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Fe(III), Cr(VI), and Fe(III) mediated Cr(VI) reduction in alkaline media using a *Halomonas* isolate from Soap Lake, Washington

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Abstract Hexavalent chromium is one of the most widely distributed environmental contaminants. Given the carcinogenic and mutagenic consequences of Cr(VI) exposure, the release of Cr(VI) into the environment has long been a major concern. While many reports of microbial Cr(VI) reduction are in circulation, very few have demonstrated Cr(VI) reduction under alkaline conditions. Since Cr(VI) exhibits higher mobility in alkaline soils relative to pH neutral soils, and since Cr contamination of alkaline soils is associated with a number of industrial activities, microbial Cr(VI) reduction under alkaline conditions requires attention.

Soda lakes are the most stable alkaline environments on earth, and contain a wide diversity of alkaliphilic organisms. In this study, a bacterial isolate belonging to the *Halomonas* genus was obtained from Soap Lake, a chemically stratified alkaline lake located in central Washington State. The ability of this isolate to reduce Cr(VI) and Fe(III) was assessed under alkaline (pH = 9), anoxic, non-growth conditions with acetate as an electron donor. Metal reduction rates were quantified using Monod kinetics. In addition, Cr(VI) reduction experiments were carried out in the presence of Fe(III) to evaluate the possible enhancement of Cr(VI) reduction rates through electron shuttling mechanisms. While Fe(III) reduction rates were slow compared to previously reported rates, Cr(VI) reduction rates fell within range of previously reported rates.

Keywords Alkaline · Chromium · Metal reduction · Soap Lake · *Halomonas*

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Introduction

Improper chromium (Cr) waste handling has led to soil and groundwater contamination throughout the United States and across the globe (James 2001; Morgado et al. 2001; Stępniewska and Bucior 2001; Khan and Puls 2003). Within pH-Eh ranges typically found in soils, chromium will exist in either the trivalent state or in the hexavalent state (Bartlett and Kimble 1976a). Between the two oxidation states,

Cr(III), in addition to being less toxic, tends to bind more tightly to soils (Bartlett and Kimble 1976a; Pettine et al. 1998) and readily precipitates (Rai et al. 1987). Unlike Cr(III), Cr(VI) is acutely toxic, is soluble, and is not likely to coordinate with or adsorb to soil constituents (Tokunaga et al. 2003; Fendorf et al. 2000; Weng et al. 2001). Cr(VI) therefore exhibits much greater environmental mobility, thus increasing its overall environmental threat (Bartlett and Kimble 1976b; Fendorf et al. 2000). This is especially true in alkaline soils, where Cr(VI) exhibits enhanced mobility compared to neutral soils (Weng et al. 2001).

For these reasons, reduction of Cr(VI) to Cr(III) is a fundamental goal of many Cr(VI) remediation strategies (Lovley and Anderson 2000; Khan and Puls 2003; Alowitz and Scherer 2002; Melitas et al. 2001) and the ability of metal reducing microorganisms to catalyze this reduction has been the subject of extensive research (Sani et al. 2002; Guha et al. 2001; Liu et al. 2002; Viamajala et al. 2003; Wielinga et al. 2001; Viera et al. 2003). While many examples of bacterial Cr(VI) reduction have been reported, virtually all were conducted at near-neutral pH values. To our knowledge, few reports of bacterial Cr(VI) reduction under alkaline conditions have been published (Khijniak et al. 2003; Ye et al. 2004; Stewart et al. 2007). Cr(VI) reduction under high pH conditions is important for certain bioremediation efforts because Cr(VI) contamination has been reported in high pH soils in association with improper tannery waste disposal (Kamaludeen et al. 2003). Chromite ore processing residue (COPR) is another major source of high pH Cr contamination (Geelhoed et al. 2002). Since Cr(VI) reduction products are least soluble at pH = 9 (Rai et al. 1987), alkaliphilic Cr(VI) reducing bacteria could potentially be useful in the remediation of these types of Cr contaminated sites.

Soda lakes represent the most stable alkaline environments on earth and the microbial diversity of such environments has been the subject of many reviews (Duckworth et al. 1996; Jones and Grant 1998; Sorokin and Kuenen 2005). Soap Lake (47°23′N, 119°30′W) is a chemically stratified soda lake located in the Grand Coulee Basin in central Washington State, and has an average pH of 9.8 (Oremland and Miller 1993; Pinkart et al. 2006). The top layer of the lake, where circulation and mixing periodically occur, is called the mixolimnion. The lower layer, perennially stagnant with no periodic mixing, is called the monimolimnion. The alkalinity of Soap Lake makes it a natural source of alkaliphilic bacteria, and recently novel alkaliphilic species and genera have been isolated from Soap Lake (Dimitriu et al. 2005; Pinkart et al. 2006; Sorokin et al. 2007), although their potential for remedial applications has yet to be determined.

As part of the NSF Soap Lake Microbial Observatory, a bacterial isolate was assessed for its ability to reduce Cr(VI) under anoxic, alkaline (pH = 9) conditions. Previous studies have reported enhanced Cr(VI) reduction in the presence of Fe(III) through electron shuttling mechanisms (Wielinga et al. 2001; Lee et al. 2003). This phenomena was also investigated under alkaline conditions as part of this study by conducting Cr(VI) reduction experiments in the presence of Fe(III), and by performing metal reduction experiments with Fe(III) only. Monod kinetic models were used to quantify metal reduction rates and assign appropriate kinetic parameters.

Materials and methods

Sample collection and bacterial isolation

Samples were collected from the mixolimnion layer of Soap Lake just below the lake surface, and stored on ice in sterile, airtight, 250 ml plastic bottles. Samples were taken back to Washington State University (Pullman, WA) and used to inoculate liquid growth media the same day. Modified Soap Lake Basal Media (SLBM), adapted from Dimitriu et al. (2005), was used as growth media and contained the following (per l): 17.5 g NaCl, 0.25 g KH_2PO_4, 0.5 g NH_4Cl, 0.1 g Bacto yeast extract, 4.0 g $Na_2B_4O_7$, and 10 g/l acetate (as $NaC_2H_3O_2$) as the primary carbon source and electron donor. Medium pH was raised to 9.0 using 10 M NaOH. Samples from the liquid enrichment were plated on alkaline (pH = 9) tryptic soy agar plates (TSA) (40 g/l) (Becton, Dickinson & Co., Sparks, MD). An individual colony was transferred separately three times onto fresh TSA plates before being transferred into 50 ml of liquid growth media. This liquid culture was allowed to reach late-log growth phase before being separated into 1.8 ml stock cultures in sterile

20% glycerol (Fisher, Fair Lawn, NJ) solution, and stored at −84°C.

Metal reduction experiments

The isolate, hereafter referred to as SL1, was used in a series of Cr(VI) and Fe(III) reduction experiments under anoxic, non-growth conditions. The decision to use a bacterium isolated under aerobic conditions in a series of anoxic experiments was a result of our failure to obtain an isolate under anoxic conditions. However, aerobic Soap Lake isolates have demonstrated anoxic metabolic capabilities including nitrite reduction (Mormile et al. 1999; Dimitriu et al. 2005). We were therefore interested in the possibility of finding an aerobic Soap Lake isolate capable of metal reduction. Since initiation of bioremediation efforts can cause aerobic soils to become anoxic (Anderson et al. 2003), understanding how native aerobes interact with metal contaminants under anoxic conditions is important for understanding microbe-metal interactions during bioremediation.

Stock cultures of SL1 were used to inoculate aerobic growth media and allowed to reach stationary phase. Cells were then pelleted by centrifugation for 10 min at 6,000*g* and washed three times with non-growth media (per l): 17.5 g NaCl, 4.0 g $Na_2B_4O_7$, 10 g/l acetate. Washed cells were suspended separately into two sterile, HCl-washed, 150 ml serum bottles at a cell density of 80.1 ± 10 mg-protein/l and a culture volume of 100 ml. Cr(VI) in the form $K_2Cr_2O_7$ or Fe(III) in the form of Fe(III) citrate was then added as the electron acceptor. Cr(VI) concentrations of 0.1 mM were used in all Cr(VI) reduction experiments. Fe(III) concentrations of 2 mM were used in Fe(III) reduction experiments, while 0.5 mM Fe(III) was used in the combined Fe–Cr experiments. Serum bottles were capped with butyl stoppers and crimped with aluminum seals. Filter sterilized (0.2 μm) ultrahigh-purity (99.999%) nitrogen gas was bubbled through media for 30 min to establish anoxic conditions, as confirmed by the resazurin test (Twigg 1945). Serum bottles were then placed on shakers rotating at 130 rpm in a 35°C temperature controlled chamber. Samples were removed periodically using nitrogen-purged syringes for metal analysis. Abiotic, metal free, and heat-killed cell controls were performed, in singlet, parallel to all metal reduction experiments.

Analytical methods

Protein concentrations were measured using a procedure based on the Coomassie assay (Bradford 1976). Since these experiments utilized bacteria adapted to a high pH environment, the traditional method of high pH digestion was not sufficient for effective cell lysis. Instead, samples were washed with non-growth media, as described above, and sonicated for 3 min using a Branson sonicator (model 102C, Danbury, CT). The sonicated sample was added to Coomassie dye (Pierce, Rockford, IL) and absorbance was measured at 595 nm. Standards of known protein concentration were made using bovine serum albumin (BSA) (Pierce, Rockford, IL). Cr(VI) was measured colorimetrically using the diphenylcarbazide (DPC) method (Bartlett and Kimble 1976a). Total Cr was measured by diluting 0.5 ml of sample in 4.5 ml of 0.5 M HNO_3 and measuring using an inductively coupled mass spectrometer (ICP-MS) (Agilent, Model 4500, Palo Alto, CA). Cr(III) concentrations were calculated as the difference between total Cr and Cr(VI) concentrations. Fe(II) concentrations were measured using the ferrozine method and acid extraction according to Lovley and Phillips (1987). Total Fe was quantified by sample digestion in a hydroxylamine hydrochloride ($NH_4OH \cdot HCl$) solution for 1 h to reduce all Fe(III) to Fe(II), and subsequent application of the ferrozine method. Fe(III) concentrations were calculated as the difference between total Fe and Fe(II) concentrations.

SEM and AFM microscopy

Bacterial samples were fixed overnight in 2.5% glutaraldehyde/2% paraformaldehyde in 0.1 M phosphate buffer, rinsed with 0.1 M phosphate buffer, post fixed with 1% osmium tetroxide (4 h), dehydrated in an ethanol series (30%, 50%, 70%, 95%, and 100%) followed by 2 exchanges of 100% acetone before treatment with hexamethyldisilizane (Ted Pella, CA). Samples were gold coated (Technics Hummer V Sputter Coater, Anatech, San Jose CA). Micrographs were collected using a Hitachi S-570 scanning electron microscope (Hitachi Scientific Instruments, Mountain View,CA) with the Quartz PCI Imaging system Software, (Vancouver, BC) at the Franceschi Microscopy and Imaging Center, Washington State University. AFM imaging was

carried out at Montana State University's (Bozeman, MT) Image and Chemical Analysis Laboratory (ICAL) with a Nanoscope IIIa Extended Multimode AFM (Veeco, Santa Barbara, CA) with a J-type scanner. Imaging in air was performed in tapping mode to reduce the tip-sample interaction and lateral forces. The sample was prepared by incubating a drop of the bacterial suspension on a mica disk for 10 min at room temperature, followed by phosphate-buffered saline (PBS) rinsing, and drying with nitrogen.

Calculation of kinetic parameters

Experiments involving Cr(VI) reduction were described using the Monod equation (1), where μ and μ_{max} are the measured and maximum specific reduction rates (mM metal/(mg-protein · day)), respectively, M is the metal concentration (mM metal), and K_S is the half-saturation constant (mM metal).

$$\mu = \frac{\mu_{max} M}{K_S + M} \quad (1)$$

To solve for $\mu_{max}$and K_S, the Lineweaver-Burke method was used (Lineweaver and Burk 1934).

Because zero-order kinetics was sufficient to describe Fe(III) reduction for the duration of these experiments, this method was used. For these experiments, metal concentration versus time curves were fit to a line using regression in EXCEL, with the slope of this line equal to the maximum specific reduction rate.

16S sequence characterization

A liquid culture of isolate SL1 was sent to Laragen, Inc. (Pasadena, CA) for 16S rRNA gene amplification and sequencing. Sequencing was performed with an ABI 377XL automated sequencer. A neighbor-joining phylogenetic tree was constructed using the approximately 800 base pair 16S rRNA gene sequence obtained from Laragen, Inc. and sequences of named *Halomonas* species obtained through BLAST searches of the GenBank database. Sequences were chosen based on a 16S rRNA sequence analysis of the family *Halomonadaceae* performed by Arahal et al. (2002a). The sequences were aligned with ClustalX 1.81 software (European Molecular Biology Laboratory, Heidelberg, Germany), the tree was created using PAUP* 4.0 (Sinauer Associates Inc., Sunderland, MA, USA) using bootstrap values based on percentage of 1,000 iterations. Tree was drawn using TreeView 1.6.6 (Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, Scotland, UK) and rooted with *Cobetia marina*, a formerly recognized member of the *Halomonas* genus (Arahal et al. 2002b).

Results and discussion

Genetic and microscopic characterization

Figure 1 is a phylogenetic tree depicting the 16S rRNA gene sequence relationship between isolate SL1 and named members of the *Halomonas* genus. Previous phylogenetic analysis of *Halomonas* species revealed two distinct groups, the first consisting of *Halomonas elongata*, *H. eurihalina*, *H. halmophila*, *H. halophila*, and *H. salina* species, and the second including *H. aquamarina*, *H. meridinia*, *H. magadiensis*, *H. veriabilis*, *H. venusta*, *H. halodurans*, and *H. subglaciescola* species (Arahal et al. 2002a). In addition, the previous study identified a number of *Halomonas* species, including *Halomonas pacifica*, *H. halodenitrificans*, *H. cupida*, *H. desiderata*, *H. campisalis*, and *H. pantelleriensis* as not belonging to any distinct phylogenetic groups, due to their relatively low level of sequence similarity. In this study, 16S rRNA sequences of the members of the two distinct groups and *H. campisalis* were included in the phylogenetic analysis along with the sequence of isolate SL1 to make meaningful comparisons with previous reports. Consistent with Arahal et al. (2002a), the two distinct phylogenetic groups are represented on the phylogenetic tree of Fig. 1, with *Halomonas campisalis* forming an out-group with a correspondingly low bootstrap value (52%). With 98% 16S rRNA gene sequence similarity to *H. salina*, SL1 appears associated with the first phylogenetic group mentioned above, however, its placement either within or outside the clade cannot be assumed, given the intermediate bootstrap value (74%), without further characterization.

Halomonas spp. often represent a major constituent of alkaliphilic organisms isolated from soda lakes in various locations (Jones and Grant 1998) including Mongolia (Khijniak et al. 2003), the Kenyan section of

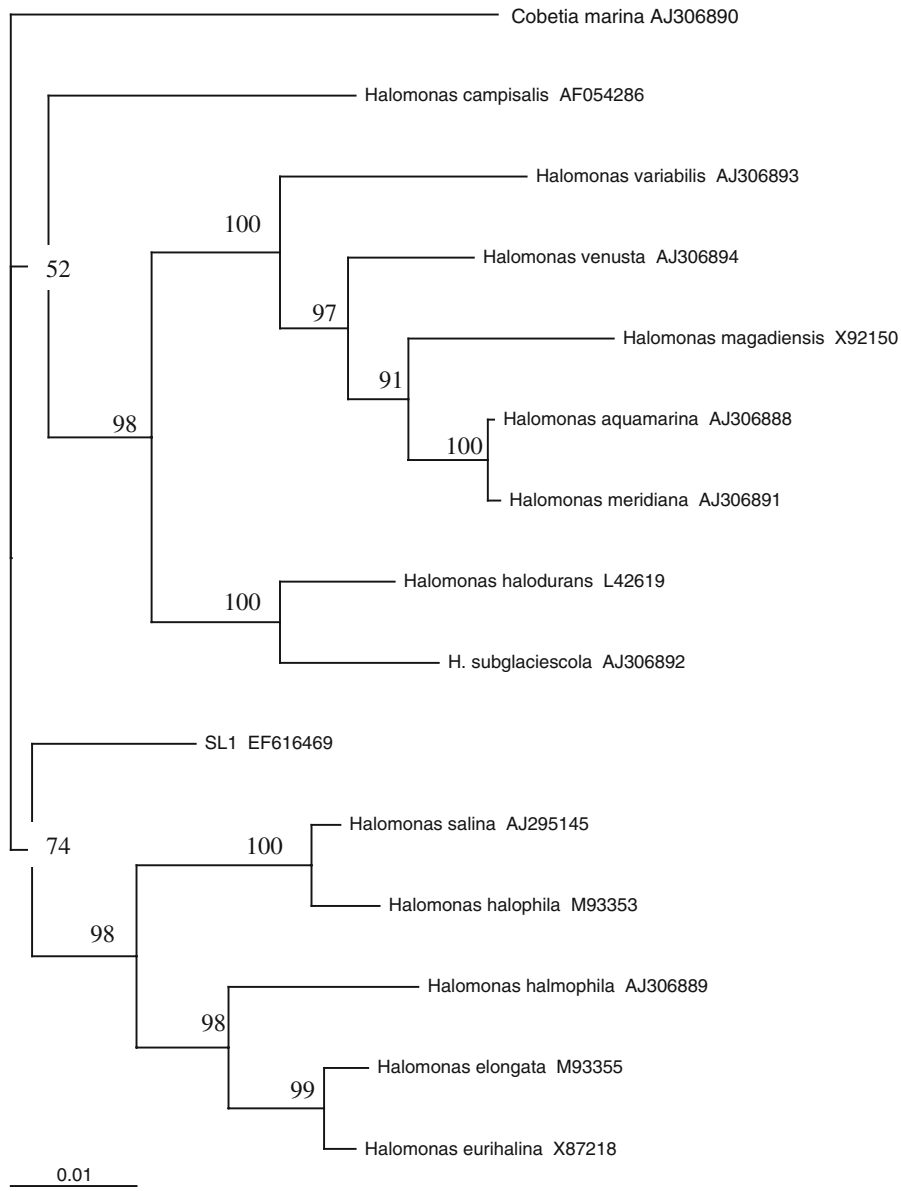

Fig. 1 Phylogenetic tree based on the 16S rRNA sequence of SL1 and the sequences of other, named *Halomonas* species. Numbers to the right of the sequence names represent GenBank accession numbers. Numbers at the nodes correspond to bootstrap values as a percentage of 1,000 iterations. *Cobetia marina*, a formerly recognized member of the *Halomonas* genus, was used as an outgroup. Bar represents 1% estimated sequence divergence

the East African Rift Valley (Duckworth et al. 1996), and Mono Lake, California, USA (Oremland et al. 2000). *Halomonas* spp. are usually associated with aerobic environments and have subsequently been used in several biotransformation studies under aerobic conditions, including the degradation of catechol (Alva and Peyton 2003) and salicylate (Oie et al. 2007). However, some *Halomonas* spp., including *H. maura*, are capable of anoxic respiration under nitrate reducing conditions (Aragandoña et al. 2006; Llamas et al. 2006; Peyton et al. 2001). Previous studies have utilized *Halomonas* isolates to study metal-microbe interactions under anoxic conditions (Francis et al. 2000; Khijniak et al. 2003), although Cr(VI) reduction by a *Halomonas* isolate has not been reported.

Both AFM and SEM imaging of SL1 revealed the presence of short rods approximately 1–2 μm in length (Fig. 2a, b, respectively). Although the substance seen binding the cells in the SEM image was unidentified, it resembled exopolysaccarides (EPS's). This was interesting since *Halomonas* derived EPS has been the subject of previous research as it has potential biotechnological applications (Llamas et al. 2006).

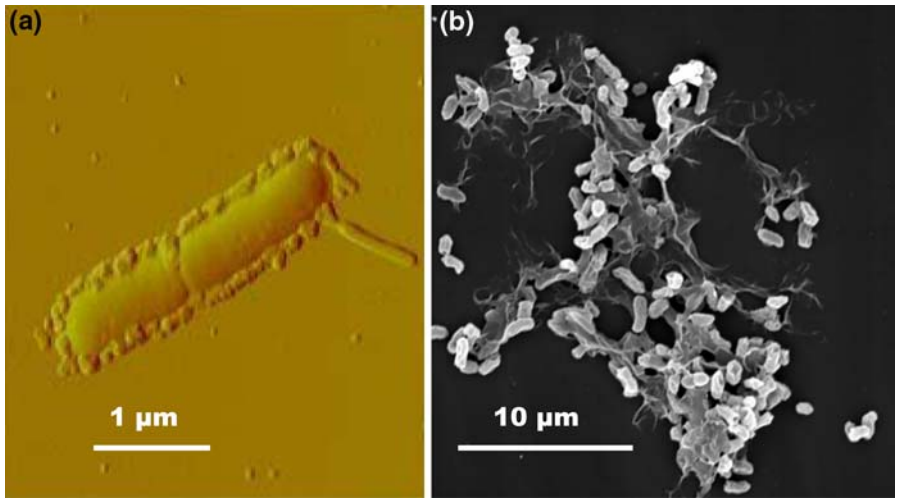

Fig. 2 (**a**) AFM image depicting *Halomonas* isolate taken from mixolimnion layer of Soap Lake. The isolate appears as short rods between 1 µm and 2 µm in length. (**b**) SEM image of the same isolate. The substance attached to the isolate was not identified

Cr(VI) and Fe(III) reduction, and Cr(VI) reduction in the presence of Fe(III)

Figure 3 depicts Cr(VI) reduction by SL1. After 25 days, most (>75%) of the 0.1 mM Cr(VI) initially present was reduced to Cr(III). The maximum specific reduction rate was found to be $1.6 \pm 0.24 \times 10^{-4}$ mM Cr(VI) · day^{-1} · $mg\text{-}protein^{-1}$ (Table 1). Comparing Cr(VI) reduction rates between different studies can be difficult, given the wide range of culturing conditions used in such studies and the effect such conditions can have on reduction rates (Guha et al. 2001). However, we were interested to know how Cr(VI) reduction rates under alkaline conditions compared to previously reported reduction rates under pH-neutral conditions with acetate as electron donor. While SL1 was able to reduce Cr(VI) up to two times faster than the *Pseudomonas* spp. used by Badar et al. (2000), the *Microbacterium* sp. used by Pattanapipitpaisal et al. (2001) exhibited a Cr(VI) reduction rate more than seven times greater than SL1 (Table 1). The reduction rate of SL1 falls between the values calculated in these two studies. This is compared to *Shewanella putrefaciens* CN32 which, with lactate as electron donor, can reduce Cr(VI) at a rate exceeding 1,000 times that of SL1 (Liu et al. 2002). The K_s value calculated in these experiments was 0.15 ± 0.01 mM Cr(VI), a value comparable to the K_s values calculated by Shen and Wang (1994) as part

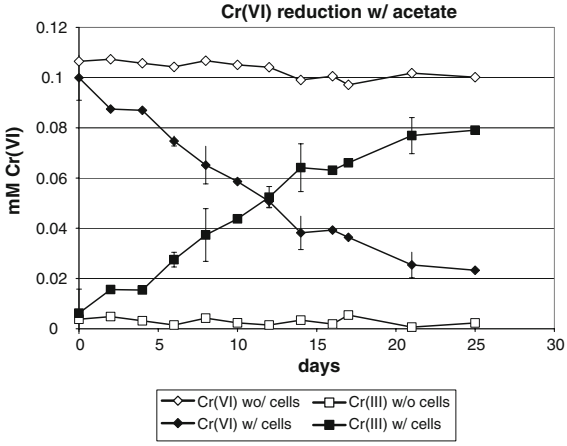

Fig. 3 Cr(VI) reduction and Cr(III) accumulation when acetate served as substrate. Error bars represent the standard deviation of experiments completed in duplicate

Table 1 Previously reported Cr(VI) reduction rates in which acetate served as electron donor

Isolate	Max. specific reduction rate[a]	Reference
Microbacterium sp. MP30	1.14E-03	Pattanapipitpaisal et al. 2001
Pseudomonas stutzeri CMG462	7.20E-05	Badar et al. 2000
Pseudomonas stutzeri CMG463	6.24E-05	
Pseudomonas synxantha K2	1.34E-04	
SL1	1.60E-04	This study

[a] In mM-Cr(VI)/(mg-protein · day)

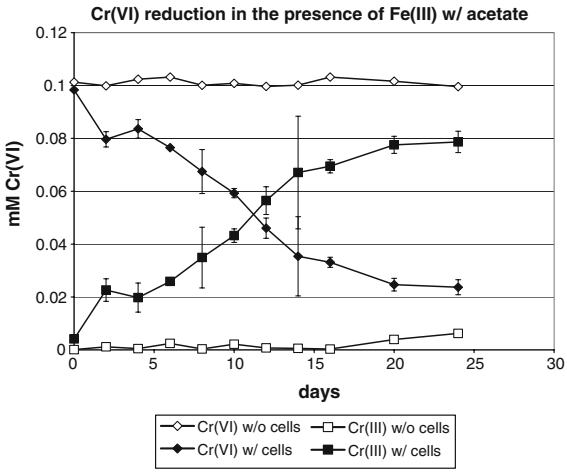

Fig. 4 Cr(VI) reduction in the presence of Fe(III) when acetate served as substrate. Error bars represent the standard deviation of experiments completed in duplicate

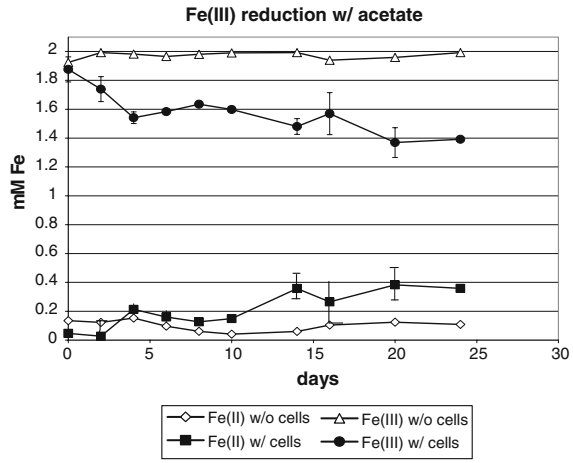

Fig. 5 Fe(III) reduction and Fe(II) accumulation when acetate served as substrate, showing a relatively small amount of Fe(III) being reduced over a 24 day period. Error bars represent the standard deviation of experiments completed in duplicate (Absence of error bars indicates error bars smaller than the data points.)

of their Cr(VI) reduction studies with *Escherichia coli* ATCC 33456.

Cr(VI) reduction in the presence of 0.5 mM Fe(III) is shown in Fig. 4. This experiment tested the possible enhancement of Cr(VI) reduction rates through the electron shuttling action of reduced Fe species, a phenomena which has been reported in previous studies, but never under alkaline conditions. Fendorf et al. (2000) and Wielinga et al. (2001) both conducted Cr(VI) reduction experiments in the presence of Fe(III) using *Shewanella alga* BrY in which they reported 10.2- and 4.5-fold increases in Cr(VI) reduction rates compared to Cr(VI) reduction rates in Fe-free controls. In these experiments, the Fe(III) did not enhance Cr(VI) reduction. An almost identical amount of Cr(VI) was reduced (approx. 76%), and the specific Cr(VI) reduction rate ($1.25 \pm 0.2 \times 10^{-4}$ mM Cr(VI) $\cdot$ day^{-1} $\cdot$ mg-protein^{-1}), although lower, was statistically identical with the Fe-free system. The large error bars at day 14 are the result of an unusually low Cr(VI) concentration measurement, probably due to experimental error, as the measured concentration recovered. Total Fe and Fe(II) concentrations remained constant (0.5 and 0 mM, respectively) throughout the experiment (data not shown). Unlike the specific reduction rate, the K_s value calculated in this experiment (0.10 ± 0.01 mM Cr(VI)), was significantly lower than the Fe-free control.

Figure 5 depicts Fe(III) reduction and Fe(II) accumulation. Zero-order kinetics described the rate of Fe(III) reduction, and therefore no K_s values were calculated. The maximum specific Fe(III) reduction rate was found to be $1.8 \pm 0.24 \times 10^{-4}$ mM Fe(III) $\cdot$ day^{-1} $\cdot$ mg-protein^{-1}, statistically identical with the Cr(VI) reduction rate. However, in terms of electron transfer rates, isolate SL1 was able to transfer electrons to Cr(VI) approximately three times faster than to Fe(III). This is because reduction of Cr(VI) to Cr(III) is a three electron transfer, compared to the one electron transferred as Fe(III) is reduced to Fe(II). This might explain why the presence of Fe(III) did not enhance Cr(VI) reduction rates.

The Fe(III) reduction rate calculated here is slow compared to previous Fe(III) reduction experiments conducted at neutral pH conditions in which acetate served as electron donor. For example, *Geobacter metallireducens* GS-15 was able to reduce Fe(III) approximately 2,500 times faster than SL1 (Liu et al. 2002). However, it is worth noting the results of a recent study in which anoxic Fe(III) reduction was carried out under alkaline conditions ($\sim$pH = 9) using soil microcosms obtained from a COPR disposal site (Stewart et al. 2007). Similar to this study, acetate was added as an electron donor to stimulate microbial metal reduction, and subsequent Fe(III) reduction proceeded slowly. On average, only $\sim$20% of the Fe(III) initially present was reduced

after 72 days of exposure. However, since no kinetic evaluations of Fe(III) were reported, quantitative comparisons with this study are not possible.

It has long been known that the toxicity of chromium depends heavily on its valence state (Mertz 1969), a fact which has lead many to consider the potentially positive role of metal reducing bacteria in chromium remediation schemes. Although many species of metal reducing bacteria are capable of reducing and immobilizing Cr(VI), progress in developing methods of microbial metal remediation has lagged behind bioremediation of organic contaminants (Lovley and Anderson 2000). This is particularly true of toxic metal bioremediation of alkaline environments, despite reports of Cr(VI) contamination of alkaline soils. In addition to the examples mentioned earlier, soil samples from underneath waste tank SX-108 located in the west-central part of the Department of Energy site at Hanford, Washington have measured Cr concentrations exceeding 500 ppm and range in pH from 7.2 up to 9.8 (Fredrickson et al. 2004).

Despite the potential importance of metal-microbe interactions in contaminated alkaline systems, practical applications of alkaliphiles and alkaliphilic enzymes are limited, with the detergent industry as their main industrial application (Horikoshi 1996; Ito et al. 1998). Alkaliphilic enzymes have also found use in the hide-dehairing step of the leather production process, an interesting fact since Cr(VI) compounds are also used in leather production, specifically in the leather tanning process (Petruzzelli et al. 1994). Reported studies involving the potential role of alkaliphiles for bioremediation purposes and their potential role in metal contaminant mobility are rare, and since metal contamination is not limited to pH neutral environments, investigating this role could prove beneficial.

Conclusions

Metal reduction has been demonstrated in alkaline media using a bacterial isolate from the mixolimnion of Soap Lake, Washington. The isolate is a member of the *Halomonas* genus, and was able to anoxically, and under non-growth conditions, couple the oxidation of acetate to the reduction of either Cr(VI) or Fe(III). While Fe(III) reduction rates were slow compared to previously reported rates, Cr(VI) reduction rates calculated here exceeded many previously reported rates. The presence of Fe(III) was not found to enhance Cr(VI) reduction rates, contrary to findings of many who have found the electron shuttling action of Fe(III) beneficial in increasing reduction rates. The results presented here represent one of the few reports of microbial metal reduction under alkaline conditions.

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