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Microbial mass-dependent fractionation of chromium isotopes

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Abstract

Mass-dependent fractionation of Cr isotopes occurs during dissimilatory Cr(VI) reduction by *Shewanella oneidensis* strain MR-1. Cells suspended in a simple buffer solution, with various concentrations of lactate or formate added as electron donor, reduced 5 or 10 μM Cr(VI) to Cr(III) over days to weeks. In all nine batch experiments, $^{53}Cr/^{52}Cr$ ratios of the unreacted Cr(VI) increased as reduction proceeded. In eight experiments covering a range of added donor concentrations up to 100 μM, isotopic fractionation factors were nearly invariant, ranging from 1.0040 to 1.0045, with a mean value somewhat larger than that previously reported for abiotic Cr(VI) reduction (1.0034). One experiment containing much greater donor concentration (10 mM lactate) reduced Cr(VI) much faster and exhibited a lesser fractionation factor (1.0018). These results indicate that $^{53}Cr/^{52}Cr$ measurements should be effective as indicators of Cr(VI) reduction, either bacterial or abiotic. However, variability in the fractionation factor is poorly constrained and should be studied for a variety of microbial and abiotic reduction pathways.

1. INTRODUCTION

Hexavalent chromium, Cr(VI), is a common contaminant in soils, groundwater and surface water (Nriagu and Niebor, 1988; Proctor et al., 2000) and is a redox-sensitive trace element in the oceans (Murray et al., 1983; Pettine, 2000; Sirinawin et al., 2000) and other uncontaminated systems. Anthropogenic sources include Cr plating, leather tanning, pigment production facilities, lumber preservation, and cooling water conditioning (Nriagu and Niebor, 1988), though weathering of ultramafic rocks can be a significant source also (Robertson, 1975; Robles-Camacho and Armienta, 2000). Cr(VI) is associated with acute health effects and with prolonged exposure is a potential cause of cancer, ulcers, and internal organ damage (U.S. Department of Health, 2000).

Cr is present in natural aqueous systems as Cr(VI) and/or Cr(III) (Ball and Nordstrom, 1998). Cr(VI) is present as the soluble, mobile chromate (CrO_4^{2-}) and hydrochromate ($HCrO_4^-$) anions. Reduction of Cr(VI) to Cr(III) renders the Cr less toxic and relatively immobile because Cr(III) adsorbs to geologic materials and/or forms solid precipitates (e.g., Jardine et al., 1999; Blowes, 2002). Natural attenuation of Cr(VI) contamination via its reduction occurs commonly. In systems without natural reduction, remediation schemes often focus on artificially induced reduction (Blowes, 2002). Quantifying reduction is thus a critical task in contaminated settings. In the oceans, redox reactions are thought to play a role in the cycling of Cr (Murray et al., 1983; Pettine, 2000).

Lighter isotopes of an element tend to have slightly greater reaction rates, and thus the reaction products tend to be enriched in lighter isotopes (e.g., Hoefs, 1997; Johnson and Bullen, 2004). In a closed or semi-closed system, the remaining, unreacted pool of the reactant species becomes progressively enriched in heavier isotopes as the reaction proceeds. This enrichment has been used to quantify reduction of the oxyanions sulfate (Strebel et al., 1990; Schroth et al., 2001), nitrate (Böttcher et al., 1990;

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McMahon and Böhlke, 1996; Sutka et al., 2004), selenate (Johnson et al., 2000; Herbel et al., 2002), and Cr(VI) (Ellis et al., 2002). It also accompanies various reactions involving organic molecules (e.g., Sherwood-Lollar et al., 2001; Kuder et al., 2005).

Ellis et al. (2002) reported the magnitude of Cr isotope fractionation induced by Cr(VI) reduction in slurries of magnetite and two different sediments. In all three experiments, reduction occurred abiotically. The magnitude of isotopic fractionation can be expressed using a fractionation factor α, defined by:

$$\alpha = \frac{R_{reac}}{R_{prod}} \quad (1)$$

where R_{reac} and R_{prod} are the $^{53}Cr/^{52}Cr$ ratios of the reactant and the reaction product flux, respectively, at one point in time. For all three experiments, $\alpha = 1.0034 \pm 0.0001$, i.e., the reaction flux was depleted in ^{53}Cr by about $3.4 \pm 0.1‰$ relative to the reactant. Given the limited scope of these experiments, fractionation factors for Cr(VI) reduction by a variety of mechanisms must be determined before Cr isotope ratios can provide a robust indication of Cr(VI) reduction.

Microbial action is an important and possibly dominant Cr(VI) reduction mechanism. Numerous Cr(VI)-reducing microorganisms have been isolated and described (Wang, 2000; Lloyd, 2003), and experiments using pure cultures and microcosms from uncontaminated and contaminated soil and sediment suggest microbes with Cr(VI) reduction capabilities are widespread in the environment (Turick et al., 1996; Lojou et al., 1998; Francis et al., 2000; Wang, 2000). Cr(VI) can be reduced indirectly by microbes that produce reductants such as Fe(II), but direct reduction, e.g., via use of Cr(VI) as an electron acceptor, is probably a dominant reduction mechanism under certain circumstances (Marsh et al., 2000; Lloyd, 2003).

Research on isotopic fractionation during sulfate, nitrate, and selenate reduction indicates that the magnitude of the fractionation depends on the metabolic pathway and metabolic state (e.g., nutrient limitation) of the microbes. Bacteria with abundant electron donors and other nutrients reduce sulfate, nitrate, or selenate more rapidly and tend to exhibit smaller isotopic fractionation factors than under less ideal conditions (e.g., Kaplan and Rittenberg, 1964; Chien et al., 1977; Herbel et al., 2000). Accordingly, if fractionation factors determined in the laboratory are to be applicable to natural settings, the bacterial media should approximate the conditions in those settings.

This paper presents measurements of Cr isotope fractionation induced by direct reduction of Cr(VI) by *Shewanella oneidensis* MR-1 in washed cell suspensions. Small electron donor concentrations were used in these experiments in order to attain conditions close to those occurring in aquifers.

2. MATERIALS AND METHODS

2.1. Bacterial strain description and cell culturing methods

Shewanella oneidensis MR-1 is a Gram-negative, mesophilic, facultative anaerobe isolated from the sediments of Lake Oneida, New York (Myers and Nealson, 1988). MR-1, the type strain (previously designated *Alteromonas putrefaciens* MR-1 and *Shewanella putrefaciens* MR-1), can reduce Cr(VI) (Myers et al., 2000), as well as nitrate, thiosulfate, iron and manganese oxides (Myers and Nealson, 1988) and other species. This organism couples Cr(VI) reduction to oxidation of the environmentally relevant electron donors lactate (Viamajala et al., 2002) and formate (Myers et al., 2000).

Batches of *S. oneidensis* MR-1 were grown to provide cells for the suspension experiments. Throughout the study, media were prepared using high-purity water ($\geqslant 18.2\ M\Omega\ cm$) and headspaces of ultra-high-purity N_2 gas. Whenever syringes were used, they were flushed with N_2 prior to use and N_2 was injected to compensate for sample withdrawals. Serum bottles were closed with thick blue butyl rubber stoppers and aluminum crimp seals. Cells were grown anaerobically at room temperature in a defined medium at pH 7.4 described by Myers and Nealson (1988, 1990). This medium was supplemented with 5–20 mM lactate as the electron donor and 10–40 mM fumarate as electron acceptor. Nutrient amendments such as casamino acids were omitted in order to avoid carryover of potential electron donors to the cell suspensions, and Cr(VI) was omitted to avoid carryover of isotopically fractionated Cr. Growth on fumarate results in the ability of MR-1 to reduce Cr(VI) and other metals without prior exposure (Myers et al., 2000; Viamajala et al., 2002).

Three batches of cells, designated A, B, and C, were grown at separate times. For each batch, a 1 L serum bottle containing degassed medium was inoculated by transfer of a single colony grown aerobically in a Petri dish containing LB Agar (Miller, 1972). The cultures were incubated on a rotary shaker table until they reached a desired density ranging between 1×10^8 and 6×10^8 cells/mL, then harvested.

2.2. Preparation and sampling of cell suspensions

In the Cr(VI) reduction experiments, cells were suspended in a simple phosphate buffer solution with salts, modified from that used by Oremland et al. (1994) for experiments with selenate reducing bacteria. The solution contained 1.9 mM K_2HPO_4, 2.5 mM KH_2PO_4, 1.0 mM $MgCl_2$, and 32 mM NaCl, and was prepared in glass bottles fitted with 0.070″ Teflon–silicone disk septa. The pH was adjusted to 7.0 using NaOH. The buffer was autoclaved, then sparged for 90 min with N_2 passed through a 0.2 μm filter. Lactate, formate, and Cr(VI) solutions were prepared from crystalline potassium formate, 85% lactic acid solution, and anhydrous sodium chromate. The formate and lactate solutions were adjusted to pH 7.0 with NaOH, and sterilized by filtration through sterile 0.2 μm filters. The chromate solution was sterilized by autoclaving. All three solutions were purged with N_2 for 30 min.

We used lactate as the electron donor for the majority of the experiments because it is used commonly in experiments with this bacterium. Formate was used in several additional experiments because it is more abundant in natural settings and we wished to begin investigating whether the isotopic fractionation depends on the type of donor. Although data on the concentrations of low molecular weight organic acids and other naturally occurring electron donors are sparse, their concentrations appear to be several micromolar or less in most aquifers (Chapelle and Lovley, 1992; Hansen et al., 2001). In more organic-rich settings, such as the upper meter of soil horizons, they may range up to 1 mM (Strobel, 2001). Total dissolved organic carbon concentrations in most groundwaters are less than 2 mg carbon per L, and they are <15 mg/L in most rivers, <5 mg/L in most lakes, and <1 mg/L in the oceans. Much of this consists of large molecules that cannot be directly metabolized by bacteria (Drever, 1997). The present study was designed to be applicable to shallow aquifers similar to that

studied by Hansen et al. (2001), where formate and acetate concentrations of less than ~4 and ~10 μM, respectively, were measured. Accordingly, we set up one experiment with 3 μM lactate and another with 7 μM formate. However, cell death apparently supplied electron donor in abundances much greater than these (see below). Several other experiments ranged up to 100 μM donor, and a single experiment was conducted with 10,200 μM lactate, a concentration comparable to that used in previous isotopic fractionation studies with sulfate and selenate reducers.

Cells were separated from growth media and transferred to the serum bottles in which experiments were run using methods designed to ensure that carryover of lactate, fumarate, and O_2 was insignificant. Preparation of cell suspensions differed slightly between the batches, with batches B and C prepared so as to decrease cell damage. To remove growth medium, aliquots of the cell cultures were centrifuged at approximately 14,000g for twenty minutes (set A) or eleven minutes (sets B and C). The supernatant was discarded, and the cells were washed three times by resuspension in degassed suspension buffer, centrifugation, and removal of supernatant. Because fluid was almost completely expelled from the pellet during each centrifugation, growth medium carried over to cell suspensions was diluted by a factor of at least 10^6, and electron donor carryover was negligible. To preserve cells during this procedure, suspension buffer was refrigerated until immediately prior to each use, and in sets B and C, ice was added to the centrifuge tubes immediately before centrifugation. In order to minimize exposure of the cells to O_2, headspaces of centrifuge tubes were kept O_2-poor by continuously injecting N_2 into the head space and minimizing the time tubes were kept open.

The final cell slurries for injection into the experiments were created by suspending them in 15 mL buffer and injecting each batch into one 100 mL serum bottle. This dense cell slurry was shaken on a rotary shaker table for at least fifteen minutes. Phase-contrast microscopy was used to obtain cell density estimates. Average cell densities, after dilution into the experiment bottles, were calculated for each set of suspensions (Table 1). Because the counts were done on diluted suspensions, the number of cells counted was small and uncertainties were large, at roughly ±50% for batch A, ±13% for batch B, and 33% for batch C. These uncertainties are those of each set as a whole. Since precise and equal volumes of dense cell slurry were added to each suspension within a set, variation between experiments created from a single batch was less than 5%. Cell densities were also monitored occasionally during the course of experiments, and variations were not observed.

The dense, stationary phase cell slurries (approximately 5–40 mL of cell suspension per bottle depending on cell density requirements) were injected into 270 mL or 100 mL serum bottles containing degassed suspension buffer. For cell batch A, a killed-cell control was prepared by autoclaving an aliquot of the dense cell slurry prior to transfer to an experiment bottle. For the batch C killed-cell control, cells were transferred live to serum bottles, which were then heated in a water bath at 60 °C for 2 h.

To start the experiments, bottles were injected with electron donor and Cr(VI) solutions. Precise determinations of amounts added (better than ±8% and ±8 nM, respectively) were made by measuring weight changes. No-cell and killed-cell controls received the same concentrations of lactate and Cr(VI) as other suspensions within their sets (35 μM lactate in set A). In set C, a second killed-cell control received no electron donor. The cell suspensions were incubated in the dark at room temperature with shaking at 150 rpm on a rotary shaker table. Samples of the suspensions were withdrawn by syringe periodically and immediately centrifuged. The supernatant was analyzed for Cr(VI) concentration, transferred to polypropylene centrifuge tubes and stored at 4 °C for isotopic analysis.

2.3. Determination of Cr(VI) concentration and Cr isotope ratios

Cr(VI) concentration was measured colorimetrically using EPA method 7196 A. Absorbance of acidified samples (~0.1 M HCl) was measured at 540 nm using a Thermo Genesys spectrophotometer with the diphenylcarbazide indicator. Reproducibility was approximately ±3% (2 σ), though at lower concentrations, baseline noise generated an uncertainty of ±0.006 mg/L (2σ). Standard additions were performed; no signal suppression due to complexation of Cr(VI) or other matrix effects was observed.

Samples were prepared for determination of the $^{53}Cr/^{52}Cr$ ratio according to the methods of Ellis et al. (2002), reviewed briefly here. We use a double isotope spike approach to correct for isotopic fractionation occurring during sample preparation and mass spectrometry (see below). A spike solution containing ^{50}Cr and ^{54}Cr in known proportions as Cr(VI) was added to aliquots of the samples containing 300–1000 ng Cr(VI). This double spike was added before sample preparation procedures to correct for any fractionation during the anion exchange purification process. Samples were then acidified to a pH between 1 and 3 and filtered through 0.2 μm nylon filters.

Cr(VI) was extracted from sample matrices and purified using a two-step anion exchange method, which separates the Cr(VI) from the sample matrix, including any Cr(III) and elements such as Fe, Ti, and V that cause isobaric interferences. Quartz-distilled HCl was used in all steps. The sample was passed through a 2.0 cm^3 bed

Table 1
Experimental conditions and calculated fractionation factors

Electron donor	Initial donor (μM)	Initial Cr(VI) (μM)	Cell batch used	Cell density[a] (10^7 cells/mL)	Cell-specific reduction rate first ~24 h (10^{-18} mol/cell/day)	Cell-specific reduction rate later[b] (10^{-18} mol/cell/day)	ε (‰)
Lactate	3.3	5.1	A	5 ± 3	42	1.2	4.1 ± 0.3[c]
Lactate	3.3	5.1	A	5 ± 3	43	1.5	4.5 ± 0.2[c]
Formate	6.8	5.1	A	5 ± 3	37	1.8	4.5 ± 0.6[c]
Lactate	35	5.1	A	5 ± 3	48	2.1	4.2 ± 0.2[c]
Lactate	35	5.1	A	5 ± 3	51	2.5	4.1 ± 0.2[c]
Formate	60	5.1	A	5 ± 3	45	2.3	4.0 ± 0.3[c]
Lactate	100	9.5	C	8.0 ± 1.0	91	7.7	4.2 ± 0.3[c]
Lactate	100	9.5	C	8.0 ± 1.0	92	7.7	4.1 ± 0.4[c]
Lactate	10,200	9.5	B	1.0 ± 0.33	450	8.8	1.8 ± 0.2[c]

[a] Cell density was uncertain but was identical within 5% for all experiments from a cell batch (see text).
[b] From about 24 h to 17 ± 1 days or end of experiment (see text).
[c] 95% confidence range derived from standard error of regression (see text).

of BioRad AG1-X8 anion exchange resin, to which the Cr(VI) adsorbed. Cations and weak acids were flushed from the column using 0.1 M HCl. The Cr(VI) was reduced to Cr(III) with sulfurous acid and eluted in 0.1 M HCl. The eluted solution was heated to convert S(IV) to sulfate, then passed through a second AG1-X8 resin column to remove the sulfate.

Blank solutions were processed through the cell suspension bottles and sample preparation methods and analyzed by ICP-MS. A 270 mL serum bottle, previously used for reduction experiments, was reloaded with degassed buffer, shaken for five days, and sampled in triplicate to determine background Cr contributions from cell suspension reagents and apparatus. In all cases, the mass of Cr detected in apparatus and method blanks was less than 3 ng, insignificant compared to the mass of Cr present in the actual experiments or processed for analysis.

Mass spectrometry was performed on a Finnigan MAT 261 multiple collector thermal ionization mass spectrometer (TIMS) at the U.S. Geological Survey, Menlo Park, CA, using procedures described in a previous publication (Ellis et al., 2002) and reviewed briefly here. The sample, 250–500 ng Cr, was mixed with 20 μg colloidal silica and 0.6 μL of a saturated boric acid solution and loaded onto a Re filament. In the mass spectrometer, filaments were slowly heated to final temperatures between 1100 °C and 1230 °C. $^{50}Cr/^{52}Cr$, $^{53}Cr/^{52}Cr$ and $^{54}Cr/^{52}Cr$ ratios were determined by simultaneous measurement of the ion beams. Fifty or more repeated integrations of 5 s were averaged, and outliers were removed. Interference from ^{54}Fe was negligible. Fe ionizes at greater temperatures, as was confirmed by measuring ^{56}Fe on a subset of the samples.

During TIMS analyses, mass-dependent isotope fractionation occurs; this induces a measurement bias that varies between samples and over time as a sample is analyzed. This is corrected via the double isotope spike approach, which has been described in detail elsewhere (Compston and Oversby, 1969; Johnson and Beard, 1999; Johnson et al., 1999; Albarède and Beard, 2004). Briefly, most of the measured ^{50}Cr and ^{54}Cr are derived from the spike solution, and the measured $^{50}Cr/^{54}Cr$ ratio reflects instrumental bias and any fractionation during sample processing. The corrected $^{53}Cr/^{52}Cr$ ratio is determined via an iterative data reduction routine, described in earlier publications (e.g., Johnson et al., 1999), that determines the bias and mathematically separates the spike from the sample. The $^{53}Cr/^{52}Cr$ ratio of each sample is expressed as a per mil deviation from the NIST SRM-979 standard:

$$\delta^{53}\mathrm{Cr}(‰) = \frac{\left(\frac{^{53}\mathrm{Cr}}{^{52}\mathrm{Cr}}\right)_{\mathrm{sam}} - \left(\frac{^{53}\mathrm{Cr}}{^{52}\mathrm{Cr}}\right)_{\mathrm{std}}}{\left(\frac{^{53}\mathrm{Cr}}{^{52}\mathrm{Cr}}\right)_{\mathrm{std}}} \times 1000 \quad (2)$$

where sam and std refer to sample and the standard, respectively. Based on results of duplicate samples and processed standards, external precision is ±0.2‰ at 95% confidence.

3. RESULTS

3.1. Cr(VI) reduction rates

Cr(VI) concentration and isotopic data are given in Table 2. Plots of concentration versus time are given in Fig. 1. Reduction proceeded slowly in most of the experiments, presumably because electron donor concentrations were small. The time required for the initial Cr(VI) concentration to decrease by a factor of 2 ranged from 4 h (in the donor-rich experiment) to 2 days. The progression of Cr(VI) concentration as a function of time does not fit a pseudo-first-order model for any of the experiments; rate constants fitting early data are much larger than those fitting later data.

The cell-specific reduction rate (SRR) was calculated for time intervals early and late in the experiments, using the following formula:

$$\mathrm{SRR} = \frac{\Delta c}{\Delta t \bullet d} \quad (3)$$

where Δc gives the change in Cr(VI) concentration over a time interval Δt, and d is the cell density. Table 1 lists SRR values for the first 24 h and for a later time period of each experiment. Invariably, the SRRs were lesser for the later time intervals. Decreases in electron donor concentration cannot explain the decreases in pseudo-first-order rate constants, as only a small fraction of the donor present was consumed. Decreases in Cr(VI) concentration probably do play a role in the decreasing SRRs. However, if decreasing Cr(VI) caused all of the SRR decrease, the data should conform to a pseudo-first-order model. The actual SRR decreases are much greater than that of such models. Reduction ceased entirely after about 10 days in most of the experiments. Thus, the data suggest the cells became progressively less active during the experiments. Suspensions could not be reactivated by addition of donor and Cr(VI). Negligible Cr(VI) reduction occurred when, on day 22, the 6.8 μM formate and two 3.3 μM lactate suspensions were re-injected with 140 μM lactate and 300 μM formate, respectively, and 10 μM Cr(VI).

Control experiments with no cells or killed cells showed no reduction of Cr(VI) (data not shown), indicating that living cells reduced the Cr(VI) in the live cell experiments and that adsorption onto cells or reduction by components of the suspension media did not cause the observed Cr(VI) concentration decreases. However, Cr(VI) reduction occurred in the no-donor-added controls for cell batches A and C (Fig. 1). This observation indicates that an unintended source of electron donor was present. Furthermore, the reduction rates of the 35 μM lactate and 68 μM formate suspensions are less than a factor of 2 greater than those of the 3.5 μM lactate and 6.8 μM formate suspensions. This suggests that the unintended donor source was dominant relative to the added donor in the latter experiments.

In the early stages of the experiments, however, the no-donor-added controls showed little reduction whereas the experiments with donor show significant reduction rates (Fig. 1b, d and f). Accordingly, the unintended donor source was not present initially in the experiments and must have been generated from within them. This suggests the source was endogenous decay (cell death) that began after hours or days of suspension. This hypothesis is supported by the fact that reduction rates in the no-donor-added controls correlated with cell density. Cell batch C, with the greatest cell density, exhibited the greatest cell-specific reduction rate in the no-donor-added control, whereas cell batch B, with the smallest cell density, had the smallest reduction rate in the control.

Moreover, in both 100 μM lactate experiments, 40% of the dissolved Cr(VI) was reduced during the first 200 minutes, whereas the corresponding no-donor-added controls showed little reduction during this time (Fig. 1d). Thus, endogenous decay did not cause the large reduction rate observed in the first several hours of these experiments; the

Table 2
Isotope ratio and concentration data

Cell batch	Electron donor	Added donor (μM)	Time (days)	Cr(VI) (μM)	δ^{53}Cr (‰)
A	Lactate	3.3	0.00	5.10	−0.31
			0.02	4.39	
			1.03	2.86	2.29
			2.79	2.17	
			4.77	1.83	3.86
			9.79	1.80	
			17.75	1.79	4.03
A	Lactate	3.3	0.00	5.06	−0.31
			0.02	4.26	
			1.03	2.76	2.32
			2.79	2.14	
			4.77	1.81	4.28
			9.79	1.57	
			17.75	1.45	5.26
A	Formate	6.8	0.00	5.06	−0.31
			0.01	4.50	
			1.03	3.04	1.89
			3.20	2.11	3.30
			9.02	1.69	
			15.22	1.69	
			15.99	1.67	4.82
A	Lactate	35	0.00	5.12	−0.31
			0.02	4.16	
			1.03	2.56	2.40
			2.79	1.90	
			4.76	1.58	4.37
			8.20	1.21	
			9.80	1.13	
			15.99	0.92	
			16.76	0.91	
			18.99	0.88	
			23.91	0.86	7.13
A	Lactate	35	0.00	5.04	−0.31
			0.02	4.19	
			1.00	2.39	2.71
			3.03	1.66	
			7.25	1.12	
			8.99	1.01	6.38
			15.05	0.47	
			15.82	0.48	
			18.04	0.47	
			22.97	0.43	9.71
A	Formate	60	0.00	5.15	−0.31
			0.01	4.48	
			1.03	2.73	2.31
			3.20	1.99	
			9.16	1.31	5.41
			15.22	1.04	
			23.14	0.88	6.61
A	Control	None	0.00	5.02	
			0.02	4.88	
			1.00	2.36	
			3.04	1.00	
			7.25	0.32	
			9.00	0.23	
C	Lactate	100	0.00	9.52	−0.31
			0.02	6.53	
			0.10	5.23	
			0.35	3.67	
			1.01	2.10	5.54
			2.05	1.24	8.41
			4.08	0.22	
C	Lactate	100	0.00	9.45	−0.31
			0.01	6.50	
			0.09	5.05	
			0.34	3.57	
			1.01	2.00	5.7
			2.05	1.18	8.33
			4.07	0.13	
C	Control	None	0.00	9.42	
			0.02	9.25	
			0.09	9.23	
			1.01	1.63	
			2.04	0.00	
B	Lactate	10400	0.00	9.43	−0.31
			0.03	8.12	
			0.09	7.42	0.2
			0.96	5.10	0.75
			4.89	4.03	1.09
			14.00	3.70	
			17.77	3.61	1.49
B	Control	None	0.00	9.46	
			0.04	9.31	
			5.82	9.17	
			15.10	8.86	
			17.77	8.77	
Isotopic analyses of Cr(VI) added to experiments					−0.40
					−0.24
					−0.15
					−0.37
					−0.38
				Mean	−0.31
				Std. Dev.	0.11

bacteria attained the observed rates by consuming only the added 100 μM lactate. Furthermore, these rates were much larger than those observed in the 35 μM lactate experiments, and this suggests the 35 μM lactate experiments had much smaller electron donor availability despite additions due to endogenous decay. The role of endogenous decay in the experiments is discussed further below.

3.2. Isotopic fractionation of chromium

In all experiments, Cr(VI) reduction was accompanied by enrichment of the heavier isotope in the remaining unreacted Cr(VI) (Table 2 and Fig. 2). In closed systems like these experiments, if the isotopic fractionation factor, α, does not vary with time, the δ^{53}Cr value of the remaining Cr(VI) evolves according to the Rayleigh relationship, which can be expressed as

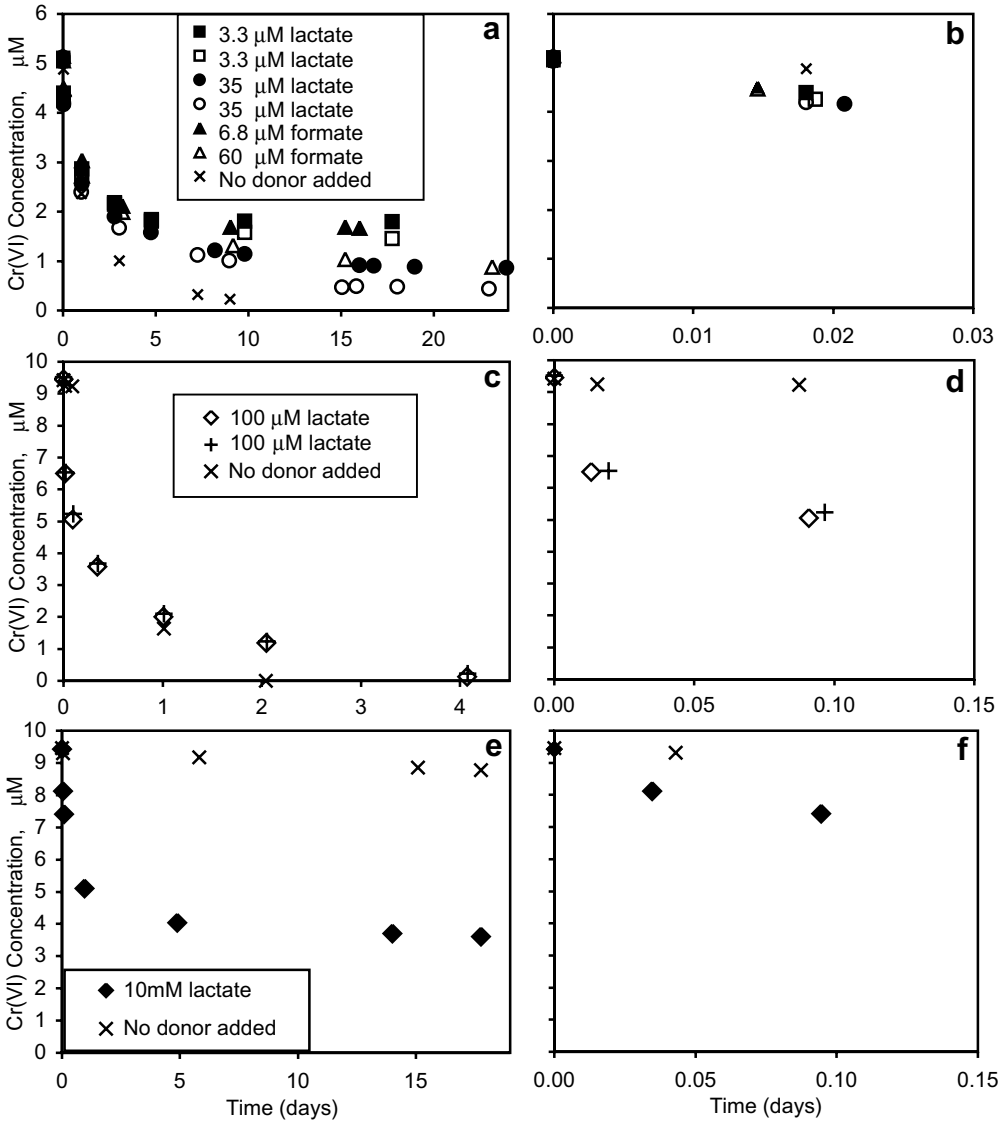

Fig. 1. Cr(VI) concentration vs. time in reduction experiments. (a) Experiments using cells from batch A with varying initial electron donor, including control experiment with no added donor. (b) Early samples from batch A experiments [symbols as in (a)]. (c) Cell batch C experiments. (d) Early samples from the batch C experiments. (e) Cell batch B experiments. (f) Early samples from batch B experiments.

$$\delta(t) = (\delta_0 + 1000)\left(\frac{c(t)}{c_0}\right)^{\frac{1}{\alpha}-1} - 1000 \tag{4}$$

where $c(t)$ and $\delta(t)$ are the concentration and isotopic composition of Cr(VI) at a certain time during the experiments, and c_0 and δ are initial values. In all experiments, Eq. (4) fits the data within the 0.2‰ analytical uncertainties. Eq. (4) can be rearranged to give

$$\ln\left(\frac{\delta(t)+1000}{\delta_0+1000}\right) = \left(\frac{1}{\alpha}-1\right) \times \ln\left(\frac{c(t)}{c_0}\right) \tag{5}$$

Best fit α values were found by fitting data to plots of $(\ln\delta + 1000)$ vs. $\ln(c/c_0)$, determining the slope of the best fit line via linear regression, and calculating α from the slope. This is equivalent to the approach advocated by Scott et al. (2004). Our results were then expressed as ε:

$$\varepsilon \equiv 1000(\alpha - 1) \tag{6}$$

This representation of the fractionation factor is convenient, as ε is very close to the difference in δ^{53}Cr between the Cr(VI) reactant and the reaction product flux, in per mil units:

$$\varepsilon \approx \delta_{react} - \delta_{prod} \tag{7}$$

Values of ε for individual experiments are given in Table 1, along with estimated uncertainties. Uncertainties were estimated from the standard error of the slope calculated by the regression routine. Because this calculation uses the deviations of measured points from the model line, it gives a rough indication of uncertainty for regressions with few data points ($n = 3$ or 4; most experiments here). In two cases, the points fell very close to the fit line by chance, and unrealistically small uncertainties resulted. These were

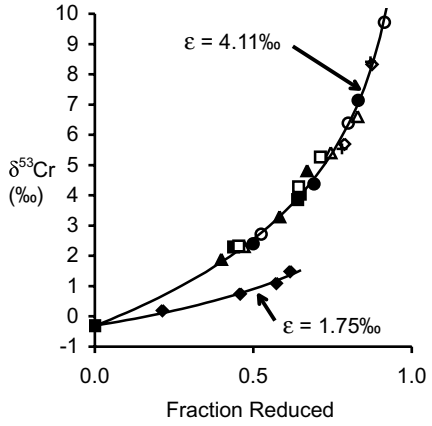

Fig. 2. Cr isotopic values vs. fraction of Cr(VI) reduced. Symbols as in Fig. 1. Data from all suspensions with $\leqslant$100 μM added electron donor fall close to a single Rayleigh model with $\varepsilon = 4.11$. Data from the 10 mM lactate suspension conform to $\varepsilon = 1.75$.

increased to 0.2‰, the minimum reasonable value when the measurement uncertainties are taken into account.

Isotopic data from all experiments are plotted in Fig. 2. For the donor-rich (10 mM lactate) experiment, $\varepsilon = 1.8$‰. For all other experiments (added lactate or formate ranging from 3.3 to 100 μM), the isotopic fractionation is within the range 4.0–4.5‰, with a best-fit value of 4.1‰ for all the data points from these experiments. Differences between experiments in this group were not statistically significant at the 95% confidence level.

Eq. (4) fits all the data for each individual experiment taken separately. For most experiments, four or more data points were generated. We calculated ε separately for early and late pairs of data points, and determined uncertainties for these values by propagating analytical uncertainties through the calculations. The uncertainty on these determinations depended on the c/c_0 difference between the points and varied between 0.3 and 0.8‰. Within these uncertainties, no change in ε occurred during the course of any experiment.

4. DISCUSSION

4.1. Endogenous decay and Cr(VI) removal mechanism

The endogenous decay that apparently caused Cr(VI) reduction in our no-donor-added controls has been observed by others in similar experiments and is not unusual for this type of experiment. Sani et al. (2002) obtained similar results from very similar donor-absent controls with four *Cellulomonas* strains. The masses of cells present in our experiments should be sufficient to support reduction by endogenous decay. If several percent of the cells' dry mass were converted to usable donor, this should be sufficient to drive the observed reduction in the donor-absent controls.

With cell batches A and C, long-term reduction rates in the no-donor-added controls were greater than in the experiments with donor added (Fig. 1). This did not result from random differences in experimental parameters; little difference was observed between duplicate experiments. We suggest, therefore, that the complete lack of electron donor in the first several hours led to accelerated cell death and release of donor compounds. Because endogenous decay apparently depends on the amount of donor present, we cannot infer, from the amounts of reduction observed in the no-donor-added controls, the amounts of reduction caused by endogenous decay in the donor-added experiments.

The presence of a poorly constrained amount of unintended electron donor diminishes the intended donor concentration differences between the various <100 μM donor experiments and presumably caused the reduction rates to be greater than those that would occur with the intended concentrations. However, the reduction rates were still quite small, and a wide range of rates was observed (Table 1). The 100 μM lactate experiments exhibited much greater SRR's than the 35 or 3.3 μM lactate experiments. Also, during each experiment, the rate decreased over time, by at least a factor of 10.

We are confident that the observed Cr(VI) removal was caused by reduction and not "luxury uptake" without any reduction. This phenomenon, whereby bacteria take up and store nutrients for later use, has been observed with phosphorus (e.g., Khoshmanesh et al., 2002). However, luxury uptake would not fractionate Cr isotopes significantly. This is because the local bonding environment for Cr should change little as a result of uptake and storage of Cr(VI) within cells. Large changes in the Cr coordination environment would be needed to drive the large isotopic effect observed here. Thus, luxury uptake cannot be the sole Cr(VI) removal mechanism because strong fractionation was observed. It could have caused part of the Cr(VI) removal, resulting in lesser isotopic fractionation than would be observed with reductive removal alone. However, the consistent ε values we measured within and between experiments would require the fraction of Cr(VI) removed by luxury uptake to be invariant between experiments and over the course of each experiment. Given the differences in cell density and reduction rates, this is extremely unlikely and we thus assume that luxury uptake was negligible.

4.2. Analogous isotopic effects: sulfate, nitrate, and selenate reduction

Reduction reactions consuming the oxyanions sulfate, selenate, nitrate, and chromate may be expected to fractionate isotopes similarly, as they involve transfer of multiple electrons and rearrangement of the oxygens bonded to the S, N, Se, or Cr. A general conclusion of studies of kinetic isotope effects induced by bacterial oxyanion reduction is that the magnitude of isotopic fractionation depends on the metabolic pathways and metabolic states of the microbes. Studies of resting cell suspensions, continuous cultures, and incubated sediment containing various sulfate reducing bacteria (Harrison and Thode, 1958; Kaplan and Rittenberg, 1964; Kemp and Thode, 1968; Chambers et al., 1975; Habicht and Canfield, 1997) have revealed that isotopic fractionation tends to increase as reduction rates

decrease. A conceptual model explaining the variability in isotopic fractionation and a corresponding mathematical model (Rees, 1973; Canfield, 2001) have been developed. A complete description of this model is beyond the scope of this paper, but we briefly review the relevant implications.

The model assumes reduction occurs in several steps, with strong fractionation in some steps of the reaction pathway and weaker fractionation in others. Changes in the relative rates of the reaction steps can result in changes in the isotopic fractionation of the overall reaction. The model predicts the isotopic fractionation of the overall reaction is equal to the sum of the fractionations induced by all reaction steps up to and including the rate-limiting step. Steps occurring after the rate-limiting step have no effect on the overall isotopic fractionation. If transport of sulfate into the cell, which is the first step in the overall reaction and which induces little isotopic fractionation, becomes rate-limiting, the overall reaction induces little isotopic fractionation. This extreme case is approached if the reduction rate within the cell is very rapid. Accordingly, large electron donor concentrations, which allow the cells to manufacture large quantities of the activated enzymes that reduce sulfate, induce both rapid reduction and small isotopic fractionation. At the other extreme, small electron donor concentrations and other factors that greatly decrease the reduction rate correlate with greater isotopic fractionation (e.g., Kaplan and Rittenberg, 1964).

This variability in isotopic fractionation also occurs during denitrification, the microbial reduction of nitrate to N_2. Inverse correlation of reduction rates and N isotope fractionation has been observed in laboratory experiments (Kohl and Shearer, 1978; Mariotti et al., 1982). Selenate reduction by growing cultures, resting cell suspensions, and unamended sediment slurries also produced variable Se isotope fractionation (Herbel et al., 2000; Ellis et al., 2003). On the other hand, reduction rate is not the sole control on the degree of isotopic fractionation. Recent work has shown that differing metabolic pathways among various sulfate reducing microorganisms induce differing degrees of S isotopic fractionation (Brüchert et al., 2001; Detmers et al., 2001).

Other metals with atomic masses similar to that of Cr exhibit isotopic fractionation induced by redox reactions (e.g., Johnson et al., 2004; Mathur et al., 2005). Extensive study of microbially mediated Fe redox reactions has revealed a complex array of factors influencing the magnitude of fractionation (e.g., Johnson et al., 2004; Balci et al., 2006). However, results of these studies have limited applicability to Cr(VI) reduction, as Fe(III)–Fe(II) reactions involve rapid, single electron transfers and equilibrium isotope effects are often dominant. With sulfate and Cr(VI) reduction, reactions include multiple electron transfers with ephemeral intermediates, so isotopic equilibration between reactants and products is greatly inhibited.

4.3. Similarities between Cr(VI) reduction and sulfate reduction

Preliminary research on the biochemical mechanisms of Cr(VI) reduction by *S. oneidensis* MR-1 suggests that Cr(VI) is similar to sulfate reduction. Myers et al. (2000) found that in fumarate-grown MR-1 cells, the enzymes responsible for Cr(VI) reduction were located in the innermost of the bacterium's three membrane layers. Accordingly, the first step in Cr(VI) reduction is transport into the cell, and the principles of sulfur and nitrogen isotopic enrichment described above should describe Cr isotope fractionation induced by Cr(VI) reduction by this bacterium. Myers et al. (2000) also found evidence that MR-1 reduced Cr(VI) using a multi-component electron transport chain. A Cr(V) intermediate was detected during Cr(VI) reduction by extracted MR-1 inner membrane, suggesting that at least two distinct reductive steps are involved. These authors further point out that since no known biological electron donors simultaneously provide three electrons, a multi-step process is expected for the microbial reduction of Cr(VI) to Cr(III). Studies of Cr(VI) reduction by other microorganisms have also noted Cr(V) intermediates (Kalabegishvili et al., 2003). Sulfate reduction has been studied in much greater detail, and similarly involves an intracellular reaction chain with multiple steps (e.g., Canfield, 2001).

Cr isotopic fractionation in our experiments is similar to that occurring during microbial sulfate reduction in two ways. First, the size of the fractionation factor relative to that induced by the corresponding abiotic reaction is similar. Abiotic Cr(VI) reduction by magnetite involves a fractionation factor (ε) of 3.4‰ (Ellis et al., 2002). The equilibrium isotopic fractionation between dissolved Cr(VI) and dissolved Cr(III) has been estimated at about 6‰ (Schauble et al., 2004). With sulfate reduction, the abiotic reaction, microbial process, and sulfate-sulfide equilibrium involve fractionations 22‰, 6 to 46‰, and 75‰, respectively (Tudge and Thode, 1950; Harrison and Thode, 1957; Canfield, 2001). Thus, for microbial reduction of both sulfate and Cr(VI), fractionation factors can be somewhat greater than those of abiotic reduction, under some conditions they are less, and they are always less than equilibrium fractionation factors. The second similarity is the lesser isotopic fractionation observed in donor-rich experiments. The 10 mM lactate experiment demonstrates that with microbial Cr(VI) reduction, as with microbial sulfate reduction, small isotopic fractionation is observed in donor-rich experiments with rapid reduction.

Reduction rates in our experiments were smaller than those occurring in most sulfate reduction studies. In those studies, the transition from experiments with small isotopic fractionation to experiments with larger isotopic fractionation occurred at roughly 3 fmol cell^{-1} day^{-1} (Kaplan and Rittenberg, 1964). The smallest rates were approximately 0.1 fmol cell^{-1} day^{-1}. Our reduction rates were less than 0.1 fmol cell^{-1} day^{-1} in all experiments except the one with very large donor concentration (10 mM lactate), in which the rate early in the experiment was 0.45 fmol cell^{-1} day^{-1}. Because most of our experiments were similar to the slowest sulfate reduction experiments, in which the strongest S isotopic fractionation was observed, we tentatively suggest that the Cr isotope fractionations we observed should be close to the maximum possible for this microbe.

4.4. Extension to field conditions

Isotopic fractionation was strong at small Cr(VI) concentrations and was not dependent on Cr(VI) concentration. Our 5.1 or 9.5 μM initial concentrations were much smaller than those used in most sulfate and nitrate reduction studies, which in some cases have found that isotopic fractionation decreases when sulfate and nitrate are scarce. These observations lead us to suggest that Cr(VI) reduction was not limited by the availability of Cr(VI), that electron donor availability controlled the metabolic state of the microbes and the isotopic fractionation, and that our results can be extrapolated to higher or lower initial Cr(VI) concentrations.

Our finding that all experiments with small donor concentrations had isotopic fractionation (ε) close to 4.2‰ suggests this is a plateau value that should apply to a range of donor-poor conditions. Although we cannot determine the exact donor concentrations in the experiments because of endogenous decay, reduction rates varied between experiments and with time in each experiment. We suggest the conditions were sufficiently oligotrophic so the observed isotopic fractionation is the maximum possible for this bacterium. If this were not the case, then we would expect to see variation in the isotopic fractionation as a function of the reduction rate.

However, our results cannot be extrapolated with certainty to natural microbial populations until certain other variables are explored. Studies of sulfate reducers (Brüchert et al., 2001; Detmers et al., 2001) suggest that metabolic differences between species are important in determining the magnitude of isotopic fractionation under natural conditions. Similarly, different metabolic pathways cause differing Zn isotope fractionation for high- and low-concentration ranges (John et al., 2007). There may be similar diversity in the metabolic pathways of Cr reducers. Whereas the Cr(VI) reduction sites in MR-1 are located in its innermost membrane (Myers et al., 2000), Cr(VI) reduction activity in other organisms may take place in the interior of the cell (Ishibashi et al., 1990), between the inner and outer membranes (Lovley and Phillips, 1994), or on the surface of the cell (Kalabegishvili et al., 2003), and at least one bacterium has been found to excrete enzymes which reduce dissolved metals extracellularly (Seeliger et al., 1998). This variability in location of reduction enzymes may cause systematic differences in Cr(VI) isotopic enrichment between different species. Accordingly, additional experiments with natural consortia or a representative array of Cr(VI) reducers are needed.

Also, Brüchert et al. (2001) suggest that physiological stresses on bacteria may increase isotopic fractionation. Specifically, they note that the largest S isotopic fractionations reported for sulfate reduction (Kaplan and Rittenberg, 1964) were produced by bacteria subjected to temperatures and electron donor types that may have caused physiological stress and unusual metabolic states that would not be applicable to natural settings. In our experiments, strong decreases in cell-specific reduction rates and complete cessation of reduction in several cases, suggest that the bacteria were under physiological stress. This may have resulted from the small donor concentrations, a lack of other nutrients, Cr(VI) toxicity, or problems with the composition of the suspension medium. Thus, it is possible that our microbes were under severe stress such that the isotopic fractionation factors determined here are greater than those that would occur under more natural conditions.

Future experiments to better determine the applicable range of Cr isotope fractionation during Cr(VI) reduction could be carried out with methods that better approximate natural environments. Continuous culture experiments like those done by Chambers et al. (1975) might enable slow reduction with steady-state cultures without extreme physiological stress. Sediment slurry experiments with no added electron donor, like those done with Se reduction by Ellis et al. (2003) make use of naturally occurring bacteria and electron donors, but may be difficult because of adsorption and slow reduction. *In situ* reduction experiments with Cr(VI) injected into aquifers could be the best option, but achieving adequate mass balance may be very difficult.

5. SUMMARY AND CONCLUSIONS

The results of this study demonstrate that fractionation of Cr isotopes can occur during bacterial reduction of Cr(VI). In eight experiments where Cr(VI) was reduced by *S. oneidensis* MR-1 cells suspended in simple buffer solutions with 3–100 μM added electron donor, the fractionation factor, expressed as ε, ranged from 4.0 to 4.5‰. Endogenous decay of cells supplied a poorly constrained amount of additional donor. A range of reduction rates was observed in these experiments, but corresponding differences in ε were not observed. In one donor-rich (10,200 μM lactate) experiment, reduction proceeded much more rapidly and weaker isotopic fractionation, with $\varepsilon = 1.8$‰, was observed.

Bacterial Cr(VI) reduction is similar to bacterial sulfate reduction in several ways. This suggests that models and theory used to understand S isotope fractionation induced by sulfate reduction should apply to Cr(VI) reduction and should improve understanding of variation in microbial fractionation factors as a function of metabolic variables.

These results support the use of Cr isotope measurements to quantify Cr(VI) reduction. Fractionation factors serve as calibration parameters in efforts to quantify reduction using isotopic data, e.g., via the Rayleigh model. Our results suggest that ε for dissimilatory Cr(VI) reduction may be close to 4.1‰ over a range of electron donor-poor conditions. This value is ~20% greater than that observed in previous abiotic experiments (Ellis et al., 2002). This demonstrates that ε is variable, but the variability observed so far is small enough to suggest effective quantification of reduction may be possible. A more complete understanding of this variability should be pursued through additional studies determining fractionation factors for other bacteria and a range of abiotic reactions under a wider range of conditions.

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