TEM investigation of U$^{6+}$ and Re$^{7+}$ reduction by *Desulfovibrio desulfuricans*, a sulfate-reducing bacterium

Huifang Xu $^a$, Larry L. Barton $^b$, Keka Choudhury $^b$, Pengchu Zhang $^c$, and Yifeng Wang $^e$

$^a$ Department of Earth and Planetary Sciences, E-mail: hfxu@unm.edu
$^b$ Department of Biology, The University of New Mexico, Albuquerque, New Mexico 87131.
$^c$ Sandia National Laboratories, MS 0750 and 1395, Albuquerque, New Mexico 87185, USA.

ABSTRACT

Uranium and its fission product Tc in aerobic environment will be in the forms of UO$_2^{2+}$ and TcO$_4^-$. Reduced forms of tetravalent U and Tc are sparingly soluble. As determined by transmission electron microscopy, the reduction of uranyl acetate by immobilized cells of *Desulfovibrio desulfuricans* results in the production of black uraninite nanocrystals precipitated outside the cell. Some nanocrystals are associated with outer membranes of the cell as revealed from cross sections of these metabolic active sulfate-reducing bacteria. The nanocrystals have an average diameter of 5 nm and have anhedral shape. The reduction of Re$^{7+}$ by cells of *Desulfovibrio desulfuricans* is fast in media containing H$_2$ an electron donor, and slow in media containing lactic acid. It is proposed that the cytochrome in these cells has an important role in the reduction of uranyl and Re$^{7+}$ is (a chemical analogue for Tc$^{7+}$) through transferring an electron from molecular hydrogen or lactic acid to the oxyions of UO$_2^{2+}$ and TcO$_4^-$.

INTRODUCTION

The solubility of uranium and Tc is dependent on their oxidation state. U$^{4+}$ and Tc$^{4+}$ are sparingly soluble. Under oxidizing environments, uranium will exist in the form of uranyl (UO$_2^{2+}$) ion that complexes with carbonate (CO$_3^{2-}$) and organic ligands. When carbonate is present in aerobic solutions, uranyl forms highly soluble metal complexes of UO$_2$CO$_3^0$, UO$_2$(CO$_3$)$_2^{2-}$, and UO$_2$(CO$_3$)$_3^{4-}$ [1]. Waste water from the nuclear industries are important because oxidative dissolution of nuclear waste and uranium fission product Tc results in the formation of very soluble oxy-ions, such as of UO$_2^{2+}$, TcO$_4^-$, and PuO$_2^{2+}$. To immobilize the uranium in water, uranium may be reduced to insoluble uraninite (UO$_2$). Recent studies have demonstrated that *Desulfovibrio desulfuricans* and other sulfate-reducing bacteria are capable of reducing uranyl and other metals by enzyme-mediated reactions [2-8]. This biological processes involving reduction of heavy metals can effectively remove them from solution. The detoxification process is known as dissimilatory reduction and occurs when electrons from molecular hydrogen (H$_2$) or lactate are transferred to oxidized metal ions such as selenate [9], or UO$_2^{2+}$ [5]. In at least one instance, the dissimilatory reduction of uranyl ion is coupled to the growth of a sulfate-reducing bacterium, *Desulfotomaculum reducens* [10].
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It is very difficult to Immobilize Tc in aerobic environment, because Tc\textsuperscript{7+} that is in the form of TcO\textsubscript{4}\textsuperscript{-} is very stable in solution. In here, we also use a sulfate-reducing bacteria of *Desulfovibrio desulfuricans* to reduce ReO\textsubscript{4}\textsuperscript{-} that is a chemical analogue for TcO\textsubscript{4}\textsuperscript{-} using electron donors of lactate and hydrogen gas respectively. If the bacteria can reduce ReO\textsubscript{4}\textsuperscript{-}, they should be able to reduce TcO\textsubscript{4}\textsuperscript{-} based on their redox potentials [1].

**CULTURE, SAMPLE PREPARATION, AND EXPERIMENTAL METHODS**

**Culture for Uranium Reduction**

Bacteria used in the experiments were *Desulfovibrio desulfuricans* DSM 642 which was grown in the following medium as described elsewhere [7]: 1000 ml distilled water; Na lactate 4 ml of a 65% solution; NH\textsubscript{4} Cl: 2.0 g; Na\textsubscript{2}SO\textsubscript{4}: 4.0 g; MgSO\textsubscript{4}: 2.0 g; K\textsubscript{2} HPO\textsubscript{4}: 0.5 g; yeast extract: 1.0g; FeSO\textsubscript{4}: 20 mg; pH adjusted to 7.4 with 20% KOH. To maintain cultures, ten ml of growth medium was added to 13 x 125 mm anaerobic tubes fitted with rubber closures. Tubes were autoclaved and flushed with purified nitrogen before they were inoculated. Using anaerobic transfer techniques, 0.1 ml of a two day culture was introduced into the stopped culture tubes. Incubation was at 35°C.

To cultivate *D. desulfuricans* for uranium reduction, 1.5 liters of growth medium was placed in a 2 liter anaerobic flask and after autoclaving was flushed with purified nitrogen gas. The inoculum for the 1.5 liters of medium was 200 ml of *D. desulfuricans*. After 2 days of incubation at 35°C, cells were removed by centrifugation at 5,800 g for 30 minutes and washed with sterile, degasses bicarbonate buffer (1.25 g/L). The biomass was resuspended in 20 ml of sterile, deionized water supplemented with 25% (w/v) of acrylamide monomer, 0.25 g (w/v) solution of potassium persulfate, and crosslinkage was accelerated by adding 2.5 ml of a 5% (w/v) solution of 3-dimethylaminopropionitrile. The cell suspension was refrigerated at 4°C until the acrylamide polymerized [8]. The gel was cut into 3 mm cubes, washed with sterile water and placed in a 125 ml serum bottle along with 100 ml of degassed solution containing 5 mM uranium (U\textsuperscript{6+}) acetate (Electron Microscopy Sciences, Fort Washington, PA), 30 mM NaHCO\textsubscript{3}, and 50 mM Tris-HCl , pH 7.6. Unless specified, chemicals were from Sigma Chemical Co., St. Louis, MO. The bottles were flushed with purified H\textsubscript{2} for 15 minutes and incubated at 35 °C. After 5 days, the reduction of yellow uranium (U\textsuperscript{6+}) was suggested from the appearance of a black film on the surface of the gel. The specimens were prepared for TEM experiment by touching C-coated formvar Cu grids to the surface of the wet acrylamide block that had black precipitates and drying the wet Cu grids in air.

**Culture for Re Reduction**
The culture of *Desulfovibrio desulfuricans* DSM 642 was grown in a lactate-sulfate medium that contained the following in a liter of distilled water: 4 ml of Na lactate, 5 g tryptone (Difco Co., Detroit, MI), 4 g yeast extract (Difco Co., Detroit, MI), 1.5 g Na$_2$SO$_4$, 1.6 g MgSO$_4$, and 0.5 g cysteine. HCl. To maintain the culture, monthly transfers were made using 10 ml of medium in 13 x 150 ml anaerobic tubes. For reduction of the element rhenium, 4 l of a culture of *D. desulfuricans* was grown in anaerobic bottles until stationary phase had been reached and cells were harvested by centrifugation. Cells were suspended in 200 ml anaerobic bottle containing 10 ml of a 50 mM Tris-HCl buffer at pH 7.6 and flushed with purified N$_2$ for 15 min. To evaluate the reduction of rhenate by cells of *D. desulfuricans*, a Warburg apparatus was used as previously described [11]. The 2 ml reaction mixture in Warburg flasks consisted of 50 mM Tris-HCl, pH 7.6 and 20 mg of cell protein, as determined by Lowry protein analysis, and the gas phase (electron donor) was purified H$_2$. The reaction was initiated by addition of 2.9 mg of potassium perrhenate (VII) (Aldrich Chem Co., Milwaukee, WI) in 0.2 ml to give a final concentration of 5 mM rhenate in the reaction. Upon completion of the hydrogen oxidation reaction was stopped after 60 minutes, the solution became black. At this time a sample was removed from the reaction mixture and placed on C-coated formvar Cu grids.

All transmission electron microscopy (TEM) and X-ray energy dispersive spectroscopy (EDS) examinations were carried out with a JEOL 2010 HRTEM with an Oxford Link ISIS EDS system. A Li-drifted Si detector with ultrathin window was used for collecting EDS spectra. Point-to-point resolution of the HRTEM is 0.19 nm, an accelerating voltage was 200 keV.

**RESULTS AND DISCUSSIONS**

**Uranyl Reduction**

Black uranium precipitates formed on the surface of the PAG after the culture was incubated for 2 days. The uranyl solution changed from yellow to colorless, and it was apparent that uranyl ion was removed from solution. TEM images indicate that the black precipitates are nanocrystals of uranium oxide which exist on the surface of the bacterial cells (Figs. 1, 2). Electron diffraction pattern from the nanocrystals indicates the nanocrystals were uraninite (Fig. 2). The average size of the nanocrystals is about 5 nm, and the crystals are anhedral. High-resolution TEM image of the uranium oxide nanocrystals indicates lattice fringes of the uraninite end product of uranyl reduction (Fig. 1). Local areas of the cell surface are rich in uraninite nanocrystals (Fig. 1).
Fig. 1 HRTEM image of the reduced uraninite nanocrystals with lattice fringes (left) and EDS spectrum from the uraninite nanocrystal (right).

Fig. 2 A bright-field TEM image of the bacteria and the reduced uraninite crystals. Inserted SAED pattern (upper right corner) is from the uraninite nanocrystals.
ReO$_4^-$ Reduction

Black precipitates are dominated by amorphous Re-oxide. The amorphous Re-oxide also coated on the surfaces of the *D. desulfuricans* cells (Fig. 3). The reduced Re may be in the amorphous forms of ReO$_2$ and Re(OH)$_4$. It is proposed that the reduced Re first occurs on the cells surfaces. We also tried electron donor of lactic acid for the reduction of Re. After 5 days, there were only small amount of reduced Re in black precipitates. The reduced Re is also amorphous. The reduction reaction is slow if using lactic acid as electron donor.

It is proposed that the reduction of uranyl is associated with cytochrome in the cell through metabolism of the cell. The function of cytochrome in the outer membrane would be to participate in detoxification and in this case would be reduction of uranyl through transferring electrons from the organic molecules to uranyl ions. Possible overall reactions occur in the uranyl reduction are:

**Cells of *D. desulfuricans***

\[
<\text{CH}_2\text{O}> + 2\text{UO}_2^{2+} \rightarrow \text{HCO}_3^- + 2\text{UO}_2(\text{s}) + 5\text{H}^+
\]

**Cells of *D. desulfuricans***

\[
\text{H}_2 + \text{UO}_2^{2+} \rightarrow 2\text{UO}_2(\text{s}) + 2\text{H}^+
\]

The basic reaction of the Re reduction is similar to that of U reduction. The reaction may be written as:

**Cells of *D. desulfuricans***

\[
3\text{H}_2 + 2\text{ReO}_4^- \rightarrow 2\text{ReO}_2(\text{s}) + 6\text{H}^+
\]

In above reactions, \(<\text{CH}_2\text{O}>> represents organic acids such as lactic acid or pyruvic acid that may serve as electron donors. Sulfate-reducing bacteria have several different molecular forms of c-type cytochromes. *D. desulfuricans* has a tetraheme (c$_3$) c-type cytochrome in the periplasmic space between the outer membrane and plasma membrane [12]. *D. desulfuricans* has two additional soluble c-type cytochromes that are either hexaheme or dodecaneheme proteins. Cytochromes have been reported in the outer membrane of *D. vulgaris* [13] and it would be consisted to assume that cytochromes are present in the outer membrane of *D. desulfuricans*. Hydrogenase is found in the periplasmic space of sulfate-reducers [12] and would provide
electrons for cytochromes in the periplasmic space with the surface of cell, reductions occurs with the accumulation of uraninite nanocrystals on the cell surface.

CONCLUSIONS

As a conclusion, polyacrylamide gel containing immobilized D. desulfuricans cells can be used to recover uranium as uraninite from uranyl-bearing solutions and waste water. The products of uranyl reduction by D. desulfuricans is uraninite which occurs as nanocrystals outside the cell and associated with the cell outer membrane. The crystals are anhedral with an average size of 5 nm. The bacteria of D. desulfuricans are also able to reduce TcO$_4^-$: The reduced forms of tetravalent Re could be amorphous ReO$_2$ and Re(OH)$_4$.

REFERENCES