Differential isotopic fractionation during Cr(VI) reduction under aerobic versus denitrifying conditions by an aquifer-derived bacterium

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Abstract

We studied Cr isotopic fractionation during Cr(VI) reduction by *Pseudomonas stutzeri* strain RCH2. Despite the fact that strain RCH2 reduces Cr(VI) co-metabolically under both aerobic and denitrifying conditions and at similar specific rates, fractionation was markedly different under these two conditions (ε ~2‰ aerobically and ~0.4‰ under denitrifying conditions).

Hexavalent chromium (in the form of chromate) is a potent toxicant, mutagen, and carcinogen (2, 3) that has contaminated aquifers at industrial sites and Department of Energy facilities. A favored approach for remediation of chromate-contaminated aquifers is *in situ* reductive immobilization. Cr(VI) can be reduced enzymatically by a diverse range of aerobic, facultative, and anaerobic bacteria (1, 15) and can also be readily reduced by certain respiration end-products, such as hydrogen sulfide or Fe(II). For optimal management of remediation of chromate-contaminated groundwater either by natural bioreduction or by engineering approaches, it is desirable to know which chromate reduction processes are dominating in the subsurface. One potential way to determine this is to use Cr isotopic ratios, provided that different reductive processes are associated with distinctive isotopic fractionation.

To date, there are few controlled laboratory studies reporting isotopic fractionation associated with chromate reduction, particularly bacterially catalyzed processes (for a recent review of Cr isotope geochemistry, see ref. 7). Ellis et al. (4) reported on isotopic fractionation of Cr during abiotic reduction by magnetite ($\alpha = 0.9965$; $\varepsilon = 3.5‰$). Kitchen et al. (10) reported ε values ranging from 2.9 to 4.7‰ in abiotic experiments involving Fe(II) and organic acids at a range of pH values. To our knowledge, the only detailed study of Cr isotopic fractionation associated with enzymatic reduction was conducted by Sikora et al. (16). In that study, cells of the dissimilatory metal-reducing bacterium *Shewanella oneidensis* MR-1 were grown anaerobically with lactate or formate and then resuspended in anaerobic, phosphate-buffered medium with 5 to 10 μM Cr(VI) as the sole added electron acceptor. Consistent isotopic fractionation ($\varepsilon = 4.1$ to 4.5‰) was observed when the added electron donor was lactate or formate at 3 to 100 μM, whereas less fractionation ($\varepsilon = 1.8‰$) was observed at a higher lactate concentration (10.2 mM).

There is clearly a need for more studies of isotopic fractionation during bacterial Cr(VI) reduction covering a range of electron-accepting conditions relevant to aquifer environments (e.g., aerobic, denitrifying, sulfate-reducing, ferric iron-reducing, fermentative). In this article, we report isotopic fractionation during Cr(VI) reduction by an aquifer-derived bacterium, strain RCH2, which can reduce chromate co-metabolically under either aerobic or denitrifying conditions (6).

Aerobic and denitrifying cell suspension results: physiology. Cell suspension assays were performed to assess isotopic fractionation during chromate reduction by strain RCH2 under both aerobic and denitrifying conditions. Strain RCH2 is closely related to *Pseudomonas stutzeri* and was isolated from groundwater from DOE's Hanford 100H site (6). Except for the inclusion of chromium isotopic measurements, cell suspension experiments were otherwise conducted as described previously (6); experimental details are provided in the Supplemental Material. Overall results for experiments with strain RCH2 cells grown and resuspended under either aerobic or denitrifying conditions were similar to those reported previously (6). Under aerobic conditions, lactate was depleted within 4 hr and the metabolite pyruvate accumulated transiently (Fig. S1). Aerobic chromate reduction was most rapid over the first 2 hr, as has been reported previously (6). The specific Cr(VI) reduction rate over the first 2 hr was ~16.6 $\mu M \cdot hr^{-1} \cdot OD^{-1}$.

Cell suspension experiments under denitrifying conditions were conducted at three different cell densities (OD_{600} ~ 0.5, 0.8, and 2; referred to as experiments Denit0.5, Denit0.8, and Denit2, respectively). In the lower cell density experiments (e.g., OD_{600} ~ 0.5; Figure S2A), strain RCH2 cells grown and resuspended under denitrifying conditions reduced Cr(VI) at a relatively constant specific rate of ~8.4 $\mu M \cdot hr^{-1} \cdot OD^{-1}$ (consistent with previous reports (6)). Pyruvate concentrations increased over time and nitrite accumulated transiently. In the higher

(OD_{600} ~ 2) cell density experiments (Figure S2B), both nitrate and nitrite were completely depleted within 2 hr and, since Cr(VI) reduction is co-metabolic with denitrification in strain RCH2 (6), Cr(VI) reduction was largely confined to this 2-hr time period. In the absence of nitrate or nitrite after 2 hr, concentrations of lactate and its metabolites pyruvate and acetate were effectively constant throughout the remainder of the experiment.

Aerobic cell suspension results: chromium isotopic fractionation. Samples were prepared for determination of Cr stable isotope composition using a ^{50}Cr-^{54}Cr double-spike technique similar to those of Ellis et al. (4) and Sikora et al. (16). Cr isotope analyses were performed on an Isoprobe multi-collector ICP-MS or a Thermo Finnigan Triton multi-collector TIMS. Experimental details for Cr chemical separation, Cr isotopic measurements, and treatment of isotopic data are provided in the Supplemental Material.

Isotopic results for aerobic cell suspensions with strain RCH2 are shown in Fig. 1A. Values of $\delta^{53}Cr$ in solution increased from 0 to ~4‰ as dissolved Cr(VI) concentrations decreased to ~10% of the initial concentration over 5 hours (Fig. 1A). Within experimental uncertainty, the Cr isotopic data fit a Rayleigh fractionation model with a fractionation factor (α) of 0.99805 ± 0.00040 (95% confidence) (corresponding to an ε value of 2 $\pm$ 0.4‰, 95% confidence).

Denitrifying cell suspension results: chromium isotopic fractionation. Under denitrifying conditions, $\delta^{53}Cr$ values of dissolved Cr(VI) did not become significantly greater with time, even when nearly 95% of the Cr(VI) in the solution had been reduced (Fig. 1B). The largest $\delta^{53}Cr$ excess was ~ 1 ‰. The data were compared with the Rayleigh model and we found a reasonable fit when using α = 0.99962 ± 0.00017 (±95% confidence) (an ε value of only 0.4 $\pm$ 0.2‰, 95% confidence). Although the denitrifying results (Fig. 1B) are compiled from three

experiments with different cell densities, they collectively indicate consistently very low isotopic fractionation.

Possible explanation for differential fractionation: differential chromate transport. The dramatic difference in Cr isotopic fractionation by strain RCH2 under aerobic and denitrifying conditions ($\varepsilon \sim 2$ and 0.4‰, respectively) is somewhat surprising in light of the similarities between Cr(VI) reduction under these two electron-accepting conditions (e.g., chromate reduction appears to be co-metabolic with respiration using the physiological electron acceptor, O_2 or nitrate, under these conditions (6)). It is possible that different proteins are catalyzing Cr(VI) reduction under aerobic and denitrifying conditions and this explains the difference in isotopic fractionation. Another possible explanation is that chromate transport into the cell, which probably results in minimal fractionation (8, 12, 16), is the rate-limiting step for chromate reduction under denitrifying conditions but not under aerobic conditions. This could result in observed differential isotopic fractionation under aerobic and denitrifying conditions even if the protein catalyzing Cr(VI) reduction is the same under both conditions. The underlying reasoning for this explanation is based on a conceptual model of isotopic fractionation (discussed in more detail elsewhere (16)) that includes the following assumptions: (a) in a multi-step reduction process, the overall isotopic fractionation is equal to the sum of fractionation from all steps up to and including the rate-limiting step, and (b) reaction steps occurring after the rate-determining step do not affect overall isotopic fractionation. Thus, if transport of chromate into the cell were involved in chromate reduction in strain RCH2 and were rate limiting under denitrifying conditions but not aerobic conditions, this would render the subsequent reduction step(s) irrelevant to overall Cr isotopic fractionation under denitrifying conditions. In effect, we hypothesize that under denitrifying conditions the cell envelope is

playing a greater role in limiting Cr isotopic equilibration between the external and internal pools of chromate than under aerobic conditions. Although there are no prior studies documenting how Cr mass-transfer limitations across cell membranes can affect Cr isotopic fractionation, there are studies reporting analogous effects during biotransformation of organic contaminants, such as toluene (8) and tetrachloroethene (12).

There is reason to believe that chromate uptake could be different under aerobic and denitrifying conditions. It has been established in a number of bacterial species, including pseudomonads, that chromate uptake occurs *via* sulfate active-transport proteins (13, 15). Furthermore, in *E. coli*, it has been shown (11) that growth under anaerobic conditions can modify either the function or expression of gene products encoded by the *cysA* operon, which includes a sulfate permease (the initial protein involved in sulfate assimilation) (9, 14). Thus, uptake of chromate through sulfate permeases could be constrained under denitrifying conditions relative to aerobic conditions in strain RCH2. Chromate can also exert transcriptional control over sulfate transporters; however, chromate induction is not relevant to our cell suspension experiments. This is because cells were grown in the absence of Cr for both aerobic and denitrifying conditions, and were resuspended in a buffer containing chloramphenicol (6), which inhibited synthesis of new proteins during the cell suspension assay in the presence of Cr.

Comparisons to Cr isotopic fractionation in *Shewanella oneidensis* MR-1. To our knowledge, the only other study of Cr isotopic fractionation during enzymatic reduction by bacteria was performed with *Shewanella oneidensis* MR-1 (16). Sikora et al. (16) observed consistent isotopic fractionation (ε = 4.1 to 4.5‰) in the presence of low concentrations of lactate or formate (3 to 100 μM) but less fractionation (ε = 1.8‰) at a higher lactate concentration (~10 mM). For strain RCH2, in experiments with 20 mM lactate, we observed ε ~

2‰ under aerobic conditions (comparable to the findings for *S. oneidensis*) but only ε ~ 0.4‰ under denitrifying conditions. The differences between isotopic fractionation under aerobic vs. denitrifying conditions in strain RCH2 are all the more remarkable in light of the similarity between isotopic fractionation for two very different systems: strain RCH2 co-metabolically reducing Cr(VI) under aerobic conditions and *S. oneidensis* anaerobically reducing Cr(VI) as the sole electron acceptor (16).

It is unknown whether isotopic fractionation for strain RCH2 (aerobic conditions) would also be comparable to that of *S. oneidensis* (ε = 4.1 to 4.5‰) if lower electron donor concentrations were used (e.g., 3 to 100 μM lactate). Logistically, this would be difficult to test because of the co-metabolic nature of Cr(VI) reduction in strain RCH2 (i.e., a very small proportion of reducing equivalents from lactate are used for chromate reduction). To illustrate, under the conditions used for aerobic studies with strain RCH2, it would only take ~1 minute to consume 100 μM lactate. Even if the cell density were reduced to extend this lactate utilization period to 2 hr, only ~ 0.25 μM of Cr(VI) would be reduced, which is not optimal for determining isotopic fractionation. Regarding *S. oneidensis* studies with low electron donor concentrations (e.g., 3 to 100 μM lactate), it is noteworthy that no-donor controls indicated that endogenous cell components (e.g., from lysed cells) may have contributed a substantial portion of the reducing equivalents for chromate reduction (16).

In terms of electron donor concentrations, the degree to which either the present study or the *Shewanella* study (16) simulates actual aquifer conditions is open to debate. Sikora et al. (16) chose electron donor concentrations that were representative of aquifers unamended with organic compounds. However, arguably, chromate reduction is more likely to be relevant to bioremediation under biostimulated conditions, which should involve much higher electron

donor concentrations. For example, under aquifer biostimulation conditions (e.g., after the addition of a commercial polylactate compound designed for slow lactate release) at the Hanford 100H field site, millimolar concentrations of acetate were detected in an aquifer for many months after initial release (5). Biostimulation conditions can also generate high cell densities (e.g., > 10^8 cells/mL; ref. 5), but not necessarily as high as the densities used in the present study (on the order of 10^9 cells/mL).

In conclusion, Cr isotopic fractionation during Cr(VI) reduction by *P. stutzeri* strain RCH2 was markedly different under aerobic versus denitrifying conditions ($\varepsilon = 2 \pm 0.4$‰ and 0.4 ± 0.2‰, respectively), yet aerobic fractionation for strain RCH2 was similar to anaerobic fractionation observed for *S. oneidensis* (16) when Cr(VI) was the sole electron acceptor. To date, these two studies constitute the only published data available for Cr isotopic fractionation during microbial chromate reduction.

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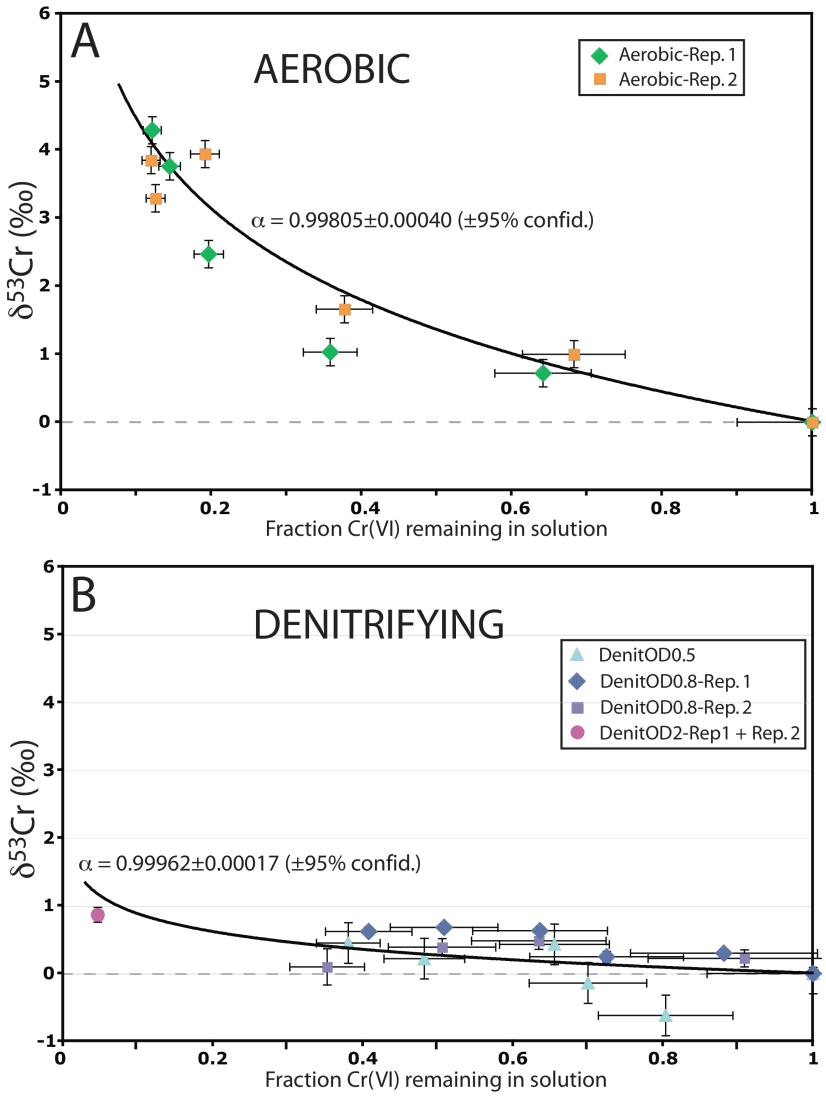
FIGURE LEGENDS

FIG 1. Plots of the fraction of Cr(VI) remaining in solution versus the isotopic composition of the Cr(VI) remaining in solution (presented as δ^{53}Cr, the ‰ deviation of the ^{53}Cr/^{52}Cr ratio relative to the starting composition). Panel (A) represents aerobic cell suspensions (Figure S1) and Panel (B) represents denitrifying cell suspensions (Figure S2). In panel (B), the purple circle represents a pooled sample for late time points (5 to 10 hr) of both replicates of the denitrifying experiment with OD_{600} = 2 (Figure S2B). Error bars represent ±95 % confidence. The α values shown are derived from the slope of the best fit lines in plots of ln(*f*) vs. $\ln\left[\frac{(\delta^{53}Cr+10^3)}{(\delta^{53}Cr_{ini}+10^3)}\right]$ (see Supplemental Material), with the uncertainties in α given at ±95 % confidence.



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