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Influence of Iron Phases on Microbial U(VI) Reduction

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ABSTRACT

The bacterial uranium(VI) reduction and its resultant low solubility make this process an attractive option for removing U from groundwater. An impact of aqueous suspending iron phase, which is redox sensitive and ubiquitous in subsurface groundwater, on the U(VI) bioreduction by *Shewanella putrefaciens* CN32 was investigated. In our batch experiment, the U(VI) concentration (5×10^{-5} M) gradually decreased to a non-detectable level during the microbial respiration. However, when Fe(III) phase was suspended in solution, bioreduction of U(VI) was significantly suppressed due to a preferred reduction of Fe(III) instead of U(VI). This shows that the suspending amorphous Fe(III) phase can be a strong inhibitor to the U(VI) bioreduction. On the contrary, when iron was present as a soluble Fe(II) in the solution, the U(VI) removal was largely enhanced. The microbially-catalyzed U(VI) reduction resulted in an accumulation of solid-type U particles in and around the cells. Electron elemental investigations for the precipitates show that some background cations such as Ca and P were favorably coprecipitated with U. This implies that aqueous U tends to be stabilized by complexing with Ca or P ions, which easily diffuse and coprecipitate with U in and around the microbial cell.

Key words : Uranium(VI) bioreduction, Shewanella putrefaciens, Iron-reducing bacterium, HRTEM, Iron

1. Introduction

Bacteria are widely distributed and probably reactive in subsurface environments. The recognition of the role that bacteria have played as geochemically reactive solids in aquatic systems stems from a large body of data that demonstrate them to be both nucleation templates for a host of authigenic minerals (Ferris et al., 1995) and potent sorbents of a wide range of dissolved metal ions (Beveridge, 1989; Warren and Ferris, 1998). Geobiological studies have shown that bacteria have played an important role in cycling elements all over the Earth's surface, especially through the interactions of cell wall surfaces with dissolved ions in the environments that produce various secondary minerals such as oxides, sulfides, carbonates, and silicates (Ehrlich, 1990; Konhauser, 1998; Kawano and Tomita, 2001).

Dissimilatory anaerobic bacteria that respire using oxidized metals must obtain energy by coupling the enzymatic reduction of oxidized species to the oxidation of organic matter (Lovley, 1991; Nealson and Saffarini, 1994). Among the dissimilatory anaerobic bacteria, the dissimilatory Fe(III)-reducing microorganisms can obtain energy for growth by electron transport to Fe(III) or other terminal electron acceptors such as U(VI) (Lovley et al., 1991). Most radionuclides, including U, are both redox active and less soluble when reduced. Therefore, bioreduction offers much promise for controlling the solubility and mobility of target radionuclides in subsurface environments through the reduction of U(VI) (the uranyl ion; UO_2^{2+}) to U(IV) (uraninite; UO_2) (Lovley et al., 1991; Lloyd et al., 2005). Such an uraninite that is bioproduced is an interesting and important nanoscale biogeological material (Bargar et al., 2008a; Lee et al., 2010).

Some dissimilatory metal-reducing bacteria can reduce solid phase Fe(III) oxides and oxyhydroxides including ferrihydrite and goethite (Roden and Zachara, 1996), hematite (Zachara et al., 1998), and magnetite (Kostka and Nealson,

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1995; Dong et al., 2000). *Shewanella putrefaciens* appears to be particularly effective at reducing crystalline Fe(III) oxide phases (Fredrickson et al., 2000). However, from a thermodynamic standpoint and because of much greater solubility, U(VI) should be reduced in preference to Fe(III) oxides, which are poorly soluble in the oxidized form (Fredrickson et al., 2000). Under suboxic conditions, U(VI), rather than Fe(III) oxides, is considered the preferred terminal electron acceptor for microbial respiration (Cochran et al., 1986). Wielinga et al. (2000) found that the presence of goethite and hematite did not significantly affect enzymatic reduction of U(VI) coupled to the oxidation of an organic electron donor. The presence of ferrihydrite, however, decreased the initial reduction rate of U(VI). In mixtures of goethite and ferrihydrite, the inhibition was related to the fraction of ferrihydrite. In these instances, it seems that the microbial reduction of Fe(III) oxyhydroxides are influenced by the type of Fe(III) phases. The bioreduction rate correlated positively with the solubility of the Fe(III) oxyhydroxides with the highest for ferrihydrite and the lowest for hematite (Bonneville et al., 2004). Hence, solubility appears to be a rate-controlling parameter in enzymatic reduction of Fe(III) oxyhydroxides.

Previous work mostly focused on elucidating mechanisms of U(VI) bioreduction under effects of iron oxyhydroxide minerals. However, very limited research has been conducted to date on the impact of dissolved metal or cationic ions on the U(VI) bioreduction. Bargar et al. (2008b) found that the inclusion of Mn^{2+} in the reaction mixture led to the production of smaller uraninite particles, but that the smaller Mn-containing uraninite particles were more resistant to re-oxidation than the larger Mn-free uraninite particles. In another example, dissolved calcium was reported to cause a decrease in the rate and extent of bacterial U(VI) reduction (Brooks et al., 2003). Thus, some concentrated cationic ions of groundwater in which U(VI) reduction takes place will undoubtedly exert control on the nature to form biogenic U(IV) phases (Abdelouas et al., 1998; Burgos et al., 2008).

In our experiment, we conducted a microbial U(VI) reduction using *Shewanella putrefaciens* CN32, a gram negative dissimilatory Fe(III)-reducing bacterium, which is thought to be widespread on the Earth's surface (Nealson and Saffarini, 1994; Haas et al., 2001). In this study, we will present some important results that were observed from microbial catalytic reduction of U(VI) to U(IV) phase in a presence of different iron species. We have also attempted to examine what background cations would strongly associate with U (IV) during the microbial reducing process.

2. Materials and Methods

2.1. Cell Cultivation

S. putrefaciens strain CN32 (ATCC BAA-1097) was obtained from the American Type Culture Collection (ATCC), USA. Strain CN32 was routinely cultured aerobically in tryptic soy broth (TSB) (30 g/L) (Difco Laboratories, Detroit, MI, USA), and stock cultures were maintained by freezing in 40% glycerol at −80°C.

The aerobically cultured CN32 cells were harvested at mid- to late-log phase by centrifugating them from 30 g/L TSB cultures. The cells were centrifuged at 4,000 rpm for 15 min. The supernatant was discarded and the cell pellets were suspended in 30 mM $NaHCO_3$ (pH 7) buffer solution and purged with O_2-free N_2. This process was repeated four times and washed cells ($> 4 \times 10^8$ cells mL^{-1}) were used as inoculum.

2.2. Bacterial U(VI) Reduction Experiments

The $NaHCO_3$ buffer solution (30 mM) was extensively flushed with N_2 to remove dissolved O_2. We dispensed 100 mL of the buffer solution containing 10 mM lactate as electron donor in 150 mL-serum bottles under N_2, which we then capped with thick butyl rubber stopper and aluminum seals (Bellco Glass, Vineland, NJ). The bottle and solution were sterilized by autoclaving at 121°C and 15 lb/sq for 20 min.

To study the effect of background electrolytes, we added aseptically filtered (0.2 μm Advantec cellulose acetate membranes) stock solutions of major cations (e.g., Ca, K, and Mg) to the serum bottles by syringe. The stock solutions had calcium chloride, potassium chloride, and magnesium chloride, each at 1 mM concentration. We separately injected 0.2 mM ferrous chloride (or manganous chloride) and 0.3 mM sodium hydrogen phosphate solutions into the serum bottles by syringe. To make aqueous ferric iron suspension, a ferrous chloride solution was permitted to be oxidized by O_2 penetration through syringe. There was an immediate color change from clear to faint brownish by the treatment,

but a precipitation of Fe-oxides did not occur. The oxidized aqueous iron was used as an electron acceptor in the microbial U(VI) reduction later.

U(VI) stock solutions were prepared by dissolving a known amount of $UO_2(NO_3)_2 \cdot 6H_2O$ (Aldrich) in a previously acidified $HClO_4$ solution to prevent cation hydrolysis. The stock solution concentrations were about 1×10^{-3} M, and U(VI) (5×10^{-5} M) was aseptically added using a purged needle and syringe. Finally, washed CN32 cells were injected into the serum bottles to a final cell protein concentration of 8 mg/L. The final pH of the solution of serum bottle was ~8.0. Measurement of solution pH was made using a Ross combination pH electrode and an Orion 920A pH/ISE/mV/EC meter. The inoculated serum bottles were then placed on a rotary-shaker (120 rpm at 25ºC) in the dark. Periodically, 2 mL samples were aseptically removed by syringe and needle through 0.2 μm cellulose acetate filters and were analyzed for soluble concentrations of major cations including U by using an inductively coupled plasma mass spectrometry (ICP/MS).

2.3. High-Resolution Transmission Electron Microscopy (HRTEM)

HRTEM was used for the investigation of microbial precipitates collected from the serum bottles. In order to examine bacterial surfaces interacted with aqueous cations, microbial samples were prepared drying aliquots of suspension on holey carbon films (a whole mount) and then immediately observed by HRTEM.

Some selected cells were cut by a diamond knife to see internal structures of them. This process was initiated by centrifugating cell suspension and then fixating the cells in 2.5% glutaraldehyde. After washing them with Na-cacodylate, they were gradually dehydrated by ethanol series and then infiltrated into Epon-812 resin. Cured blocks of the resin sample were sectioned by the Diatome 35º diamond knife. Ultrathin sections (50 to 70 nm in thickness) were mounted on copper grids with Formvar support film coated with carbon. Samples were examined by a JEOL JEM 2100F high-resolution field emission TEM at 200 kV. An energy dispersive X-ray spectrometer (EDS) was also used to analyze their chemical compositions.

3. Results

During the microbial reduction of U(VI), initial aqueous U(VI) concentration (5×10^{-5} M) decreased steadily to a very low level of no detection (Fig. 1). A similar pattern was not noted in an uninoculated control. During CN32 respiration, some cations injected (e.g., Ca) were associated with aqueous U(VI), partly affecting U(VI) bioreduction rates. Among the background cations, iron species greatly affected the microbial U(VI) reducing rate.

Amorphous Fe(III) oxides have been usually synthesized by neutralizing a $FeCl_3 \cdot 6H_2O$ solution with high NaOH concentration (e.g., 1 M NaOH) (Bonneville et al., 2004). Such a method using high alkali solution is frequently used to make amorphous Fe(III) colloids. In natural environment, homogeneous nucleation of ferric iron phases can occur in bulk solution when the supersaturation exceeds a certain critical value. Such phases are initially very soluble (Schwertmann and Cornell, 2000). A Fe(III) phase converted from aqueous Fe(II) in our experiment was a very amorphous and near-soluble form. Hereafter, we will use “Fe(III) phase” instead of the “very amorphous or near-soluble Fe(III) phase”.

In the microbe-uranium experiment, U(VI) concentration rarely decreased from the beginning in the presence of

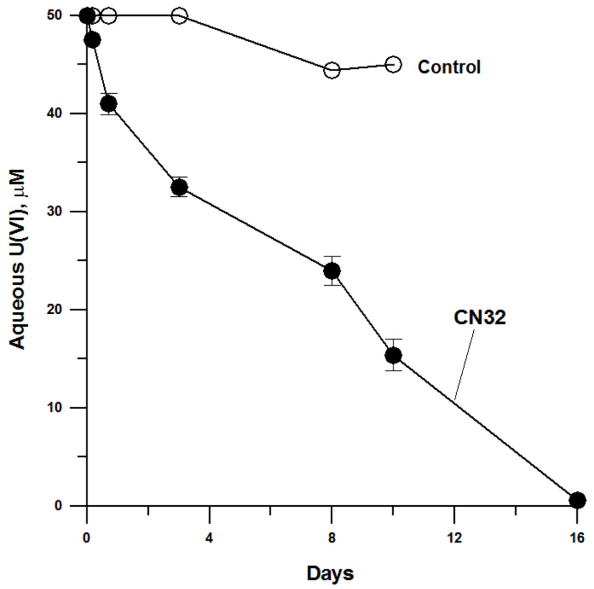

Fig. 1. U(VI) reduction by *Shewanella putrefaciens* strain CN32 in a presence of aqueous cations (Na, Ca, K, and Mg) in 30 mmol/L, pH 8.1 HCO_3 buffer.

Fe(III) phase (Fig. 2). However, as iron phase was Fe(II) in the system, the U(VI) ions was largely removed. These results indicate that there may be an effective role of iron species on the microbial catalytic U(VI) reduction. As observed in Fig. 3, a serum "bottle A" that contained Fe(III) phase was exhibiting faint brownish yellow color, but its color changed to nearly colorless as shown in "bottle B". This

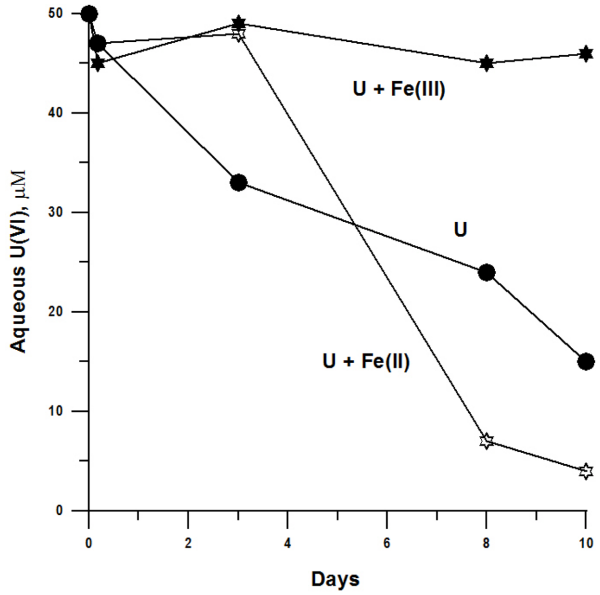

Fig. 2. U(VI) reduction by *S. putrefaciens* and influence of aqueous iron species (0.2 mM).

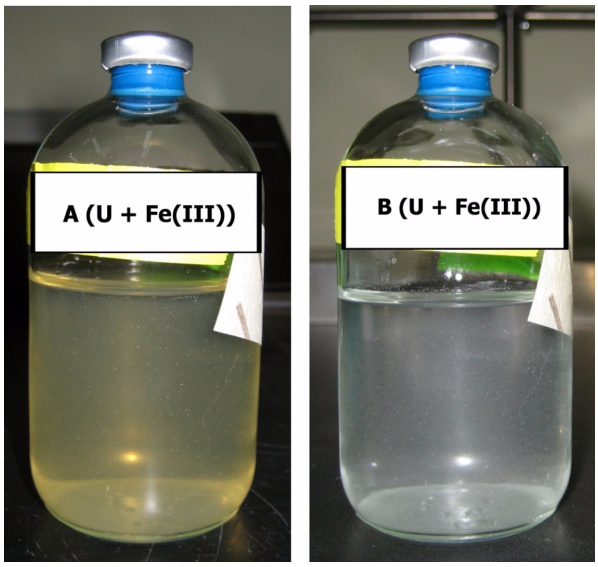

Fig. 3. (A) A serum bottle containing uranyl ions and ferric iron and (B) bottle (A) incubated by *S. putrefaciens* for 2 weeks. There was a color change from brownish yellow to colorless by microbial iron reduction.

reveals that Fe(III) phase was progressively reduced to a Fe(II) phase by bacteria (Roh et al., 2007). In that case, there almost not occurred a U(VI) decrease or reduction (Fig. 2).

Resulting from the U(VI) bioreduction, insoluble U nanoparticles were nucleated and formed on bacterial surfaces (Fig. 4). The coating degree of U precipitates on bacteria was different from each other. Fig. 5A shows a single bacterim whose surface was coated with particulate U, which was derived from a continuous attachment of U onto the cell. In a high-resolution electron image, the U particles on the surface were randomly distributed and their sizes were ranged from several to hundred nanometers in diameter (Fig. 5B). The selected-area electron-diffraction (SAED) pattern (Fig. 5C) indicates that the U particles were nearly mineralized to uraninite (UO_2) with a good crystallinity (Lee et al., 2010). The primary particle size of the uraninite was about 2-3 nm in diameter. The orientation of the particles was not uniform, but their intimate contact between them seems to cause their large aggregation on the surface (Figs. 5B and C).

An intra-cellular U precipitation was also examined by HRTEM (Fig. 6). A sectioned sample shows a precipitation of U accumulated in and around a cell. In addition, characteristic U nanoparticles were found to be aligned along the cytoplasmic membrane of the cell (Fig. 6A),

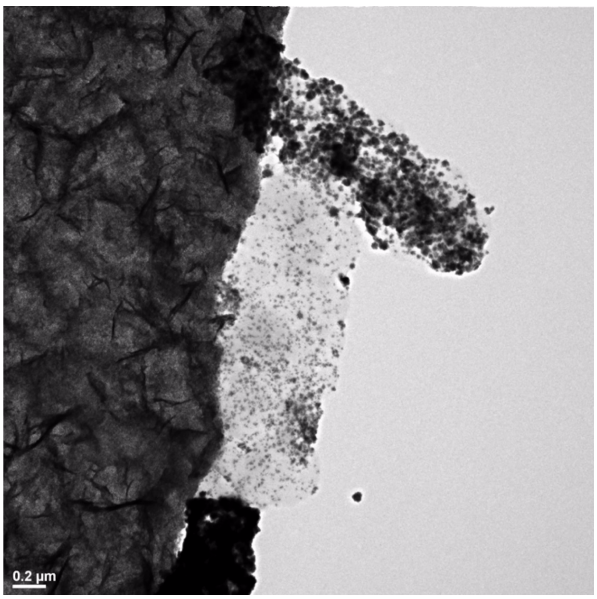

Fig. 4. HRTEM image showing several *S. putrefaciens* cells coated with U precipitates.

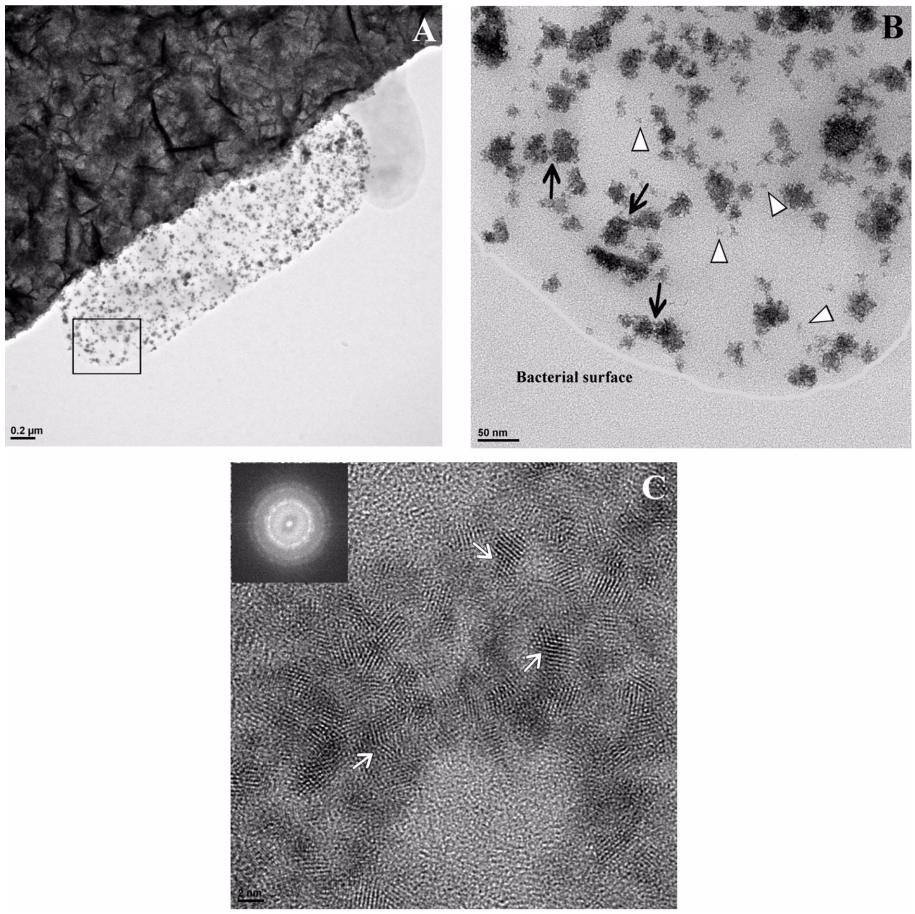

Fig. 5. HRTEM photomicrographs showing (A) a cell with U precipitates on its surface, and (B) an enlarge view of a rectangular area of (A). Spearheads indicate some tiny U particles, and arrows point out aggregated U particles. (C) HRTEM lattice fringes and SAED pattern of precipitated U that has particle sizes of 2-3 nm in diameter. Arrows mark some interfaces between primary particles.

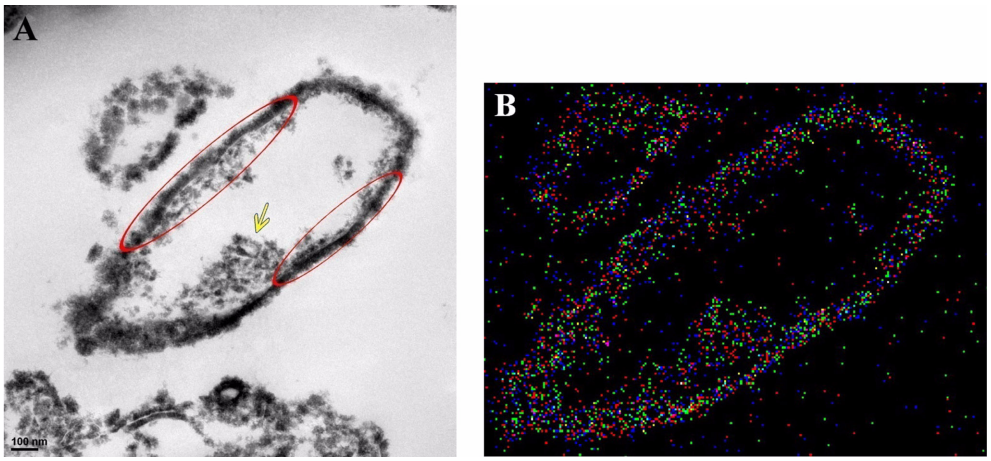

Fig. 6. (A) A HRTEM image of thin sectioned *S. putrefaciens* cells with U precipitates in and around the cell. The U nanoparticles (arrow) accumulated and (circles) arrayed as a form of flat-type are shown. (B) An electron elemental map of the (A) image shows a coprecipitation of U, Ca, and P components (Red dots: U, blue dots: Ca, green dots: P).

indicating enzymatic layers which are responsible for U(VI) reduction. Fig. 6B is an elemental map showing intra-cellular Ca and P coprecipitated with U in and around the cell.

4. Discussion

4.1. Influence of Iron Species on U(VI) Bioreduction

In a presence of ferrous iron, U(VI) concentration rapidly decreased by microbial reducing process (see Fig. 2), showing a positive effect of Fe(II) ions on U(VI) bioremoval. Liger et al. (1999) studied a reduction of U(VI) by ferrous iron and found no indication of homogeneous reduction of U(VI) by the ferrous irons. In our microbial experiment, however, biogenic U(VI) reduction was propagated by the help of aqueous ferrous iron. In that case, it is possible to predict that Fe(II) ions can transfer electrons to U particles by attaching onto them. This process can be similar to the heterogeneous U(VI) reduction occurring on the surface of Fe-bearing mineral (Ilton et al., 2004; Lee et al., 2009). Besides, Fe(II) ions could be attracted onto bacterial surfaces (Urrutia et al., 1998; Liu et al., 2001). Some attached Fe(II) ions on the surface could make a favorable complex with U(VI) on the cell. By contrast, when Fe(III) coexisted with U(VI), it may play a negative role on the U(VI) bioreduction, limiting a reducing chance of U(VI) (see Fig. 2).

In most natural settings, iron (hydr)oxides generally predominate when O_2 infiltrates groundwater (Nealson and Saffarini, 1994; Wielinga et al., 2000). However, groundwaters frequently have considerable soluble iron and, moreover, iron-bearing minerals/rocks constantly release ferrous or ferric irons during the weathering process. The released iron phases could exist as near-soluble or very amorphous forms initially for a while in subsurface environments, and it will take time for them to be changed to a stable solid phase. If a precipitation of iron does not rapidly occur in natural environments, microbe could use the suspending Fe(II) or Fe(III) phases in groundwater.

Previously, most researchers have conducted microbial U(VI) reduction together with solid-type iron-(hydr)oxide phases (e.g., ferrihydrite and goethite). They reported that microbial reduction of Fe(III) and U(VI) has occurred simultaneously (Truex et al., 1997; Fredrickson et al., 2000; Istok et al., 2004; Komlos et al., 2008). In our experiment, however, as the Fe(III) phase existed in a very amorphous form together with aqueous U(VI), there did not occur a simultaneous reduction, but a preferred Fe(III) reduction. This means that a solubility of iron(III) phase is likely to function as an important factor for U(VI) bioreduction. In this case, the suspending Fe(III) phase can play a strong competitive inhibitor to U(VI) during the microbial U(VI) bioreduction.

If oxidized irons existed as a colloidal form, a direct interaction between iron and bacteria would be very facilitated. It is amenable for the Fe(III) phase to be electrostatically attracted onto cells, overcoming a physiological problem arising from precipitable crystallized Fe(III)-oxides. Ferric iron is known to be bound tenaciously to bacterial surfaces (Warren and Ferris, 1998). This strong binding could lead the ferric iron to be a preferential terminal electron acceptor over U(VI).

4.2. U Precipitation and Solidification

The bioreduced U product resulting from the microbial activity for aqueous U(VI) was examined by HRTEM. The newly-formed U particles were less than 3 nm in diameter and appeared as aggregated forms. Initial U precipitates were gradually aggregated and solidified to larger particles. It has been known that biogenic uraninite is localized in a periplasmic space (Payne et al., 2004; Wall and Krumholz, 2006). The distribution of U particles may distinctively represent characteristic sites of periplasmic cytochromes of the CN32 bacterium. Locations of arrayed cytochromes can be found at flat-type precipitates of U (see Fig. 6A).

Geobiological studies have shown that bacteria play an important role in subsurface mineralization, especially through the interactions of cell wall surfaces with dissolved aqueous ions (Beveridge, 1989; Konhauser, 1998). In our experiment, some background cations such as Ca and P were coprecipitated with bioreduced U particles (see Fig. 6). This type of microbial coprecipitation may occur when there is an affinity between exposed functional groups of the cell surfaces such as carboxyl, hydroxyl, and phosphoryl groups with specific ions in the external fluid (Fein et al., 1997; Fowle and Fein, 2000; Kawano and Tomita, 2001). Complexation of cations by these surface functional groups is interpreted to be the mechanism underlying the sorptive properties of bacteria while the surficially sorbed cations are thought to furnish the sites for subsequent nucleation and precipitation of minerals (Walker et al., 1989; Warren and Ferris, 1998). Nominally uraninite always contains cation impurities, e.g. Ca, Pb, REE (Janeczek and Ewing, 1992). Natural stable uraninite has been frequently found that contains ~11wt %

CaO (Burns, 1999). Uraninites formed in the field are, thus, likely to contain structural impurities which probably increase the stability of the uraninite. In our microbial system, the coprecipitation of 3 components (e.g., U, Ca, and P) suggests that it is possible for U to complex with those impurities (e.g., Ca and P) in geomicrobial conditions. Importantly, this process may make U precipitates to be more stable and insoluble in natural environments (Lee et al., 2010).

5. Conclusion

A study for U(VI) reduction by *S. putrefaciens*, an iron-reducing bacterium, was conducted in a specific laboratory condition, where U(VI) was interacted with iron species. By the microbial U(VI) reduction, initial concentration (50 μM) of U(VI) continued to decrease to a non-detectable level. However, as amorphous Fe(III) phase coexisted with dissolved U(VI), the U(VI) reduction and removal were suppressed, while a microbial Fe(III) reduction was much more facilitated. On the other hand, when iron was present as Fe(II) ions, the U(VI) was easily bioreduced and removed from the solution with large enhancement. In the microbial system, some dissolved cations were coprecipitated with U in and around the cells. This shows that aqueous U may be chemically stabilized by complexing with Ca or P ions, precipitating around the cell.

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