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Bacterial Reduction of Chromium

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ABSTRACT

A mixed culture was enriched from surface soil obtained from an eastern United States site highly contaminated with chromate. Growth of the culture was inhibited by a chromium concentration of 12 mg/L. Another mixed culture was enriched from subsurface soil obtained from the Hanford reservation, at the fringe of a chromate plume. The enrichment medium was minimal salts solution augmented with acetate as the carbon source, nitrate as the terminal electron acceptor, and various levels of chromate. This mixed culture exhibited chromate tolerance, but not chromate reduction capability, when growing anaerobically on this medium. However, this culture did exhibit chromate reduction capability when growing anaerobically on TSB. Growth of this culture was not inhibited by a chromium concentration of 12 mg/L. Mixed cultures exhibited decreasing diversity with increasing levels of chromate in the enrichment medium. An *in situ* bioremediation strategy is suggested for chromate contaminated soil and groundwater.

Index entries: Bioremediation; chromium; chromium-reduction; chromium-tolerance; chromate; mixed culture; diversity.

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INTRODUCTION

More than 300,000 metric tons of chromite ore are processed each year in the US for use in stainless-steel production, electroplating, leather tanning, wood preservation, drilling muds, magnetic recording tape, photographic films, pigments, and many other purposes *(1)*. These uses of chromium have resulted in chromium-contaminated soils and ground water at some production sites *(2)*. At such sites, chromium exists primarily in two valence states: Cr^{3+} and Cr^{6+}. Of these two states, trivalent chromium is the dominant species in the natural environment *(3)*. This species is relatively immobile in ground water owing to complexation with organics or precipitation as an hydroxide *(4)*. This form of chromium is also an essential trace element in the human diet: the glucose tolerance factor has been identified as a complex of Cr^{3+} with three amino acids *(5)*.

In contrast to trivalent chromium, hexavalent chromium (Cr^{6+}) is found in the natural environment almost exclusively as a result of human activities *(6)*. Further, unlike the trivalent form, this species is soluble and very mobile in ground water *(7)*. Hexavalent chromium is typically found in ground water systems as chromate, which is an oxyanion that is actively transported into cells by the sulfate transport system *(8)*. Because chromate is a carcinogen *(9)* capable of directly causing lesions in DNA as well as indirectly generating oxygen radicals, the maximum allowable concentration of chromium in drinking water is 0.1 mg/L (100 ppb) *(10)*. Effective remediation methods must be developed for sites contaminated with chromium. Methods that have previously been tested to remediate these sites include excavation, pump and treat, *in situ* vitrification, and chemical treatment with a reductant *(11)*. We are exploring biological reduction of chromate to the less hazardous trivalent state as an alternative remediation strategy.

In this article, the diversity of bacteria enriched in chromate-containing medium under denitrifying conditions and the relative rates of chromate reduction of two mixed cultures are reported. Further, the application of a bacteriological treatment strategy for *in situ* remediation is discussed.

METHODS

Two enrichment cultures, designated culture A and culture B, were employed in this study. Mixed Culture A was enriched from surface soil obtained by Idaho National Engineering and Environmental Laboratory (INEEL) from an eastern US site that had been contaminated by chromite ore tailings used as landfill. The soil Cr^{6+} concentration was approx 250 mg Cr/kg soil. Soil dilutions (10^{-1} g/mL) were made using isotonic phosphate buffer *(12)* and were inoculated into sealed serum vials containing tryptic soy broth (Difco Laboratories, Detroit, MI) and nitrogen gas in the headspace. The mixed culture was harvested and added to a packed-bed

reactor filled with sterile polymeric biocatalyst support beads (Bio-Sep™, DuPont, Newark, DE). After operation of the reactor under anaerobic conditions with various Cr^{6+} concentrations, the beads were removed and stored at 4°C prior to their use.

Mixed culture B was enriched from subsurface soil obtained by Pacific Northwest National Laboratory (PNNL) from the edge of a dichromate contamination plume *(13)* on the US Department of Energy's Hanford facility in southeastern Washington. The soil used was a 30.5-cm long, 10 cm diameter (1-ft long, 4 in. diameter), core from 14.5 m (47.5 ft) below the surface in well 199-H5-15. The water table is 12.2 m (40 ft) below the surface at this location. After the core was removed, ground water analyses from the well had a mean value of 0.055 mg/L (55 ppb) total chromium *(14)*. The core was obtained aseptically on November 8, 1995, and stored anaerobically (in an inert argon atmosphere) in a plastic cylinder at 4°C until March 29, 1996. The cylinder was opened in a laminar flow hood and 10 g of soil transferred into each of 12 165-mL serum bottles that had been autoclaved (121°C for 15 min). During the soil-transfer process, the serum bottles were continuously purged with filtered (0.2-µm) prepurified nitrogen. Ninety milliliters of a filter-sterilized minimal salts medium augmented with 200 mg/L acetate and 100 mg/L nitrate were added to each of the serum bottles. Potassium chromate was added to three bottles to obtain a concentration of 20 mg/L Cr^{6+}, to three bottles to obtain a concentration of 5 mg/L Cr^{6+}, and to three bottles to obtain a concentration of 0.5 mg/L Cr^{6+}. Three autoclaved bottles containing only 100 mL of the filter-sterilized minimal salts medium augmented with 200 mg/L acetate and 100 mg/L nitrate were used as a control. The 15 serum bottles were capped and pressurized to 5 psig with filtered prepurified nitrogen. After 3 d, 100 µL were aseptically withdrawn from each serum bottle and serially diluted in 0.1% peptone sterile blanks to perform heterotrophic plate counts on tryptic soy agar (Difco Laboratories, Detroit, MI) under aerobic conditions at 30°C *(15)*. Diversity, based on colony morphology and cultural characteristics, was noted for each countable plate. Two distinctly different colonies were found in each bottle containing 20 mg/L Cr^{6+}, and they were labeled culture B strain B and culture B strain C.

Ten milliliters of tryptic soy broth (Difco Laboratories, Detroit, MI) were autoclaved (121°C for 15 min) in each of 18 test tubes. After cooling to room temperature in a laminar flow hood, filtered (0.2 µm) prepurified nitrogen gas was bubbled through the broth for 15 min to remove oxygen. Potassium chromate was added to nine of the tubes to a concentration of approx 15 mg/L. Six tubes (three with potassium chromate and three without) were inoculated with the mixed culture A by placing five polymeric biocatalyst support beads (Bio-Sep™, DuPont, Newark, DE) removed from the INEEL packed-bed reactor into each of the tubes and vortexing for 30 s. Six tubes (three with potassium chromate and three without) were inoculated with pure culture B, strain B, by transferring a single, isolated colony

growing aerobically on tryptic soy agar into each of the tubes and vortexing for 30 s. Six tubes (three with potassium chromate and three without) were inoculated with pure culture B, strain C, in a similar manner. The tubes were capped, pressurized to 5 psig with filtered prepurified nitrogen, and incubated at 27°C until used as sources for inoculation of the batch reactors.

Batch reactors consisted of 125-mL serum vials. One hundred milliliters of tryptic soy broth (Difco Laboratories) were autoclaved (121°C for 15 min) in each of 18 125-mL serum vials (batch reactors). Immediately after removal from the autoclave, each reactor was purged with filtered (0.2 μm) prepurified nitrogen gas in the headspace for 5 min in a laminar flow hood. Potassium chromate was added to nine of the reactors to a concentration of approx 15 mg/L. The reactors were capped and pressurized to 5 psig with filtered prepurified nitrogen. Three reactors with potassium chromate and three reactors without potassium chromate were not inoculated and used as abiotic controls. Five milliliters were transferred from each mixed culture A tube described above (with or without potassium chromate as appropriate) into each culture A reactor (with or without potassium chromate, as appropriate). Mixed culture B reactors were inoculated by transferring 2 1/2 mL from a culture B, strain B, tube and 2 1/2 mL from a culture B, strain C, tube described above (with or without potassium chromate, as appropriate) into each culture B reactor (with or without potassium chromate, as appropriate). Each transfer was performed in a laminar flow hood by disposable sterile hypodermic syringe and needle after sterilizing the tube septum or reactor septum with 70% ethanol.

The 18 reactors in the preceding paragraph constitute a completely random experimental design with a 2 × 3 factorial arrangement of treatments. The treatments were randomly applied to the reactors, which were then identified by the numbering scheme summarized in Table 1.

The 18 reactors were placed on a gyratory shaker operated at 200 RPM in a 27°C incubator. Each reactor was vortexed for 30 sec and sampled at approx 6-h intervals. Each 5-mL sample was obtained by disposable sterile hypodermic syringe and needle after sterilizing the reactor septum with 70% ethanol. The samples were transferred to sterile 10-mL disposable test tubes. Three and one-half milliliters were then transferred to a plastic cuvet, and absorbance was immediately measured spectrophotometrically at 600 nm (Milton Roy Genesys 5 spectrophotometer) using type I reagent-grade water as a blank.

Chromate (Cr^{6+}) concentration in each sample was measured spectrophotometrically. Two hundred microliters of each sample were transferred by digital pipet (Eppendorf) to a sterile 10-mL disposal test tube containing 9.80 mL of type I reagent-grade water. The contents of a Hach Chrom Ver3 pillow (0.09 g diphenylcarbazide) was added to the tube, the tube shaken for 30 s, and absorbance measured between 5 and 20 min later

Table 1
Experimental Design[a]

Treatment B Initial Concentration Cr^{6+} (mg/L)	Treatment A, Inoculum								
	Sterile			Culture A			Culture B		
0	1	7	13	3	9	15	5	11	17
12	2	8	14	4	10	16	6	12	18

[a]The completely random experimental design employed a 2 × 3 factorial arrangement of treatments, Treatment A, inoculum, was applied at three levels: sterile; mixed culture A from DuPont Bio-Sep™ beads; and mixed culture B enriched from Hanford subsurface soil. Treatment B, initial hexavalent chromium concentration, was applied at two levels: 0 and ~12 mg/L. Treatments were randomly assigned to 125 mL reactors containing 100 mL of TSB, and the reactors were then identified with numbers from 1–18 as indicated in the matrix above.

at 542 nm (Milton Roy Genesys 5 spectrophotometer) using similarly treated samples from the aseptic reactors without chromate as a blank. Chromate concentration (mg/L Cr^{6+}) was calculated for each sample based on a five-point calibration curve developed over the range 0.1–20 mg/L Cr^{6+} undiluted sample in a TSB matrix.

In the final sample taken from each reactor, total Cr in the supernatant of the centrifuged (10,000*g* for 5 min) samples was measured by direct aspiration into an inductively coupled argon plasma simultaneous emission spectrometer (Thermo Jarrell Ash ICAP-61).

Results

Soil from the highly contaminated eastern site was enriched in TSB under anaerobic conditions with one level of chromate concentration, 20 mg/L. As reported previously by Turick and Apel *(16)*, three species were recovered from the enriched mixed culture (culture A). This mixed culture exhibited chromate reduction during the enrichment process.

Soil from the Hanford subsurface was enriched on minimal salts medium augmented with acetate and nitrate under anaerobic conditions with four different chromate concentration levels. None of the enrichment cultures exhibited significant chromate reduction during the enrichment process. Heterotrophic plate counts indicated that both the stationary-phase populations (Fig. 1) and the population diversity (Fig. 2) decrease with increasing Cr^{6+} concentration over the range 0–20 mg/L. The two species constituting the mixed cultured enriched at 20 mg/L Cr^{6+} are referred to as mixed culture B.

When grown on TSB under anaerobic conditions in the presence of Cr^{6+} mixed culture A and mixed culture B exhibit significantly different growth rates (Fig. 3) and chromate reduction rates (Fig. 4). Mixed culture A

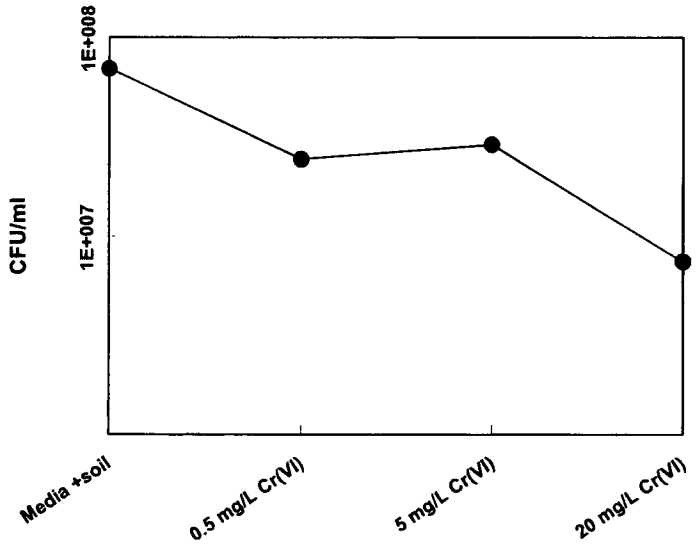

Fig. 1. Heterotrophic plate count from Hanford core 199-H5-15. 10^{-1} dilution of soil into minimal salts medium augmented with 200 mg/L acetate and 100 mg/L nitrate, followed by 3 d of anaerobic growth at 30°C. Spread plates on full-strength TSA were counted after 2 d of aerobic growth at 30°C. All dilutions and spread plates were done in triplicate.

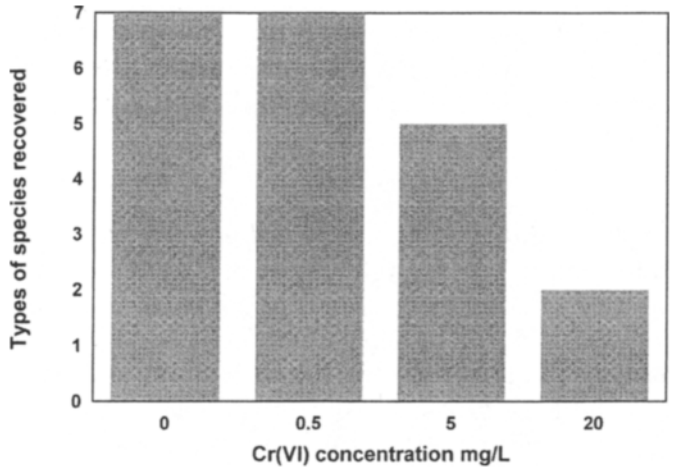

Fig. 2. Diversity of species in Hanford soil inoculum as a function of medium chromate concentration. The same diversity was noted on all replicates of countable spread plates. Diversity was based on colony morphology and cultural characteristics.

reduced all chromate in <12 h, whereas mixed Culture B required more than 24 h to reduce all of the Cr^{6+} to Cr^{3+}. After the Cr^{6+} concentration was reduced to levels below the detection limits, the total Cr in the supernatant of centrifuged samples was found to be equal to the initial Cr^{6+} concentration, within the limits of the methods employed. Thus, although Cr^{3+} was

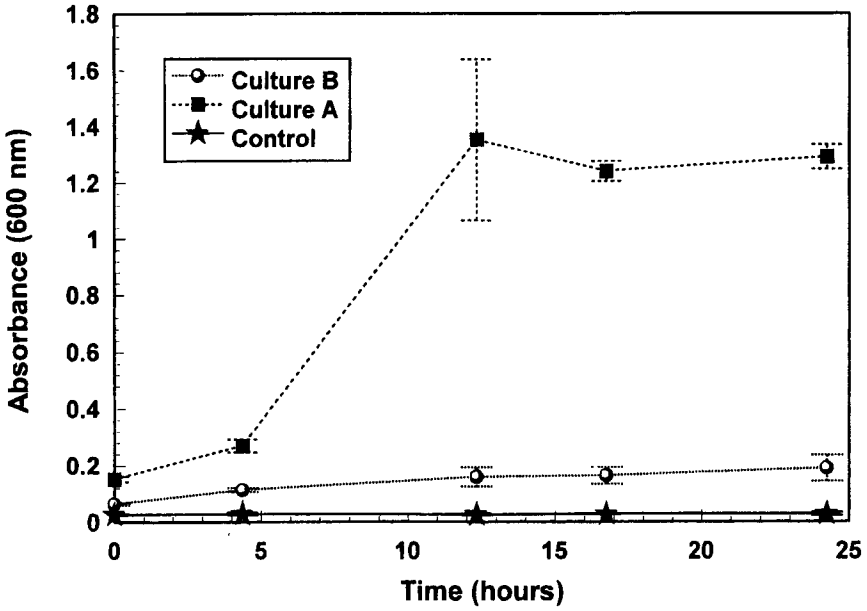

Fig. 3. Microbial growth rates of two mixed cultures growing anaerobically on TSB. ★ Abiotic control, ■ culture A, O culture B. Error bars are 1 SD.

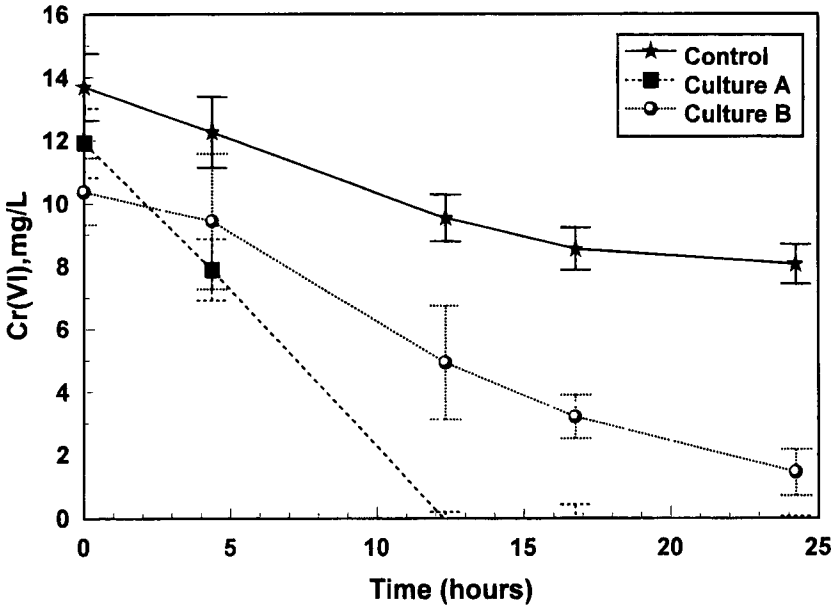

Fig. 4. Chromate reduction rates of two mixed cultures grown anaerobically on TSB. ★ Abiotic control, ■ culture A, O culture B. Error bars are 1 SD.

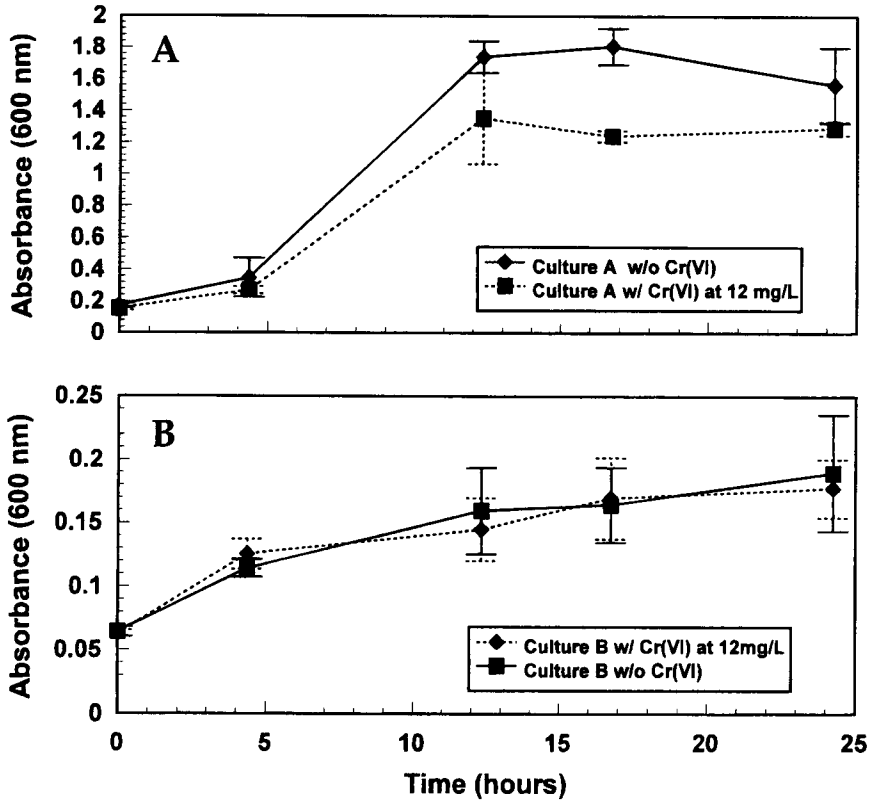

Fig. 5. Chromate inhibition. **(A)** mixed culture A, **(B)** mixed culture B. ♦ No chromate in medium, ■ Chromate present in medium. Error bars are 1 SD.

not directly measured, a mass balance on Cr^{6+} suggests that it had been reduced to Cr^{3+} and that the Cr^{3+} remained soluble at neutral pH.

In Fig. 5, the microbial concentration of each of the cultures, as indicated by absorbance at 600 nm, is shown as a function of time. Further, the bacteria were either grown with or without Cr^{6+} in solution during growth. Mixed culture A exhibited inhibition by 12 mg/L Cr^{6+} (Fig. 5A), whereas mixed culture B did not exhibit inhibition at this concentration of Cr^{6+} (Fig. 5B).

DISCUSSION

This research demonstrates that it is possible to enrich for chromium-reducing bacteria from soil at chromium-contaminated sites. This suggests an *in situ* bioremediation strategy: determine conditions limiting growth of the autochthonous chromium-reducing bacteria, and then provide conditions that support their growth. When the hazardous Cr^{6+} has been reduced to the relatively benign Cr^{3+} withdraw support and allow the bacterial populations to return to their prior state.

As indicated above (Fig. 3), absorbance at 600 nm was used as an indicator of the microbial concentration in the reactor. Other methods that would have allowed direct comparison of the biomass concentration in the various reactors were considered. For example, a portion of the reactor broth could have been filtered and the total suspended solids could have been determined. However, production of exopolysaccharides by both mixed cultures prohibited measurement of the biomass concentration using such filtration-based techniques. Alternatively, total protein concentration was considered a measure of biomass concentration. However, the use of TSB as the carbon source precluded the use of protein assay for measurement of biomass. Therefore, although absorbance at 600 nm is indicative of the biomass concentration, this parameter cannot be directly related to dry cell weight.

Because of this inability to measure the total biomass concentration directly in the reaction vessels, it is possible that the chromate reduction rate exhibited by the two cultures, when considered on a per unit dry cell weight basis, is different than the relative chromate reduction rates indicated by absorbance. That is, were it possible to express the chromate reduction rate in terms of mg of Cr reduced/mg dry cell weight/hour, culture B might be found to reduce Cr at a rate greater than culture A.

Acetate was used as the carbon source for the enrichment from Hanford soil because of prior success in the use of acetate as a nutrient for other *in situ* bioremediation activities at this site. Mixed culture B exhibited chromate resistance, but not chromate reduction, when grown on acetate. Although chromate reduction is exhibited when growing on TSB, this complex carbon source is not suitable for *in situ* application for economic and technical reasons.

In addition to biomass measurement limitations described above, the complex carbon source also precludes the use of ion chromatographic methods for chromium speciation. Future research on the biological reduction of chromium will be facilitated by finding a simple carbon source on which the cells will exhibit chromate reduction. The success of the *in situ* strategy for application of this bioremediation technique is also dependent on the determination of such a simple, inexpensive, and pumpable carbon source.

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