FINAL REPORT

Phase II - Biogeochemistry of Uranium Under Reducing and Re-oxidizing Conditions: 
An Integrated Laboratory and Field Study ($20,575 extension) 
Grant # DE-FG02-05ER64030

Submitted to the DOE NABIR Program 
Biogeochemical Dynamics Research Element

by

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1. Executive Summary

Our understanding of subsurface microbiology is hindered by the inaccessibility of this environment, particularly when the hydrogeologic medium is contaminated with toxic substances. Past research in our labs indicated that the composition of the growth medium (e.g., bicarbonate complexation of U(VI)) and the underlying mineral phase (e.g., hematite) significantly affects the rate and extent of U(VI) reduction and immobilization through a variety of effects. Our research was aimed at elucidating those effects to a much greater extent, while exploring the potential for U(IV) reoxidation and subsequent re-mobilization, which also appears to depend on the mineral phases present in the system. The project reported on here was an extension ($20,575) of the prior (much larger) project. This report is focused only on the work completed during the extension period. Further information on the larger impacts of our research, including 28 publications, can be found in the final report for the following projects:

1) Biogeochemistry of Uranium Under Reducing and Re-oxidizing Conditions: An Integrated Laboratory and Field Study Grant # DE-FG03-01ER63270, and

2) Acceptable Endpoints for Metals and Radionuclides: Quantifying the Stability of Uranium and Lead Immobilized Under Sulfate Reducing Conditions Grant # DE-FG03-98ER62630/A001

In this Phase II project, the toxic effects of uranium(VI) were studied using *Desulfovibrio desulfuricans* G20 in a medium containing bicarbonate or 1, 4-piperazinediethane sulfonic acid disodium salt monohydrate (PIPES) buffer (each at 30 mM, pH 7). The toxicity of uranium(VI) was dependent on the medium buffer and was observed in terms of longer lag times and in some cases, no measurable growth. The minimum inhibiting concentration (MIC) was 140 µM U(VI) in PIPES buffered medium. This is 36 times lower than previously reported for *D. desulfuricans*. These results suggest that U(VI) toxicity and the detoxification mechanisms of G20 depend greatly on the chemical forms of U(VI) present and the buffer present in a system.

Phase II of this project was supported at a cost of $20,575 with most funds expended to support Rajesh Sani salary and benefits. Results have been published in a peer reviewed journal article. The abstract and citations is given below.
2. Publications (Published)

2.1 Publication - Toxic effects of uranium on *Desulfovibrio desulfuricans* G20


2.1.2. Abstract: The toxic effects of uranium(VI) were studied using *Desulfovibrio desulfuricans* G20 in a medium containing bicarbonate or 1, 4-piperazinediethane sulfonic acid disodium salt monohydrate (PIPES) buffer (each at 30 mM, pH 7). Uranium(VI) toxicity was dependent on the medium buffer and was observed in terms of longer lag times and in some cases, no measurable growth. The minimum inhibiting concentration (MIC) was 140 µM U(VI) in PIPES buffered medium. This is 36 times lower than previously reported for *D. desulfuricans*. In all cases in which G20 grew in the presence of U(VI), the final cell protein yield was equivalent to that of the U(VI)-free control. In 24 h, *D. desulfuricans* G20 (40 mg/L total cell protein) removed 50 µM U(VI) from solution in PIPES buffer as compared to 96 µM U(VI) in bicarbonate buffer under anaerobic, nongrowth conditions. Even though the solubility of U(VI) was significantly lower in PIPES buffer than in bicarbonate buffer, U(VI) was much more toxic in PIPES buffer than in bicarbonate buffer. Analysis of thin sections of G20 treated with 90 µM U(VI) in medium containing PIPES buffer revealed that only a very small fraction of cells had reduced uranium (U) precipitates in the periplasmic spaces. In the presence of bicarbonate buffer, however, reduced U was observed not only in the periplasm, but also in the cytoplasm. Selected area electron diffraction patterns and crystallographic analysis of transmission electron microscope lattice fringe images confirmed the structure of precipitated U in the cell periplasm and cytoplasm as being that of uraninite. These results suggest that U(VI) toxicity and the detoxification mechanisms of G20 depend greatly on the chemical forms of U(VI) present.
3. Presentations – National Meetings


TOXIC EFFECTS OF URANIUM ON DESULFOVIBRIO DESULFURICANS G20

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Abstract—The toxic effects of U(VI) were studied using Desulfovibrio desulfuricans G20 in a medium containing bicarbonate or 1,4-piperazinediethane sulfonic acid disodium salt monohydrate (PIPES) buffer (each at 30 mM and pH 7). Uranium(VI) toxicity was dependent on the medium buffer and was observed in terms of longer lag times and, in some cases, no measurable growth. The minimum inhibiting concentration was 140 μM U(VI) in PIPES-buffered medium. This is 36-fold lower than that reported previously for D. desulfuricans. For all cases in which D. desulfuricans G20 grew in the presence of U(VI), the cell protein yield was equivalent to that of the U(VI)-free control. In 24 h, D. desulfuricans G20 (total cell protein, 40 mg/L) removed 50 μM U(VI) from solution in PIPES buffer, as compared to 96 μM U(VI) in bicarbonate buffer under anoxic, nongrowth conditions. Even though the solubility of U(VI) was significantly lower in PIPES buffer than in bicarbonate buffer, U(VI) was much more toxic in PIPES buffer than in bicarbonate buffer. Analysis of thin sections of D. desulfuricans G20 treated with 90 μM U(VI) in medium containing PIPES buffer revealed that only a very small fraction of cells had reduced U precipitates in the periplasmic spaces. In the presence of bicarbonate buffer, however, reduced U was observed not only in the periplasm but also in the cytoplasm. Selected-area electron diffraction patterns and crystallographic analysis of transmission-electron microscopic lattice fringe images confirmed the structure of precipitated U in the cell periplasm and cytoplasm as being that of uraninite. These results suggest that U(VI) toxicity and the detoxification mechanisms of D. desulfuricans G20 depend greatly on the chemical forms of U(VI) that are present.

Keywords—Bioavailability Heavy metal High-resolution transmission-electron microscope Lag time Selected-area electron diffraction

INTRODUCTION

Many activities associated with the mining, extraction, and processing of U for nuclear fuel and weapons, as well as with the processing of spent fuel, have generated substantial quantities of waste materials contaminated with U and other radionuclides. In many cases, past practices relating to the handling and storage of such waste materials have resulted in extensive subsurface contamination. Uranium is present in soils, sediments, and groundwater at U.S. Department of Energy sites [1–3]; in addition, a recent report from the United Nations Environment Programme has indicated that depleted U, from ammunition involved in military activities in 1995, has contaminated groundwater and soils in Bosnia (the full report is available at http://postconflict.unep.ch/publications/BiLDU_report.pdf). Such weapons also were used in Iraq, where an estimated 820 tons of depleted U were used in 1991. It has been stated by Craft et al. [4] that depleted U can be toxic to many human body systems. Most importantly, normal functioning of the kidney, brain, liver, and heart can be negatively affected by depleted U exposure (see [4] and references therein). Besides the detrimental effects of U to human health, U toxicity also has been reported for other organisms and microorganisms [5–7].

The hexavalent form of U is highly soluble and, thus, is mobile in groundwater, potentially reaching sensitive receptors (e.g., drinking-water supplies). The fate and transport of U in groundwater may depend significantly on the activity of subsurface bacteria. Dissimilatory metal-reducing bacteria (DMRB) can decrease the solubility of U via enzymatic reduction, which may stop or slow the movement of this radionuclide. One group of DMRB, known as sulfate-reducing bacteria (SRB), is present in many contaminated subsurface sites [8–11]. Stimulating the growth of SRB has potential remedial value for U- and heavy metal–contaminated aquifers. One of the key features of SRB over other groups of DMRB, such as iron-reducing bacteria and fermentative bacteria, is that SRB can decrease the solubility of other, less redox-active metals, such as Cd(II), Cu(II), Hg(II), Ni(II), Pb(II), and Zn(II), using sulfide (the end product of sulfate reduction). Sulfate-reducing bacteria also can decrease the solubility of redox-active metals, such as Cr(VI), Te(VII), and U(VI), using enzymatic mechanisms [1,12–14].

Whereas SRB can catalyze a variety of heavy metal transformations, it has been demonstrated that toxic levels of heavy metals may inhibit or prevent bacterial growth [14–17]. The efficient management of bacterial processes in ex situ–engineered treatments, or effective manipulation of indigenous bacterial communities to stimulate in situ activity in the presence of toxic heavy metals, requires knowledge regarding the toxic effects of various heavy metals on bacteria. Thus, the response of SRB to U has important implications for understanding U in terms of reactivity, fate, and transport to human receptors. Previous reports concerning the toxicity of U(VI) to SRB [18–21] have used microbial media containing either phosphate or bicarbonate buffer. In these studies, because of the formation...
of uranyl phosphate precipitates or uranyl bicarbonate complexes, only high concentrations of U were reported to be toxic to SRB. To examine the toxic effects of U(VI) on a model organism, Desulfovibrio desulfuricans G20, metal toxicity medium (MTM) [15] was used with two buffer systems (bicarbonate and 1,4-piperazinediethane sulfonic acid disodium salt monohydrate [PIPES], each at 30 mM and pH 7). Metal toxicity medium was developed to minimize the abiotic precipitation of heavy metals in an SRB growth medium. The toxic effects of U(VI) in these two buffer systems were examined in terms of inhibition of total cell protein, decrease in U(VI) reduction rates, longer lag times, and in some cases, no measurable growth of D. desulfuricans G20. Additional objectives were to better understand the U(VI) detoxification mechanisms used by D. desulfuricans G20 and to characterize the transformation products of U in two buffer systems. These additional objectives were achieved using transmission-electron microscopy (TEM) and selected-area electron diffraction ring patterns.

MATERIALS AND METHODS

Bacteria and cultivation conditions

The D. desulfuricans G20 (referred to hereafter as G20) used in the present study was a gift of J. Wall (University of Missouri–Columbia, Columbia, MO, USA) and was derived from D. desulfuricans G100A [22]. The G20 was maintained in MTM [15]. Medium components were of analytical grade and purchased from Fisher Scientific (Pittsburgh, PA, USA) with the following exceptions: Yeast extract and tryptone were obtained from Difco Chemical Company (Detroit, MI, USA), and PIPES and sodium sulfate were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). Uranium was purchased as UO₂Cl₂·3H₂O from Bodman Industries (Aston, PA, USA). Water was supplied from a Barnstead/Nanopure system (Hayward, CA, USA) and had a nominal resistivity of 17.6 MΩ·cm. All glassware was washed with 2 N HNO₃.

Uranium toxicity experiments

Volumes of 100 ml of MTM with PIPES or bicarbonate buffer, each at 30 mM and pH 7 in 150-ml serum bottles, were autoclaved. To observe the effects of U(VI) on the redox potential (Eₒ) during the incubation of G20, filtered resazurin, which is colorless at 0.5 mg/L in pH 7 medium with an Eₒ of −100 mV or less [23], was added to the medium before autoclaving. A filtered (pore size, 0.2 μm; Gelman Acrodisc, San Diego, CA, USA) anaerobic stock solution (42 mM) of UO₂Cl₂·3H₂O was diluted with 0.2 μM from 1 M stock solution and with 100 μM UO₂Cl₂·3H₂O. The total volume of inoculated reaction mixture in each serum bottle was 10 ml. All bottles were incubated at room temperature (25°C) and 125 rpm and were sampled for U(VI). Each treatment was conducted in duplicate, with the initial sample taken anaerobically within 5 min after inoculation. Samples (0.2 ml) were taken using disposable syringes, which were purged with N₂ to avoid introducing O₂ into the serum bottles. In addition to cell- and lactate-free controls, heat-killed cell controls were included.

Analytical methods

Total cell protein and soluble U. Total cell protein in cultures was determined using a quantitative colorimetric Coomassie assay method (Pierce, Rockford, IL, USA) as described previously [24]. The absorbance of each solution was measured at 595 nm and compared to a standard curve generated for bovine serum albumin. Samples for U(VI) were filtered (pore size, 0.2 μm) unless otherwise mentioned, and concentrations were measured as described previously [25,26] with a kinetic phosphorescence analyzer-11 (KPA-11; Chemcheck Instruments, Richland, WA, USA). Calibration was done using UO₂Cl₂·3H₂O solutions of 0 to 160 nM. Because the KPA-11 allows detection of U(VI) concentrations as low as 0.04 nM with a precision of ±5%, the estimated detection limit in the present study (using 1,000-fold dilutions) was 40 nM.

Soluble sulfate and sulfide. Samples for sulfate were filtered (pore size, 0.2 μm), and concentrations were determined using a Dionex ion chromatograph (DX-500 equipped with conductivity detector-20 with an IonPac AS11-HC4-mm column and conductivity detection; Sunnyvale, CA, USA). Elution was carried out using a sodium hydroxide gradient (1–100 mM). The detection limit was 3 mg/L for each anion. The filtered (pore size, 0.2 μm) samples for soluble sulfide were diluted with zinc acetate solution (10% w/v), and sulfide concentrations were determined spectrophotometrically using the methylene blue method (Hach, Loveland, CO, USA) [27]. The absorbance was measured at 665 nm and compared to a standard curve generated for known concentrations of sodium sulfide. The detection limit for sulfide was estimated to be 3 nM. Sulfide in the headspace was not determined in these experiments.
Uranium toxicity to *Desulfovibrio desulfuricans* G20

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Fig. 1. (A) The effects of uranium(VI) concentration on the growth of *Desulfovibrio desulfuricans* G20 as measured by total cell protein and (B) soluble uranium(VI) concentrations in metal toxicity medium containing bicarbonate buffer (30 mM, pH 7). Symbols show the mean of duplicate analyses. Error bars indicate the standard deviation.

Fig. 2. (A) The effects of uranium(VI) concentration on the growth of *Desulfovibrio desulfuricans* G20 as measured by total cell protein and (B) soluble uranium(VI) concentrations in metal toxicity medium containing 1,4-piperazinediethane sulfonic acid disodium salt monohydrate buffer (30 mM, pH 7). Symbols show the mean of duplicate analyses. Error bars indicate the standard deviation.

Transmission-electron microscopy

Because of the O₂-sensitive nature of the samples, the entire embedding procedure as well as thin sectioning were conducted in an anaerobic glove box (Ar:H₂, 95:5; Coy Laboratory Products, Grass Lake, MI, USA). The precipitates, resulting from batch experiments of G20 with 90 μM U(VI) in MTM containing PIPES (incubation of 25 d) or bicarbonate buffer (incubation of 15 d), were washed in anoxic deionized water and fixed in 2.5% glutaraldehyde. This was followed by gradual dehydration in an ethanol series and infiltration in LR White embedding resin (Sigma, St. Louis, MO, USA). Samples embedded in solid resin blocks were sectioned (thickness, 70 nm) on a microtome (Leica Ultracut UCT; Leica Microsystems, Bannockburn, IL, USA), and sections were mounted on 200-mesh copper grids coated with formvar-support film sputtered with carbon. Sections were examined using a model 2010 high-resolution transmission-electron microscope equipped with a LaB₆ filament operating at 200 kV with resolution of 0.19 nm (JEOL, Peabody, MA, USA). Elemental analysis was performed using an Oxford energy dispersive spectrometry system equipped with a SiLi detector coupled to the transmission-electron microscope and analyzed with ISIS software (JEOL). Images were digitally collected and analyzed using a digital micrograph (Gatan, Pleasanton, CA, USA). The d-spacings obtained from the selected-area electron diffraction ring patterns were evaluated by Desktop Microscopist software (La cuna Beaverton, OR, USA).

Statistical analysis

Each set of experiments was carried out in duplicate and repeated three times. In each batch experiment, duplicate treatment profiles were similar in total cell protein, U(VI), sulfate, and sulfide concentrations; however, the length of the lag time was somewhat variable. Similar variability in lag times of *D. desulfuricans* and *Shewanella oneidensis* among different experiments has been observed for metals such as Cr, Cu, Ni, Pb, and Zn [14,17,28]. One-way analysis of variance was used to determine any statistically significant differences in G20 lag times among treatments with and without U(VI). The threshold level of statistical significance for the present study was *p* = 0.05.

RESULTS

Effects of U(VI) on growth of G20

The effects of U(VI) at 0 to 425 μM on the growth of G20, as measured by total cell protein in MTM containing bicarbonate buffer (30 mM, pH 7), are shown in Figure 1A. It can be seen that except for a small lag time with 425 μM U(VI), no significant inhibition in G20 growth was observed. All treatments with U(VI) attained the same final cell protein yields, equivalent to those of U(VI)-free treatments. Measured soluble U(VI) concentrations for the cultures are given in Figure 1B, which shows that U(VI) concentrations decreased sharply during the active growth of G20 and that no removal of U(VI) from solutions was observed in either the G20-free or heat-killed cell controls.

Figure 2A presents growth profiles of G20 in MTM containing PIPES buffer (30 mM, pH 7) at 0 to 220 μM U(VI). In contrast to the bicarbonate buffer system, these results show...
that U(VI) at low concentrations was toxic to G20 and that increases in U(VI) concentration caused longer lag times and, in some cases, no measurable growth. Using analysis of variance \((p = 0.05)\), with 43 and 90 \(\mu\)M soluble U(VI), the lag times of G20 increased to 9 and 15 d, respectively \((p = 0.0001)\), as compared to a lag time of 1 d for both 0 and 11 \(\mu\)M U(VI). Lag times correlated with observations of the color change of resazurin. The U(VI)-free serum bottles became colorless from blue via pink within 2 h, indicating an oxidation–reduction potential \(E_{\text{ox}}\) of \(-100\) mV or less [23], whereas with U(VI), the medium became colorless in between 6 and 10 h at all concentrations tested.

Measured aqueous U(VI) concentrations for the PIPES-buffered cultures are given in Figure 2B, which shows that U(VI) concentrations decreased slightly for all cultures for approximately 5 d and then remained nearly constant until growth began. At 43 and 90 \(\mu\)M U(VI), after the onset of growth (9 and 15 d, respectively), a rapid removal of U(VI) from solutions was observed. However, with initial U(VI) concentrations of 140 \(\mu\)M or greater, no significant U(VI) removal was observed. A slight decrease in soluble U(VI) concentration in the G20-free controls might have resulted from the adsorption of U(VI) to the glass serum bottles. It has been reported that the sorption of heavy metals to serum bottles may reach 4 to 6% in the pH range of 6 to 7.5 [6,29]. As noted by Francis et al. [30], other hydrolysis and condensation reactions among U(VI) and medium components also may have been involved.

**Effect of U(VI) on sulfate reduction**

Sulfate concentrations in cultures containing bicarbonate or PIPES buffer decreased rapidly during active growth, as shown in Figure 3. In MTM containing PIPES buffer, 43 and 90 \(\mu\)M U(VI) increased the lag time of G20, and after the active growth phase, sulfate concentrations were similar to those of U(VI)-free controls (Fig. 3). In contrast, it can be seen that in the presence of bicarbonate buffer, U(VI) up to 425 \(\mu\)M had little effect on sulfate reduction or lag time. In addition, no decrease in sulfate concentration was observed in heat-killed G20, G20-free controls, or PIPES-buffered treatments with U(VI) at 140 \(\mu\)M or greater (data not shown).

**Effects of PIPES or bicarbonate buffer on U(VI) reduction by G20**

Taken together, Figures 1 through 3 suggest that U(VI) toxicity to G20 under growth conditions was greatly influenced by the buffer present in the growth medium. We therefore hypothesized that removal of U(VI) from solutions by washed G20 under nongrowth conditions also would depend on the buffer present in the reaction solution. To test this hypothesis, G20 cells were grown in MTM containing bicarbonate or PIPES buffer (each at 30 mM and pH 7). Washed G20 cells (total cell protein, 40 mg/L) were suspended in bicarbonate or PIPES buffer (each at 30 mM and pH 7) with 100 \(\mu\)M uranium(VI) and appropriate controls. It can be seen from Figure 4 that after 24 h, G20 removed 95 and 50% of U(VI) from solutions in reaction solutions containing bicarbonate and PIPES buffers, respectively. After a very small initial decrease, removal of U(VI) from solutions was not observed in the G20-free, lactate-free, and heat-killed controls. These results further indicated that even under nongrowth conditions, the rate of removal of U(VI) from solutions by G20 was influenced significantly by the buffer present in the reaction solutions.

The toxicity of a metal to a microorganism depends on its solubility, speciation, and chemical properties [31–33]. In the present study, U(VI) solubility in different buffer systems was quantified using membrane filters of various pore size. In the presence of PIPES buffer alone or in MTM containing PIPES buffer, more than 65% of the U(VI) could not pass through either 1-kDa (pore size, 0.6 nm) or 5-kDa (pore size, 3 nm) cutoff membrane filters (Fig. 5). However, in bicarbonate buffer alone or MTM containing bicarbonate buffer, more than 95% of the U(VI) passed through the 1- and 5-kDa cutoff membrane filters. Figure 5 also shows that the addition of 1 mM lactate to the PIPES buffer resulted in an increase in U(VI)
solubility. Taken together, these results suggest that as expected, U(VI) had a greater solubility in the bicarbonate buffer than in the PIPES buffer system. Thermodynamic speciation calculations using MINTEQA2 (U.S. Environmental Protection Agency, Cincinnati, OH) [34] showed that MTM containing PIPES buffer had a number of uranyl species, such as UO₂(OH)⁺, UO₂(OH)₂, UO₂H₂O, and uranyl lactate complexes. In bicarbonate buffer alone or in MTM containing bicarbonate buffer, U(VI) was mostly complexed as UO₂(OH)₂ and UO₂(CO₃)₂⁺. Because of a lack of suitable parameters, however, these calculations did not include the possible effects of U(VI) complexion with tryptone and yeast extract present in MTM.

TEM, energy-dispersive x-ray spectroscopy, and selected-area electron diffraction pattern

Figure 6 shows TEM images of unstained thin sections of G20 treated with 90 μM U(VI) in MTM containing bicarbonate buffer. After detailed analysis of thin sections, 80% of the cells had U precipitates associated with them, and of these cells, 30% had U precipitates in the cytoplasmic spaces (Fig. 6C and D). Selected-area electron diffraction pattern and crystallographic analysis of TEM lattice-fringe images (d-spacings of 0.164, 0.193, 0.273, and 0.316 nm, consistent with UO₂; Joint Committee for Powder Diffraction Studies 41-1442) confirmed the structure of precipitated U in the cytoplasm as being that of uraninite. These d-spacings were consistent with those obtained previously for U reduced by D. desulfuricans and Geobacter metallireducens [18,35]. High-resolution TEM images also showed that individual particle diameters of U precipitates that formed in the cytoplasm were in the range of 3 to 5 nm and occurred as discrete and aggregated particles. Our results corroborate those of Suzuki et al. [11,36], who also observed nanometer-sized particles of UO₂ resulting from bacterial U(VI) reduction.

In contrast to the bicarbonate buffer systems, the results obtained from TEM images of unstained thin sections of G20 culture treated with 90 μM U(VI) in MTM containing PIPES buffer differed in at least two aspects (Fig. 7). First, 50% of the cells had no reduced U associated with them (Fig. 7A). Second, in sharp contrast to the bicarbonate-buffered system, no cells were observed that had U precipitates in the cytoplasmic spaces.

DISCUSSION

Uranium toxicity

It has been shown that the toxicity of a metal depends on solubility, speciation, and chemical properties as well as on geochemical factors (e.g., complexation, pH, and precipitation) [31,33,37]. Toxicity in bacteria is believed to result from displacement and/or substitution of essential ions from cellular sites and from blocking functional groups of important biochemical molecules, such as enzymes, polynucleotides, and essential nutrient transport systems [38,39]. This can cause denaturation and inactivation of enzymes as well as disruption of cell organelle–membrane integrity [40].

For U in particular, it has been shown under laboratory
conditions that certain DMRB, such as *G. metallireducens* and *Shewanella putrefaciens* [41] as well as *Desulfomaculum reducens* [42], can grow anaerobically on U(VI) as a terminal electron acceptor. Pietzsch et al. [21] reported that *Desulfovibrio* sp. strain UFZ B490 also could grow anaerobically on U(VI) as a terminal electron acceptor; however, further detailed study revealed that *Desulfovibrio* sp. strain UFZ B490 reduced U(VI) but did not get sufficient energy for growth [20]. Furthermore, 1,000 and 425 were observed under growth [47] and nongrowth conditions. During the present study with 43 and 90 \( \mu M \) U(VI) in PIPES buffer, in contrast to bicarbonate buffer, the extent of sulfate removal before the onset of growth was very low and became negligible when the U(VI) concentration was increased to 140 \( \mu M \). This was likely caused by the decrease in G20 metabolic activity as a result of U(VI) toxicity to G20.

It is conventionally believed that the toxicity of a given metal greatly depends on its solubility. However, in the present study, highly soluble U(VI) as uranium carbonate complexes (Fig. 5) showed no toxicity to G20, and less soluble U(VI) complexes in PIPES buffer (Fig. 5) exerted severe toxicity to G20. These results prompt significant consideration of the metal complexes involved in metal toxicity to microorganisms. It also has been suggested that to have a physiological or toxic effect, most heavy metals have to enter the cell [38]. However, in the present study, TEM results showed that in a bicarbonate buffer, more U entered the cells compared to the level in a PIPES-buffered system, but that the uranium carbonate complexes were less toxic to G20. In general, however, the mechanisms of U(VI) toxicity and inhibition in microbiological systems, especially with SRB, are not understood and deserve further study.

### Uranium detoxification

In previous reports [1,51], the enzymatic reduction of U(VI) to U(IV) has been suggested as a primary microbial detoxification mechanism. In the present study with PIPES buffer, in addition to extracellular U precipitates (as described by Lovley and Phillips [18]), G20 also had reduced U precipitates in the periplasmic spaces. This likely is because U is soluble in its oxidized state, U(VI), and is available to react with electron-transfer proteins localized in the periplasm. Uranium(VI), after accepting electrons, precipitates as uraninite in the periplasmic space because of its low solubility. This distribution of biogenic uraninite is consistent with the current understanding of electron-transfer mechanisms in metal-reducing bacteria (i.e., the uranium reductase activity is associated with the periplasmic space) [52,53].

In contrast to the PIPES buffer system, in bicarbonate buffer, reduced U precipitate was observed not only in the periplasm but also in the cytoplasm. These results may indicate the U that precipitated in the periplasm may have entered the cytoplasm, or in addition to metal reductase activity in the periplasm, G20 might have U(VI)-reducing activity in the cytoplasm (Fig. 8). It does not seem likely that reduced, solid-

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### Table 1. Toxicity of uranium(VI) to sulfate-reducing bacteria reported in literature

<table>
<thead>
<tr>
<th>Phosphate buffer (mM)</th>
<th>Bicarbonate buffer (mM)</th>
<th>NOEC(^a) of uranium(VI) (( \mu M ))</th>
<th>MIC(^b) of uranium(VI) (( \mu M ))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>11</td>
<td>140</td>
<td>Present study</td>
</tr>
<tr>
<td>2.2</td>
<td>0</td>
<td>2,000</td>
<td>5,000</td>
<td>[19]</td>
</tr>
<tr>
<td>1.5</td>
<td>30</td>
<td>5,000</td>
<td>—</td>
<td>[42]</td>
</tr>
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<td>3.7</td>
<td>0</td>
<td>1,000</td>
<td>—</td>
<td>[19]</td>
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<tr>
<td>2.9</td>
<td>0</td>
<td>1,000</td>
<td>—</td>
<td>[47]</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>5,000</td>
<td>—</td>
<td>[18]</td>
</tr>
</tbody>
</table>

\(^{a}\) NOEC = no-observed-effect concentration.

\(^{b}\) MIC = minimum inhibiting concentration.

\(^{c}\) — = not reported.
phase U could enter the cytoplasm, because the cytoplasmic membrane would act as a barrier. A more likely explanation is that the cytoplasm contains U(VI)-reducing components. It was suggested that electrons for U(VI) reduction are transferred solely by the periplasmic protein cytochrome $c_5$ [19]. When the corresponding gene in *D. desulfuricans* G20 was knocked out, more than 90% of U(VI) reduction from externally supplied H$_2$ was blocked. However, only a 50% decrease was found in the reduction of U(VI) with lactate or pyruvate as the electron donor [19]. This indicates that although cytochromes are involved in metal ion reduction, other unknown proteins likely play a significant role as well. In general, however, these U(VI) reduction mechanisms are not well understood, and further research is needed.

**CONCLUSION**

The results of the present study clearly show that at the concentrations tested in the presence of bicarbonate buffer, U(VI) toxicity to G20 was not observed. In the presence of PIPES buffer, however, U(VI) toxicity to G20 was demonstrated by longer lag times (e.g., 15 d for 90 μM), and in some cases, no measurable growth was observed (e.g., ≥140 μM U(VI)). Once growth began, however, cultures ultimately attained the same total cell protein concentration as that in the U(VI)-free control, indicating that toxic effects were temporary or nonpermanent. Transmission-electron microscopy provided interesting insights regarding U(VI) inhibition and detoxification mechanisms in G20 in bicarbonate and PIPES buffer systems. The results showed that in the presence of PIPES buffer, very few cells had reduced U associated with them (mainly in the periplasm). However, in the presence of bicarbonate buffer, the reduced U resided in the cell periplasm and cytoplasm. These results suggest that in addition to periplasmic metal-detoxification mechanisms, SRB also have other uncharacterized cytoplasmic metal-detoxification mechanisms.

For SRB with U(VI) in the absence of strong complexants, chelators, precipitants, and reductants, we observed a NOEC of 11 μM and a MIC of 140 μM. Comparison of our U(VI) toxicity results with literature values indicates that under certain conditions, U(VI) is much more toxic than previously thought, because earlier studies used media that contained significant amounts of metal-precipitating and/or -complexing agents (e.g., phosphate or bicarbonate). The results also indicate that MTM is a sensitive medium for measuring metal toxicity to SRB and should be considered further as a potential reference medium for evaluating natural and industrial waters. Whereas the use of MTM and a pure culture of G20 may overestimate U(VI) toxicity in natural environments where chemical complexants and other microorganisms are present, these results have fundamental relevance to U(VI)-contaminated systems and efforts at using microorganisms to remediate U(VI) contamination.

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