

Metal Respiratory Pathway-Independent Cr Isotope Fractionation during Cr(VI) Reduction by *Shewanella oneidensis* MR-1

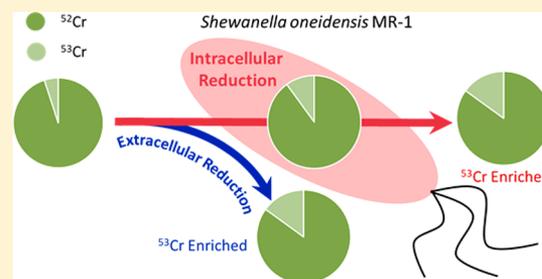
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S Supporting Information

ABSTRACT: Cr isotope fractionation during microbial reduction processes is commonly recognized as a promising tool in biogeochemistry and bioremediation. However, the mechanism of Cr isotope fractionation during microbial reduction is poorly understood. In this work, the relationship between the bacterial respiratory pathway and Cr isotope fractionation was investigated in *Shewanella oneidensis* MR-1. For comparison with the wild type, a mutant strain ($\Delta omcA/\Delta mtrC$) with a deficiency in extracellular Cr(VI) reduction was constructed by deleting the *omcA* and *mtrC* genes, which encode the terminal reductase during extracellular reduction. The magnitudes of Cr isotope fractionation (ϵ) for Cr(VI) reduction by the wild type and $\Delta omcA/\Delta mtrC$ were $-2.42 \pm 0.68\%$ and $-2.70 \pm 0.22\%$, respectively. Surprisingly, the ϵ values were not significantly different between the two strains. This suggests that isotope fractionation is independent of the metal respiratory pathway during Cr(VI) reduction by *S. oneidensis* MR-1. Moreover, a three-step Cr isotope fractionation model that includes uptake, reduction, and efflux was proposed to exist during intracellular Cr(VI) reduction by *S. oneidensis* MR-1. The developed model provides a better understanding of Cr isotope fractionation during microbial reduction.



INTRODUCTION

Discharge of chromium into the environment severely threatens ecological and human health.¹ Cr(VI) and Cr(III) are the dominant species of chromium contaminants in the natural aquatic environment. Cr(VI) is usually present as soluble, mobile chromate (CrO_4^{2-}) and hydrochromate ($HCrO_4^-$) anions, whereas Cr(III) usually forms insoluble hydroxide precipitates.² The toxicity of the former is 100-fold higher than that of the latter.³ Therefore, reduction of Cr(VI) to Cr(III) is quite environmentally significant and has become a common remediation strategy.⁴ Many bacterial species in the subsurface are capable of reducing Cr(VI) to Cr(III).^{5–7} Therefore, bioremediation is a promising approach for mitigating Cr pollution.

Dissimilatory metal-reducing bacteria (DMRB) make up a class of diverse bacteria that can couple intracellular oxidation of electron donors with extracellular reduction of electron acceptors.⁸ As a model of DMRB, *Shewanella oneidensis* MR-1 can transfer electrons to various extracellular electron acceptors, including Cr(VI). It has been reported that *S. oneidensis* MR-1 can reduce Cr(VI) using lactate as the electron donor for both extracellular and intracellular reductions of Cr(VI).^{9–13} During extracellular reduction, *S. oneidensis* MR-1 uses the metal respiratory (Mtr) pathway, which is primarily composed of OmcA, MtrC, MtrA, MtrB, and CymA.⁸ The proteins OmcA and MtrC act as terminal reductases of Cr(VI) extracellular reduction in *S. oneidensis* MR-1.⁹ The enzymes involved in the

extracellular reduction of Cr(VI) show no specificity because they can also reduce other electron acceptors, such as insoluble iron oxide and methyl orange, among others.^{14,15} In addition, some Cr(VI) can be transported into cells via the sulfate transport protein and then intracellularly reduced by nitrite reductase.^{11,16} Specific enzymes, i.e., the transport protein and reductase, are involved in intracellular reduction of Cr(VI).

Chromium has four stable isotopes in nature with different masses (^{50}Cr , ^{52}Cr , ^{53}Cr , and ^{54}Cr).¹⁷ Because of the different masses of Cr isotopes, Cr isotope fractionation usually occurs during Cr redox processes. The Cr isotopic ratios have potential application for identifying and monitoring chromate reduction processes during environmental remediation.^{18–20} Microbial Cr isotope fractionation has been found to occur during the process of Cr(VI) reduction by bacterial species, including *S. oneidensis* MR-1.^{21,22} Most previous studies primarily focused on the effects of different experimental conditions on Cr isotope fractionation during microbial Cr(VI) reduction, such as the types and concentrations of electron donors.^{21,22} The magnitudes of Cr isotope fractionation varied from -4.5% to -1.8% when the concentration of the electron donor, lactate, was in the range of $3.3 \mu M$ to $10 mM$

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during microbial Cr(VI) reduction.²¹ When the electron donor concentration increased, less Cr isotope fractionation was observed. Moreover, the magnitudes of Cr isotope fractionation exhibited only a slight difference during Cr(VI) reduction by a metabolically diverse group consisting of *Geobacter sulfurreducens* PCA, *Pseudomonas stutzeri* DCP-Ps1, and *Desulfovibrio vulgaris*.²² However, thus far, the mechanism of Cr isotope fractionation during microbial Cr(VI) reduction by *S. oneidensis* MR-1 has not been clarified, in particular with regard to the contribution of extracellular and intracellular reduction processes to Cr isotope fractionation.

In this study, *S. oneidensis* MR-1 and its $\Delta mtrC/\Delta omcA$ mutant, which showed a deficiency in the pathway of extracellular Cr(VI) reduction,^{9,23} were used to elucidate the possible Cr isotope fractionation mechanisms during reduction by *S. oneidensis* MR-1. The Cr(VI) reduction process and product precipitation location were investigated using the wild type (WT) and a mutant; then, Cr isotope fractionation during Cr(VI) reduction by the two strains was analyzed. To the best of our knowledge, this is the first attempt to study the influence of reduction pathways on Cr isotope fractionation using a mutant of a Cr(VI)-reducing bacterium.

MATERIALS AND METHODS

Strains and Culture Conditions. *S. oneidensis* MR-1 (ATCC 700550) and the mutant $\Delta mtrC/\Delta omcA$ were kindly provided by K. H. Nealson (University of Southern California, Los Angeles, CA).²⁴ Single colonies of *S. oneidensis* MR-1 and the mutant strain from the freshly streaked plates were inoculated into Luria-Bertani (LB) medium and cultured at 30 °C to produce overnight cultures. Then, the overnight cultures were transferred to 100 mL of LB medium and cultured aerobically at 30 °C for 16 h to the late stationary phase. The cultures were collected upon centrifugation at 4 °C and 6000g for 10 min. After being washed twice with minimal growth medium (MM) at 4 °C, the concentrated cultures were injected into serum bottles with MM in an anaerobic glove chamber (Whitley DG250, Don Whitley Scientific, Shipley, U.K.) for Cr(VI) reduction.

MM was prepared using high-purity water (18.2 M Ω cm) obtained using a Milli-Q Reference water purification system (Millipore, Bedford, MA). The MM used in this work can be found elsewhere²⁵ and consists of 0.46 g/L NH₄Cl, 0.225 g/L K₂HPO₄, 0.225 g/L KH₂PO₄, 0.117 g/L MgSO₄·7H₂O, 0.225 g/L (NH₄)₂SO₄, and 10 mL of a mineral mix with modifications [containing 1.5 g/L NTA, 0.1 g/L MnCl₂·4H₂O, 0.04 g/L FeSO₄·7H₂O, 0.17 g/L CoCl₂·6H₂O, 0.1 g/L ZnCl₂, 0.04 g/L CuSO₄·5H₂O, 0.005 g/L AlK(SO₄)₂·12H₂O, 0.005 g/L H₃BO₃, 0.09 g/L Na₂MoO₄, 0.12 g/L NiCl₂, 0.02 g/L NaWO₄·2H₂O, and 0.10 g/L Na₂SeO₄]. Sodium lactate was added as an electron donor at a final concentration of 3 mM. The medium was flushed with a high-purity mixed gas (80/20 N₂/CO₂) until the dissolved O₂ concentration was <0.1 ppm. Then, the medium was adjusted to pH 7.2, and 5 mM NaHCO₃ was added. Afterward, the medium was dispensed into anoxic serum bottles under the same gas atmosphere. The serum bottles were sealed with butyl rubber stoppers and crimped with aluminum caps. After being autoclaved (121 °C for 20 min), the serum bottles with MM were supplemented with Wolfe's vitamin.²⁶

Cr(VI) Reduction. K₂CrO₄ (metal basis, 99.99%, Aladdin Chemical Co. Ltd., Shanghai, China) from an anaerobic filter-sterilized 100 mM stock solution was added to the sterilized

medium to give a final concentration of 100 μ M. The Cr(VI) reduction experiments were initiated by adding the prepared concentrated cells, as mentioned above, to a final OD₆₀₀ of 0.2. The cell densities of WT and $\Delta mtrC/\Delta omcA$ were $(2.98 \pm 0.23) \times 10^8$ and $(2.79 \pm 0.18) \times 10^8$ colony-forming units/mL, respectively. All serum bottles were incubated at 30 °C in the dark, and all experiments were conducted in duplicate. Samples were taken in an anaerobic glove chamber at predetermined time points followed by centrifugation at 12000g for 10 min. Afterward, the supernatants were transferred into a clean tube and stored at -20 °C before further analysis. To balance the gas pressure loss of the sample collection, an equal volume of mixed gas (80/20 N₂/CO₂) was injected into the serum bottles using a sterile syringe prior to sampling.

Analyses and Calculations. After centrifugation of the sample, bacterial cells of the WT and mutants were fixed in 2.5% glutaraldehyde overnight after being exposed to Cr(VI) for 30 h. Then, the fixed cells were dehydrated and infiltrated with an epoxy resin. Ultrathin sections (50–100 nm thick) were obtained using an ultramicrotome and placed in copper grids. The fixed cells were prepared for analysis via transmission electron microscopy (TEM) (JEM-2011, JEOL Co.) at 200 kV. Energy dispersive X-ray spectroscopy (EDX) was performed to probe the components of the elements at specific cellular locations using an EDX system (Oxford Instruments, Abingdon, U.K.) equipped with a Si(Li) detector attached to a JEM-2011 TEM instrument.

The residual Cr(VI) concentration in the supernatant of the samples was analyzed via the diphenylcarbazide method using an ultraviolet–visible (UV–vis) spectrophotometer (UV-2401 PC, Shimidazu Co.) at 540 nm.²⁷ To determine the Cr isotope compositions in the samples, Cr purification was first performed using previously described techniques.²⁸ Detailed information regarding the purification procedures can be found in the Supporting Information. Isotopic measurements were performed using a Neptune Plus multicollector inductively coupled plasma mass spectrometer (MC-ICP-MS) (Thermo Fisher Scientific, Bremen, Germany) using a double-spike method, as previously described.²⁸ The ⁵⁰Cr–⁵⁴Cr double-spike method was used to correct instrumental mass bias and fractionation during sample preparation. The double-spike solution has ⁵⁰Cr/⁵²Cr, ⁵³Cr/⁵²Cr, and ⁵⁴Cr/⁵²Cr ratios of 17.5254, 0.163256, and 12.1379, respectively. Data are expressed using the δ -notation units relative to the National Institute of Standards and Technology (NIST) standard reference material (SRM) 979:

$$\delta^{53}\text{Cr} = \left[\frac{(\text{}^{53}\text{Cr}/\text{}^{52}\text{Cr})_{\text{sample}}}{(\text{}^{53}\text{Cr}/\text{}^{52}\text{Cr})_{\text{SRM 979}}} - 1 \right] \times 1000\% \quad (1)$$

The long-term precision was 0.06‰ (2SD) for $\delta^{53}\text{Cr}$ of the spiked SCP solution (internal laboratory standard) (SCP Science, Baie-D'Urfe, QC).

To express the magnitude of fractionation, a fractionation factor, α , is usually used as follows:

$$\alpha = \frac{R_{\text{prod}}}{R_{\text{react}}} \quad (2)$$

where R_{prod} and R_{react} are the ⁵³Cr/⁵²Cr ratios of Cr reduced at a particular time and in the reactant pool, respectively.

In the Rayleigh model, the $\delta^{53}\text{Cr}$ values and fraction of reduced Cr(VI) can be expressed using the following equation:

$$\delta_t = (\delta_0 + 1000)f^{\alpha-1} - 1000 \quad (3)$$

where δ_0 and δ_t are the $\delta^{53}\text{Cr}$ values of the Cr(VI) sample at the beginning and at time t , respectively, and f is the fraction of Cr(VI) reduction. For the sake of convenience, the α value is converted to ε , which represents the magnitude of Cr isotope fractionation using the following equation:

$$\varepsilon = (\alpha - 1) \times 1000\text{‰} \quad (4)$$

The details for determining the uncertainties of $\delta^{53}\text{Cr}$ and ε are provided in the Supporting Information.

RESULTS AND DISCUSSION

Cr(VI) Reduction by *S. oneidensis* MR-1. In addition to the intercellular reduction process, previous studies have demonstrated that MtrC and OmcA cytochromes in *S. oneidensis* MR-1 can function as the terminal Cr(VI) reductases in extracellular Cr(VI) reduction.⁹ To investigate Cr(VI) reduction using different electron transfer pathways, we compared the performances of the WT and $\Delta\text{omcA}/\Delta\text{mtrC}$ mutant strains. Control experiments with heat-killed cells showed that there was an insignificant Cr(VI) decrease, as shown in Figure 1A. This indicated that adsorption onto cells

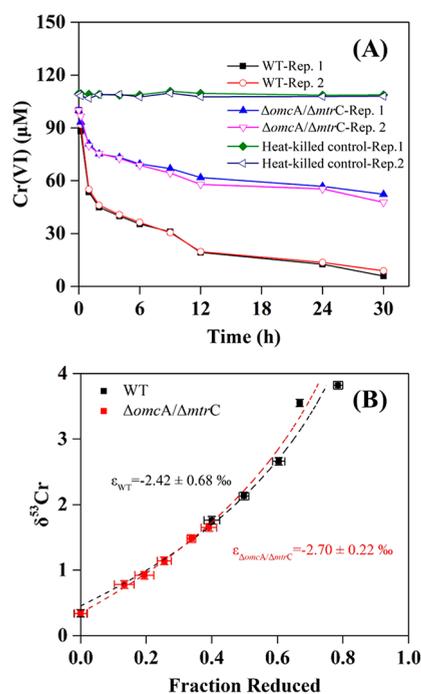


Figure 1. (A) Cr(VI) reduction by *S. oneidensis* MR-1 wild type (WT) and $\Delta\text{omcA}/\Delta\text{mtrC}$. (B) $\delta^{53}\text{Cr}$ values of the remaining Cr(VI) vs the fraction reduced and the Rayleigh model fitting.

or reduction by components of media did not contribute to the observed decreases in Cr(VI) concentration. The reduction rate of the WT was $11.12 \pm 0.38 \mu\text{M Cr(VI) h}^{-1}$ (mean \pm standard deviation; $n = 2$) for the first 4 h, whereas the reduction rate of the mutant strain was $4.91 \pm 0.48 \mu\text{M Cr(VI) h}^{-1}$ ($n = 2$) (i.e., 44.2% of that of the WT). This result is consistent with a previous report that $\Delta\text{omcA}/\Delta\text{mtrC}$ had a Cr(VI) reduction rate substantially lower than that of the WT.⁹ Moreover, the cell-specific reduction rates for WT and $\Delta\text{omcA}/\Delta\text{mtrC}$ were 3.58×10^{-15} and $1.69 \times 10^{-15} \text{ mol cell}^{-1} \text{ day}^{-1}$, respectively, for the first 4 h and 1.05×10^{-15} and $0.85 \times 10^{-15} \text{ mol cell}^{-1} \text{ day}^{-1}$,

respectively, after 24 h. Because of the elevated concentration of the electron donor and cell density, these values are approximately 10 times higher than those in a previous study.²¹ The cellular locations of reduced precipitates were determined using TEM. Extracellular matrix-associated precipitates were found on the cell surface of the WT (Figure 2A) after reduction

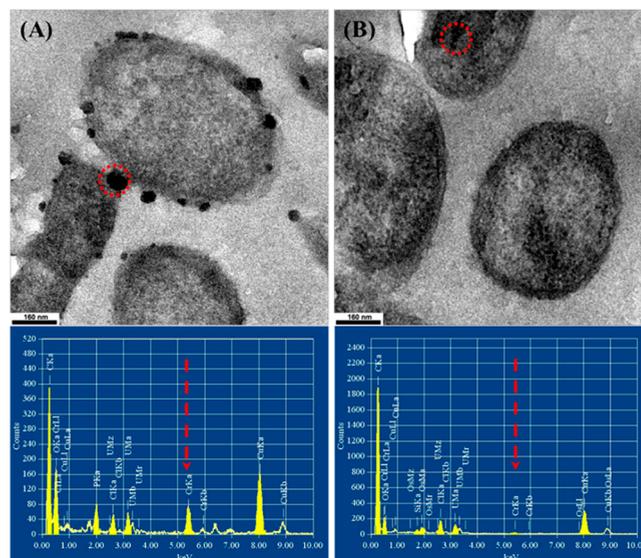


Figure 2. TEM image of the product during Cr(VI) reduction by (A) the wild type (WT) and (B) $\Delta\text{omcA}/\Delta\text{mtrC}$ and EDX-based elemental analysis of the product. The elemental analyses are from the circled spots, and the virtual arrow indicates the signal of the Cr element.

of $100 \mu\text{M Cr(VI)}$ for 30 h. Different from the results for WT cells, this phenomenon was not found in $\Delta\text{omcA}/\Delta\text{mtrC}$ under the same experimental conditions (Figure 2B). Furthermore, EDX-based elemental analysis confirmed the formation of Cr-rich precipitates (Figure 2A). The Cr element was detected in the spot located in the cell interior of $\Delta\text{omcA}/\Delta\text{mtrC}$ (Figure 2B), which suggests that Cr(VI) can be transported into the cell interior and reduced intracellularly.

Cr Isotope Fractionation during Cr(VI) Reduction by Different Strains. To investigate Cr isotope fractionation during Cr(VI) reduction in different electron transfer pathways, the $\delta^{53}\text{Cr}$ values of the residual Cr(VI) in batch reduction experiments were determined. The $\delta^{53}\text{Cr}$ value, which corresponds to $\Delta\text{omcA}/\Delta\text{mtrC}$ and WT, increased from the initial value of 0.34 to a higher value, as shown in Figure 1B. To further determine the ε values in the experiments, Rayleigh distillation models were used to fit the data. Although there was a substantial difference in their Cr(VI) reduction kinetics, the ε values surprisingly showed no significant difference between the two strains, i.e., $-2.42 \pm 0.68\text{‰}$ and $-2.70 \pm 0.22\text{‰}$, respectively (Figure 1B). The results suggest that Cr isotope fractionation is Mtr pathway-independent in *S. oneidensis* MR-1. In addition, there is no statistically significant difference in the magnitudes of Cr isotope fractionation in a diverse group of bacteria, such as *G. sulfurreducens* PCA, *P. stutzeri* DCP-PS1, and *D. vulgaris*, in which both extracellular and intracellular Cr(VI) reduction occur.²² This further implies that Cr isotope fractionation may be independent of the extracellular reduction pathway in bacteria.

Cr Isotope Fractionation Processes in *S. oneidensis* MR-1. Microbial Cr isotope fractionation is determined by the biochemistry processes of microbial Cr(VI) reduction, but the exact mechanisms underpinning this process have rarely been investigated.^{21,22,28} As indicated by previous studies, Cr(VI) can be reduced extracellularly but also transported into the cell interior and then reduced in the cytoplasm.^{9,10,12} Myers et al.¹² first reported that cytoplasmic proteins had Cr(VI) reductase activity in anaerobically grown MR-1 cells. An investigation of chromate–nitrite interactions in *S. oneidensis* MR-1 further suggested that the nitrite reduction pathway is involved in intracellular reduction of Cr(VI).^{10,11} Because of the structural similarity of chromate (CrO_4^{2-}) and sulfate (SO_4^{2-}) anions, it has been demonstrated that transmembrane transport of Cr(VI) across the cell membrane occurred via the sulfate transport pathway in various bacteria, including *S. oneidensis* MR-1.^{16,29} Furthermore, the protein ChrA in *Shewanella* sp. strain ANA-3 was confirmed to be an efflux transporter of Cr(VI), which was also suitable for *S. oneidensis* MR-1.³⁰ Our results indicate that isotope fractionation is independent of the Mtr pathway during Cr(VI) reduction by *S. oneidensis* MR-1. On the basis of the results from our study as well as the literature, herein we propose a Cr isotope fractionation model during intracellular reduction by *S. oneidensis* MR-1. As shown in Figure 3, Cr isotope fractionation in *S. oneidensis* MR-1 can

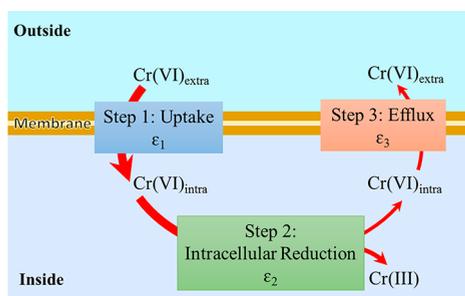


Figure 3. Schematic diagram of Cr isotope fractionation during intracellular Cr(VI) reduction in *S. oneidensis* MR-1. Isotope effects are denoted by the ϵ terms.

be divided into three steps. First, chromate is transported across the cell membrane into the cell interior via the sulfate transport pathway. Second, some chromate is reduced intracellularly. Third, chromate is transported outside of the cell simultaneously by the efflux protein ChrA.³⁰ Therefore, the reaction chain consisting of a combination of these three steps would determine the observed Cr isotope fractionation, among which intracellularly enzymatic Cr(VI) reduction that reconfigures Cr bonds plays an important role.²² Accordingly, a relatively strong isotopic fractionation can be observed if intracellularly enzymatic Cr(VI) reduction steps are rate-limiting.²² On the contrary, an overall weaker isotopic fractionation was observed in our study, implying that transport or efflux of Cr(VI) during its microbial reduction could be the rate-limiting step under the conditions tested. This model is helpful in understanding the isotopic fractionation during microbial Cr(VI) reduction. Nevertheless, the reaction kinetics of each step and values of the isotope fractionation parameters warrant further studies with gene manipulation to improve our understanding of the contribution of each step to the overall Cr isotope fractionation during microbial reduction.

■ ASSOCIATED CONTENT

📄 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.7b00471.

Details of materials and methods in the measurement of the Cr(VI) concentration, chemical separation for isotopic measurements, and determination of the uncertainties of $\delta^{53}\text{Cr}$ and ϵ (PDF)

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Notes

The authors declare no competing financial interest.

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