VI) production was obtained, but significant activity was observed microscopically in a number of treatments, 2.5 mL aliquots of following treatments were transferred into fresh minimal medium after 11 days:

a) Nitrate reducing treatments with 4.0 mM NO_3^- and 5 mM Cr(III) were transferred into i) nitrate reducing treatments with 4.0 mM NO_3^- and 5 mM Cr(III) and ii) same treatment plus yeast extract (YE).
b) Nitrate reducing treatments with 4.0 mM NO_3^-, 5 mM Cr(III), and 1 mM NTA were transferred into i) nitrate reducing treatments with 4.0 mM NO_3^-, 5 mM Cr(III), and 1 mM NTA, ii) the same treatment plus yeast extract (YE), iii) nitrate reducing treatments with 4.0 mM NO_3^- and 5 mM Cr(III) and iv) same treatment plus YE.
c) Aerobic enrichments with initially 1% O_2 headspace concentration amended with 5 mM Cr(III) were transferred to i) 1% O_2 plus 5 mM Cr(III) and ii) the same treatment plus YE.
d) Aerobic enrichments with initially 5% O_2 headspace concentration amended with 5 mM Cr(III) were transferred to i) 5% O_2 plus 5 mM Cr(III) and ii) the same treatment plus YE.
e) Aerobic enrichments with initially 5% O_2 headspace concentration amended with 5 mM Cr(III) and 1 mM NTA were transferred to i) 5% O_2 plus 5 mM Cr(III) and 1

mM NTA ii) the same treatment plus YE, iii) 5% O_2 plus 5 mM Cr(III) iv) the same treatment plus YE.

Microscopic observation of the transfers revealed the highest abundance of cells in yeast extract amended treatments. Two days after the transfer significant cell numbers were detected in all yeast extract amended setups indicating that cell growth was promoted by the addition of yeast extract. Four days later (i.e. 6 days after the transfer) however, the number of motile cells in all setups seemed to have decreased. The abundance of cells other than motile cells was impossible to estimate accurately due to the presence of precipitates. Cr(VI) analysis (detection limit approximately 5 ug L^{-1}) did not indicate a significant extent of Cr(III) oxidation in any of the treatments.

The initial enrichments were also analyzed once again, 15 days after inoculation. Phase contrast microscopy revealed again more motile cells in Cr(III)-free NTA controls and Cr(III) plus NTA containing treatments indicating the enrichment of nitrate reducing NTA degrading organisms. Cr(VI) analysis (detection limit approximately 5 ug L^{-1}) revealed concentrations around 10 ug L^{-1} in the 20 % O_2 control treatments with and without NTA. No significant concentrations of Cr(VI) were detected in any other treatment.

Overall, the number of cells in all treatments appeared to have decreased compared to day 04, day 08, and day 11 microscopic observations. The attempt to quantify cell numbers using dark membrane filters and DAPI staining was unsuccessful, thus all estimates are semi-quantitative. It appeared though that the abundance and richness of cells in all treatments had decreased after 15 days. However, even after 2 weeks, active protists were observed in the 5% O_2 plus 5 mM Cr(III) treatment indicating that the Cr(III) precipitates were not immediately toxic to all organisms in the inocula.

Gradient Tubes: Four days after inoculation with a soil suspension from the School Street Marsh soil, a reddish color developed around the area of inoculation. After 7 days, the whole overlay was of reddish color indicating a decrease in redox potential. No clear turbid bands were formed except immediately below the surface of the overlay in the aerobic gradient tubes. The turbid bands in these treatments were associated with a zone of clearing and decreased viscosity just above these bands, indicating the possibility of agarose degradation. Aerobic agar and agarose degrading bacteria had been observed several times throughout the course. The oxygen-free but nitrate amended gradient tubes also changed color from blueish to reddish but no clear or turbid bands were observed. Uninoculated control tubes remained blue throughout the duration of the course.

Conclusions

The enrichments established did not provide sufficient evidence of Cr(III) oxidation within the three weeks of the miniproject.

The gradient tube enrichment strategy although it has the potential of providing visual clues, did not appear to be appropriate for the use with a very low solubility electron donor such as $Cr(OH)_3$. Cr(III) concentrations throughout the overlay of the gradient tubes might be extremely low due to diffusion limitations. The low concentrations might not allow for growth of Cr(III) oxidizing bacteria sufficient to result in clearly visible turbid bands. In addition, the use of agarose in the tubes provides an electron donor alternate to Cr(III), which in the aerobic enrichments apparently resulted in the enrichment of agarose degrading bacteria.

The batch microcosm enrichments appear to have potential for the enrichment of Cr(III)-oxidizing bacteria, however the three week time frame of the miniproject was not long enough to perform a final assessment. It appears that the addition of NTA to the enrichments promotes growth of nitrate reducing and oxygen respiring NTA degraders. Moreover, the apparent increase in Cr(VI) concentrations in the 20 % O_2 controls makes the assessment of the enrichments more complicated. An increase in Cr(VI) in any of the inoculated treatments would have to be significantly higher than in the abiotic controls. A number of enrichments will be shipped to my home university and will be observed periodically in order to monitor the further potential of these enrichments.

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Appendix 1:

Microcosm Preparation:

Stock Solutions:

NaCl		80 g L^{-1}
10 x mineral base	(NaCl	10 g L^{-1}
	$MgCl_2$ x 6 H_2O	4 g L^{-1}
	$CaCl_2$ x 6 H_2O	1 g L^{-1}
	KH_2PO_4	2 g L^{-1}
	KCl	5 g L^{-1}
NTA		50 mM (0.957 g/100 mL)
$CrCl_3$ x 6 H_2O		500 mM (6.66 g/50 mL)
$NaNO_3$		40 mM (0.17 g/50 mL)
$NaHCO_3$		1 M (17 g/200 mL)
Deionized water		

Autoclave all solutions separately, autoclave the $NaHCO_3$ solution under N_2/CO_2 (90:10) atmosphere in sealed serum bottles.

For aerobic enrichments, combine all solutions as follows:

Deionized water	727 mL
NaCl	100 mL
10 x mineral base	100 mL
$NaHCO_3$	30 mL (adjust pH to 7.1 – 7.3 using 1.0 N HCl or 0.5 N Na_2CO_3)
$NaNO_3$	10 mL
12 vitamin mix	1 mL
vitamin B_{12}	1 mL
trace element solution (SL 10)	1 mL

dispense 48.5 mL into sterile 120 mL serum bottles, add $CrCl_3$, and NTA as follows (add equivalent amount of deionized water where NTA or $CrCl_3$ is omitted)

Treatment	1 % O_2	5 % O_2	10 % O_2	20 % O_2	20 % O_2 ctrl
5 mM $CrCl_3$ + 1 mM NTA	0.5 mL $CrCl_3$ stock + 1.0 mL NTA stock	0.5 mL $CrCl_3$ stock + 1.0 mL NTA stock	0.5 mL $CrCl_3$ stock + 1.0 mL NTA stock	0.5 mL $CrCl_3$ stock + 1.0 mL NTA stock	0.5 mL $CrCl_3$ stock + 1.0 mL NTA stock
5 mM $CrCl_3$	0.5 mL $CrCl_3$ stock	0.5 mL $CrCl_3$ stock	0.5 mL $CrCl_3$ stock	0.5 mL $CrCl_3$ stock	0.5 mL $CrCl_3$ stock
1 mM NTA	1.0 mL NTA stock	1.0 mL NTA stock	1.0 mL NTA stock	1.0 mL NTA stock	1.0 mL NTA stock

Inoculate bottles with 0.5 mL of soil sample. Purge headspace with N_2/CO_2 (90:10) gas (except 20 % O_2 treatment) and seal with blue butyl rubber stopper. Remove appropriate amount of headspace gas and replace with appropriate amount of air (i.e. 35 mL for 10% O_2, 17.5 mL for 5% O_2, and 3.5 mL for 5% O_2). Air was injected through 0.2 um sterile filter. Incubate statically at 30 °C.

For nitrate reducing enrichments:

Prepare basic medium in Widdel Flask but omit 90 mL of deionized water in initial preparation. Cool down under N_2/CO_2 (90:10) gas stream, add all solutions from anoxic stock solutions (prepared by sparging solutions with N_2/CO_2 (90:10) for > 15 min before sealing and autoclaving). Dispense 44 mL of medium into serum bottles under N_2/CO_2 (90:10) gas stream. Seal with blue butyl rubber stoppers. Add an extra 4.5 mL of anoxic $NaNO_3$ solution to 4 mM NO_3^- treatments and 4.5 mL of anoxic deionized water for 0.4 mM NO_3^- treatments. Add NTA and $CrCl_3$ solutions from anoxic stocks as following.

Treatment	0.4 mM NO_3^-	4 mM NO_3^-	0.4 mM NO_3^- ctrl
5 mM $CrCl_3$ + 1 mM NTA	0.5 mL $CrCl_3$ stock + 1.0 mL NTA stock	0.5 mL $CrCl_3$ stock + 1.0 mL NTA stock	0.5 mL $CrCl_3$ stock + 1.0 mL NTA stock
5 mM $CrCl_3$	0.5 mL $CrCl_3$ stock	0.5 mL $CrCl_3$ stock	0.5 mL $CrCl_3$ stock
1 mM NTA	1.0 mL NTA stock	1.0 mL NTA stock	1.0 mL NTA stock

Inoculate under N_2/CO_2 (90:10) purge and incubate statically at 30 °C.

Appendix 2:

Gradient Tube Preparation:

Metal-Agarose Plug (100 mL)

10 x mineral base	10 mL
NaCl stock	10 mL
Agarose	0.4 g
Resazurin	1 mL (40 mg/L stock)
$NaNO_3$ stock	1 mL
Deionized water	76 mL

Melt in autoclave, Let cool to 60 °C. Add 1 mL of $CrCl_3$ solution. Add 3 mL of $NaHCO_3$ stock and adjust pH to 7. Dispense 3 mL into Balch tubes, purge headspace with N_2/CO_2 (90/10), seal with blue butyl rubber stoppers, autoclave.

Oxygenated overlay (500 mL):

10 x mineral base	50 mL
NaCl stock	50 mL
Agarose	1.0 g
Resazurin	5 mL (40 mg/L stock)
$NaNO_3$ stock	5 mL
Deionized water	373.5 mL

Melt in autoclave, Let cool to 60 °C. Add 15 mL of $NaHCO_3$ stock and adjust pH to 7. Add each 0.5 mL of vitamin B_{12}, 12 vitamins, and trace element solution. Dispense 20 mL into Balch tubes, cap with 20 mm caps.

Anoxic overlay (200 mL):

10 x mineral base	20 mL
NaCl stock	20 mL
Agarose	0.4 g
Resazurin	2 mL (40 mg/L stock)
$NaNO_3$ stock	20 mL
Deionized water	131.4 mL

Purge with N_2/CO_2 (90/10) gas. Seal, melt in autoclave, Let cool to 60 °C. Add 6 mL of $NaHCO_3$ stock and adjust pH to 7. Add each 0.5 mL of vitamin B_{12}, 12 vitamins, and trace element solution. Dispense 20 mL into Balch tubes while purging with N_2/CO_2 (90/10) gas, crimp seal using blue butyl rubber stoppers and aluminum crimp seals.