Reduction of Hexavalent Uranium from Organic Complexes by Sulfate- and Iron-Reducing Bacteria

RAJAGOPALAN GANESH,^{1,2†} KEVIN G. ROBINSON,^{1,2*} GREGORY D. REED,^{1,2}
AND GARY S. SAYLER^{2,3}

Department of Civil and Environmental Engineering,¹ Department of Microbiology,² and Center for Environmental Microbiology,³ The University of Tennessee, Knoxville, Tennessee 37996

Received 23 December 1996/Accepted 1 September 1997

The influence of organic-hexavalent-uranium [U(VI)] complexation on U(VI) reduction by a sulfate-reducing bacterium (*Desulfovibrio desulfuricans*) and an iron-reducing bacterium (*Shewanella alga*) was evaluated. Four aliphatic ligands (acetate, malonate, oxalate, and citrate) and an aromatic ligand (tiron [4,5-dihydroxy-1,3-benzene disulfonic acid]) were used to study complexed-uranium bioavailability. The trends in uranium reduction varied with the nature and the amount of U(VI)-organic complex formed and the type of bacteria present. *D. desulfuricans* rapidly reduced uranium from a monodentate aliphatic (acetate) complex. However, reduction from multidentate aliphatic complexes (malonate, oxalate, and citrate) was slower. A decrease in the amount of organic-U(VI) complex in solution significantly increased the rate of reduction. *S. alga* reduced uranium more rapidly from multidentate aliphatic complexes than from monodentate aliphatic complexes. The rate of reduction decreased with a decrease in the amount of multidentate complexes present. Uranium from an aromatic (tiron) complex was readily available for reduction by *D. desulfuricans*, while an insignificant level of U(VI) from the tiron complex was reduced by *S. alga*. These results indicate that selection of bacteria for rapid uranium reduction will depend on the organic composition of waste streams.

Microbially mediated precipitation has been proposed as a viable technique for selective uranium removal from aqueous solutions (32). Certain iron- and sulfate-reducing bacteria can enzymatically reduce highly soluble hexavalent uranium [U(VI)] to the sparingly soluble tetravalent form [U(IV)]. Reduced uranium then abiotically precipitates as uraninite (UO_{2(s)}) (23, 34). Attempts to develop this process for uranium removal from various waste streams are in progress (33, 41, 44). For example, groundwaters contaminated with uranium have been successfully treated by using this technique (23, 33). A few studies have evaluated the impact of inorganic ions found in wastewaters on uranium removal by this mechanism. In general, high concentrations of bicarbonate, sulfate, and nitrate ions appear to negatively impact enzymatic removal of uranium (41, 44). Phillips et al. (41) found that an increase in bicarbonate ion concentration from ~30 to 100 mM significantly decreased the rate of uranium reduction by microorganisms. Robinson et al. (44) noted that the presence of ~5,000 mg of nitrate or sulfate ions per liter did not appreciably affect uranium reduction; however, these ions at concentrations of 10,000 to 50,000 mg/liter decreased both the rate and extent of U(VI) removal from solution.

In addition to the inorganic ions evaluated, the presence of organic ligands may influence bioreduction of uranium in wastewaters. Waste streams from radionuclide-processing facilities generally contain several simple and complex organic ligands (16, 37). Organic compounds, through carboxyl, hydroxyl, or other functional-group interactions, spontaneously form coordinate-covalent bonds with metal cations, resulting in organic-metal complexes. Several studies relating the impact of organic-metal complexation to microbial degradation indi-

cate that biodegradation of organic ligands by heterotrophic bacteria and metal bioavailability to assimilatory metal-reducing bacteria are often affected by the presence of organic-metal complexes (14, 18, 45). For example, Firestone and Tiedje (15) studied the biodegradation of nitrilotriacetic acid (NTA) complexed to several metals by using a *Pseudomonas* species. The bacteria rapidly degraded NTA from Ca(II), Mg(II), Mn(II), and Na complexes; however, NTA chelated with Ni(II) was not degraded. In a separate study, *Pseudomonas fluorescens* rapidly degraded citrate from Ca(II), Ni(II), and Fe(III) complexes; however, degradation from an Fe(II) complexes was slower (28). Also, enzymes of *Azotobacter vinelandii* were found to reduce Fe(III) from citrate and azotochelin complexes at different rates during iron assimilation (27). In general, the types of complexes formed, their lability, and the functional group(s) involved in complexation have been proposed as parameters of organic-metal complexes affecting biodegradation (5, 17, 18).

Bioavailability of organic ligands and metals from metal-organic complexes appears to depend not only on the nature of the complexes formed but also on the type of bacteria present. Different *Pseudomonas* species degrade citrate from metal complexes at different rates (38). Two assimilatory metal-reducing bacteria, *P. aeruginosa* (13) and *A. vinelandii* (27), were found to reduce ferric iron from citrate complexes at different rates. The relationship between bacterial metabolism and the mechanism(s) of organic or metal utilization from complexes has not been well established. However, inhibition of the transport system which facilitates intracellular metabolism (4, 52) and structural incompatibility of the chelate with the active sites of the target enzymes (28) have been reported as reasons for the observed differences in the bioavailability of organic-metal complexes.

Several strains of metabolically diverse iron- and sulfate-reducing bacteria can reduce U(VI) in organic-free bicarbonate solutions (32, 34). It is not known if the presence of organic ligands can affect U(VI) reduction by these bacteria due to transport limitations, structural incompatibility, or other mech-

* Corresponding author. Mailing address: Department of Civil and Environmental Engineering, 219A Perkins Hall, University of Tennessee, Knoxville, TN 37996-2010. Phone: (423) 974 0722. Fax: (423) 974 2669. E-mail: kgr@utk.edu.

† Present address: Biopraxis, Inc., San Diego, CA 92191-0078.

TABLE 1. Organic ligands used to evaluate U(VI) biotransformation

Ligand	Nature of ligand	Functional group(s) involved in U(VI) complexation	Stability constant (log K)	Reference
Monodentate acetate	Aliphatic	Carboxyl	3.21	1
Bidentate				
Malonate	Aliphatic	Carboxyl	5.6	43
Oxalate	Aliphatic	Carboxyl	6.5	43
Bi- or polynuclear				
Citrate	Aliphatic	2 carboxyl, 1 hydroxyl	18.9	42
Tiron	Aromatic	Hydroxyl	55.0	25

anisms. Uranium in wastewater often is associated with several simple and complex organic compounds (16, 37). Any impact on the rate of U(VI) reduction due to organic complexation will affect treatment system design by this technique. Hence, it is essential to select bacteria which can most efficiently reduce U(VI) in waste streams containing a variety of organic compounds.

This research was performed to evaluate the availability of U(VI) for microbial reduction in solutions containing organic complexants. One aromatic and four aliphatic organic ligands were used to study the impact of metal-ligand binding on enzymatic U(VI) reduction by two metabolically diverse bacteria.

MATERIALS AND METHODS

Organisms and culture conditions. *Desulfovibrio desulfuricans* (ATCC 29577), a sulfate-reducing bacterium, and *Shewanella alga*, a primary iron-reducing bacterium (7), were used to evaluate U(VI) reduction in solution. Both of these organisms, under anaerobic conditions, can enzymatically reduce U(VI) to the tetravalent form in organic-free bicarbonate solutions with lactate or H₂ gas as the sole electron donor (7, 34). *D. desulfuricans* was grown in a well-defined medium (34) containing lactate as the primary carbon-electron source and sul-

fate as the electron acceptor. *S. alga* was grown in a medium containing lactate as the carbon-electron source and ferric citrate as the sole electron acceptor (7). Standard anaerobic techniques were used to prepare growth medium and for maintenance of culture conditions throughout this research (26). Briefly, the growth medium was heated to near boiling, purged with a high-purity gas mixture (5% H₂, 10% CO₂, 85% N₂) for 10 min to remove dissolved oxygen, anaerobically transferred in 60-ml volumes to 150-ml serum bottles, and autoclaved to ensure sterility prior to inoculation with cells (21, 26, 32).

Ligand concentration and uranyl ion speciation. Five organic ligands which form monodentate, bidentate, or bi- or polynuclear complexes with uranyl ion were selected for study (Table 1). Four of the ligands selected were aliphatics, which complexed with uranyl ion primarily through carboxyl ion interactions. One ligand, 4,5-dihydroxy-1,3-benzene disulfonic acid (tiron), was aromatic and complexed with uranium exclusively through hydroxyl ion interactions. Thermodynamic stability constants were obtained from the literature (39, 40, 49) and from an empirical relationship developed in our laboratory to approximate stability constants for selected UO₂(OH)-organic ligand species (21). These constants were used in association with the computer speciation program MINTEQA2 (2) in the selection of test ligand concentrations and prediction of uranyl ion speciation in each experiment. Two concentrations of each aliphatic ligand were chosen such that most (>98%) or one-half (~50%) of the U(VI) initially added directly complexed the target ligand. The remaining U(VI) formed organic [UO₂(OH)-organic ligand] and inorganic [(UO₂)₃(OH)₅] uranyl hydroxide species. In the aromatic-ligand (tiron) solutions, greater than 98% of the U(VI) initially formed ternuclear (UO₂)₃(OH)₂(tiron)₃ species at the higher tiron dosage. At the lower ligand concentration, ~50% of the U(VI) added formed ternuclear tiron complexes. The concentration of organic ligands and the percent distribution of various uranium species predicted by MINTEQA2 before microbial reduction are shown in Table 2.

Sample preparation. Batch experiments were performed under nongrowth conditions (7, 21, 34) to evaluate bioreduction of uranyl ion complexed to organic ligands. Test solutions contained H₂ gas as the sole electron donor, uranyl ion as the primary electron acceptor, each target ligand as the primary complexing agent, and sodium nitrate as the background electrolyte (ionic strength, 0.17 M). Initially, stock solutions (1 liter) were prepared by adding each test ligand and sodium nitrate to distilled, deionized water. The pH of the stock was then adjusted to 6.0 by using HNO₃ or NaOH (0.1 M). Subsequently, the solutions were filter sterilized (0.2-μm-pore-size filter), deoxygenated by purging with an anaerobic gas mixture (5% H₂, 10% CO₂, 85% N₂), and dispensed in 9-ml volumes into 25-ml anaerobic pressure tubes. Each tube was then closed with a butyl rubber stopper and sealed with an aluminum cap. A 0.5-ml deoxygenated uranyl nitrate solution [~2-g/liter U(VI) stock] was injected into each tube, resulting in an initial U(VI) concentration of ~100 mg/liter. Solutions were then mixed (100 rpm) at a constant temperature (32°C) for 48 h to allow equilibrium speciation to occur. H₂ gas was then added to the headspace of each pressure tube by injecting 20 ml of an 80% H₂-20% CO₂ gas mixture. The samples were then shaken gently but thoroughly by hand for ~1 min at room temperature to

TABLE 2. Ligand concentrations and distribution of various species, as predicted by MINTEQA2, before microbial inoculation in test solutions^a

Ligand and concn (M)	UO ₂ -organic ligand species	UO ₂ (OH)-organic ligand species	UO ₂ -inorganic species
Acetate			
0.17	UO ₂ (CH ₃ COO) ₃ ⁻¹ (91.2) ^b	UO ₂ (OH)CH ₃ COO (2.5)	
0.03	UO ₂ (CH ₃ COO) ₂ (5.8) UO ₂ (OH)CH ₃ COO ⁻¹ (23.1) UO ₂ (OH)CH ₃ COO ₂ (8.8) UO ₂ (OH)CH ₃ COO ⁺¹ (4.3)	UO ₂ (OH)CH ₃ COO (23)	UO ₂ OH ⁺ (1.6) (UO ₂) ₃ (OH) ₅ ⁻¹ (38)
Malonate			
0.01	UO ₂ (C ₃ H ₄ O ₄) ₂ (98)	UO ₂ (OH)(C ₃ H ₄ O ₄) (1.2)	
0.0075	UO ₂ (C ₃ H ₄ O ₄) ₂ (33.9) UO ₂ (C ₃ H ₄ O ₄) (5.5)	UO ₂ (OH)(C ₃ H ₄ O ₄) (9.3)	UO ₂ OH ⁺ (1.7) (UO ₂) ₂ (OH) ₂ (1.0) (UO ₂) ₃ (OH) ₅ ⁻¹ (48.4)
Oxalate			
0.01	UO ₂ (C ₂ H ₄ O ₄) ₃ (22.8) UO ₂ (C ₂ H ₄ O ₄) ₂ (75.2)	UO ₂ (OH)(C ₂ H ₄ O ₄) (1.6)	
0.00075	UO ₂ (C ₂ H ₄ O ₄) ₂ (30.7) UO ₂ (C ₂ H ₄ O ₄) (11.9)	UO ₂ (OH)(C ₂ H ₄ O ₄) (36.5)	UO ₂ OH ⁺ (1.2) (UO ₂) ₃ (OH) ₅ ⁻¹ (18.8)
Citrate			
0.005	[UO ₂ (C ₆ H ₈ O ₇) ₂] (100)		
0.0002	[UO ₂ (C ₆ H ₈ O ₇) ₂] (49.7)		(UO ₂) ₃ (OH) ₅ ⁻¹ (49.7)
Tiron			
0.0004		(UO ₂) ₃ (OH) ₂ tiron ₃ (100)	
0.0002		(UO ₂) ₃ (OH) ₂ tiron ₃ (50)	(UO ₂) ₃ (OH) ₅ ⁻¹ (49.7)

^a The initial pH was 6.0 in all test solutions.

^b Each value in parentheses is the percent distribution of that uranium species.

FIG. 1. U(VI) reduction in test solutions containing low and high concentrations (Conc.) of aliphatic organic ligands after inoculation with *D. desulfuricans*.

allow H_2 gas dissolution into each test solution. *D. desulfuricans* or *S. alga* cells grown in nutrient-rich culture medium were collected by centrifugation ($7,500 \times g$, 20 min, $4^\circ C$), washed twice with a uranium-free anaerobic solution (21), and inoculated into the test samples. Each pressure tube initially contained $\sim 5 \times 10^8$ cells/ml of test solution. All samples were then incubated at $32^\circ C$, and U(VI) biotransformation was evaluated over time.

Analytical techniques. Uranium levels in test solutions were measured with a kinetic phosphorescence analyzer. This instrument uses a pulsed nitrogen dye laser and a complexing reagent to measure only U(VI) levels in solution (6). Microbial reduction of complexed uranium was evaluated by monitoring the decrease in the U(VI) concentration over time in the unfiltered test solution after cell inoculation. Subsamples (~ 2 ml) from each pressure tube were collected and appropriately diluted with deoxygenated, deionized water, and the U(VI) level was analyzed under anaerobic conditions (21, 32). Internal standards for calibration were prepared such that the same amounts of organic ligand, sodium nitrate, and other sample constituents were present during uranium analysis (21). Acetate and citrate levels were measured by high-performance liquid chromatography with a Spherisorb C_8 column (21) and an LC90 UV detector set at a 210-nm wavelength. Malonate and oxalate were measured by ion-exchange chromatography with an Ion-Pak AS4A-SC anion-exchange column and an ED40 Dionex conductivity detector. Tiron was measured with a Beckman DU-70 spectrophotometer at a 300-nm wavelength (21).

Statistical analysis. Duplicate measurements of U(VI) concentrations in two tubes for each time period were used for analyses. The U(VI) reduction data were analyzed by using a one-way analysis of variance and a nonparametric Cox and Stuart test (12). The differences in U(VI) reduction rates were determined at a confidence level of 95%.

RESULTS AND DISCUSSION

U(VI) reduction by *D. desulfuricans*. Results indicated that *D. desulfuricans* reduced U(VI) from each of the aliphatic complexes evaluated (Fig. 1). In the higher-concentration acetate, malonate, and oxalate solutions [test ligands initially complexed $>98\%$ of the U(VI)], the bacteria reduced more than 95% of the U(VI) initially present after 96 h of incubation. In the higher-concentration citrate solution $\sim 85\%$ of the U(VI) was reduced after 96 h. The trend in uranium reduction for the higher-concentration citrate solution suggested that further reduction can be achieved by extending the incubation period. Significant amounts ($>90\%$) of uranium were also reduced in solutions containing the lower aliphatic ligand concentration [$\sim 50\%$ U(VI) initially complexed to the test ligands]. Results suggested that complex formation with aliphatic ligands did not appear to affect the availability of U(VI) as an electron acceptor to the sulfate-reducing bacteria.

TABLE 3. V_i of U(VI) by *D. desulfuricans* in aliphatic solutions

Ligand (concn [M] used)	V_i [mg of U(VI)/mg cell-h]
Acetate (0.17)	1.6×10^{-3}
Acetate (0.003)	1.9×10^{-3}
Malonate (0.01)	9.0×10^{-5}
Malonate (0.00075)	3.4×10^{-4}
Oxalate (0.01)	8.9×10^{-5}
Oxalate (0.00075)	1.8×10^{-4}
Citrate (0.005)	6.0×10^{-5}
Citrate (0.0002)	1.4×10^{-4}

Although a substantial amount of U(VI) was reduced in each test solution, the rate of reduction varied with the type and relative amount of U(VI)-organic complex formed. The differences observed in U(VI) reduction trends were statistically significant ($\alpha = 0.05$). The U(VI) reduction rate kinetic coefficients in the presence of each test ligand were evaluated by using a linearized form of the Michaelis-Menten expression (3, 24, 50). The initial rate of reduction (V_i), which represents the U(VI) reduction rate immediately after cell inoculation, was used to compare the impact of solution constituents (organic complexants) on U(VI) biotransformation (Table 3). The V_i values were normalized to the amount of U(VI) (milligrams) utilized by a unit of cell mass (milligram-cell) by using a conversion of 10^7 cells/mg of biomass (48). It was observed that in solutions containing the higher concentration of aliphatic ligand, the V_i of U(VI) from acetate complexes was ~ 20 -fold higher than that observed in the presence of multidentate aliphatic ligands. This indicated that U(VI) was not as readily available as an electron acceptor from multidentate aliphatic complexes as it was from monodentate (acetate) complexes for *D. desulfuricans*. When the concentrations of multidentate aliphatic ligands were reduced such that $\sim 50\%$ of the U(VI) formed only multidentate complexes, the V_i increased two- to fivefold.

Rapid reduction of U(VI) from selected organic complexes was not due to degradation of these ligands by *D. desulfuricans*. No significant decrease ($\alpha = 0.05$) in organic-ligand concentration was measured, even after 96 h of incubation, in U(VI)-containing solutions (Fig. 2). Note that the bacterium reduced

FIG. 2. Concentrations (Conc.) of organic ligands during U(VI) bioreduction by *D. desulfuricans*. Ligands initially complexed $>98\%$ of the U(VI).

FIG. 3. U(VI) reduction in test solutions containing low and high concentrations (Conc.) of an aromatic organic ligand (tiron) after inoculation with *D. desulfuricans*.

>85% of the U(VI) during this period. The very high concentration of acetate (11,500 mg/liter) utilized in the experiment rendered this ligand analysis difficult. However, evidence in the literature indicates that *D. desulfuricans* does not use acetate as a carbon or electron source (11, 31).

Slower reduction of U(VI) in the presence of multidentate aliphatic ligands was not due to toxicity of these organics to the bacterium. This was verified in a separate experiment in which the bacterium, under resting-cell conditions, readily reduced a poorly complexing compound (thiosulfate) in the presence of each test ligand (21). The results suggested that complexation by multidentate aliphatic ligands was responsible for the slower U(VI) reduction by *D. desulfuricans*, and the extent of impact depended on the amount of U(VI) organically complexed in solution. This impact, however, appeared to differ from that observed during Fe(III) reduction by *D. desulfuricans*. Coleman et al. (10) observed that multidentate aliphatic complexation by NTA did not appreciably affect the trend in Fe(III) reduction by *D. desulfuricans*. The bacteria reduced equal amounts (~ 3.5 mM) of Fe(III) from poorly crystalline Fe(III)oxide and Fe(III)-NTA complexes after 36 h of incubation. Further investigation is required to better understand the mechanism(s) of metal utilization from aliphatic complexes by the sulfate-reducing bacteria.

The impact of aromatic complexation on U(VI) bioreduction was evaluated by using tiron as the test ligand. Figure 3 shows U(VI) levels in tiron solutions after inoculation with *D. desulfuricans*. The bacteria reduced significant levels of U(VI) (>95%) in solutions containing high, as well as low, concentrations of tiron. Although U(VI) from multidentate aliphatic complexes was not readily available for reduction, *D. desulfuricans* rapidly reduced U(VI) from the multinuclear aromatic complex evaluated. In solutions in which >98% of the U(VI) initially formed ternuclear complexes with tiron, the V_i of U(VI) [5.7×10^{-4} mg of U(VI)/mg cell-h] was approximately 50% lower than that observed in acetate solutions but was more than an order of magnitude higher than that in the presence of multidentate aliphatic ligands. A similar impact on the relative rates of U(VI) reduction was also observed in solutions containing lower concentrations of tiron. The V_i of

FIG. 4. U(VI) reduction in test solutions containing low and high concentrations (Conc.) of aliphatic organic ligands after inoculation with *S. alga*.

U(VI) [4.6×10^{-4} mg of U(VI)/mg cell-h] was slightly lower than that observed in solutions containing the lower concentration of acetate but was 1.5- to 3-fold higher than that observed at lower concentrations of multidentate ligands. This suggested that the negative impact on U(VI) reduction due to complexation with the aromatic ligand (tiron) was much less than that observed with multidentate aliphatic ligands.

U(VI) reduction by *S. alga*. Figure 4 shows U(VI) levels in unfiltered aliphatic test solutions after inoculation with *S. alga*. The trends in uranium reduction were very different from those observed when *D. desulfuricans* was used. The extent of U(VI) reduction by *S. alga* in several aliphatic solutions was much lower than that by *D. desulfuricans* observed. When >98% of U(VI) was initially complexed to acetate, approximately 80% of the added U(VI) was reduced after 96 h of incubation. In solutions containing multidentate aliphatics, more than 95% of the U(VI) was reduced during this period. However, in solutions containing lower concentrations of aliphatic ligands, the bacteria reduced only 50 to 80% of the U(VI) initially introduced.

The V_i in test solutions varied with the type and level of U(VI)-aliphatic complexes present (Table 4). When >98% of the U(VI) was complexed to the test organics, the V_i of U(VI) in the presence of multidentate ligands was more than an order of magnitude higher than that observed in monodentate aliphatic (acetate) solutions. The slower reduction from acetate solutions does not appear to be due to toxicity of this ligand,

TABLE 4. V_i of U(VI) by *S. alga* in aliphatic solutions

Ligand (concn [M] used)	V_i [mg U(VI)/mg cell-h]
Acetate (0.17)	2.0×10^{-5}
Acetate (0.003)	1.5×10^{-5}
Malonate (0.01)	1.1×10^{-3}
Malonate (0.00075)	5.7×10^{-5}
Oxalate (0.01)	1.25×10^{-3}
Oxalate (0.00075)	5.2×10^{-5}
Citrate (0.005)	1.3×10^{-3}
Citrate (0.0002)	1.1×10^{-5}

FIG. 5. U(VI) reduction in test solutions containing low and high concentrations (Conc.) of an aromatic organic ligand (tiron) after inoculation with *S. alga*.

since this bacterium was isolated by using a medium containing a high concentration of acetate (7). Furthermore, differences in U(VI) reduction were not due to metabolism of the test ligands by this bacterium. Insignificant amounts of each ligand were utilized by this bacterium during U(VI) reduction (data not shown). Also, *S. alga* does not couple oxidation of acetate and citrate to metal reduction, even under growth conditions (7, 30). It should be noted that, when multidentate ligand levels were lowered such that only ~50% of the U(VI) initially added was complexed, the rate of reduction decreased by more than an order of magnitude (Table 4). This observation suggested that if *S. alga* degraded the test ligands (thereby decreasing their concentration), U(VI) reduction rates would have been retarded rather than enhanced. Therefore, it appears that chemical speciation of uranyl ion in solution rather than inhibition-degradation of organic ligands dictates U(VI) availability to *S. alga*. It has been well documented that the activity of selected yeast cells and *Thiobacillus ferrooxidans* was lower in the presence of uncomplexed uranyl ions (UO_2^{2+}) but was not affected when uranyl ions were complexed to strong complexants such as carbonate or citrate (46–48, 51). In addition, it has been reported that iron-reducing microorganisms from anaerobic sediments reduced Fe(III) from multidentate aliphatic (NTA) complexes more rapidly than from uncomplexed, poorly crystalline Fe(III) oxide (29, 36).

S. alga did not reduce U(VI) from aromatic ternuclear tiron complexes (Fig. 5). Less than 5% of the U(VI) present was reduced in solutions in which the predominant U(VI) species was in the tiron complex (>98%), as well as in solutions containing only ~50% tiron-U(VI) complexes. The reduction trends observed in various experiments indicated that multidentate aliphatic complexes were better sources of the U(VI) electron acceptor for *S. alga* than were monodentate aliphatic complexes (acetate), aromatic complexes (tiron), or uranyl hydroxide species.

It should be noted that tiron formed a polynuclear complex with U(VI) and the stability constant for complexation was higher than values reported for the other organic complexes evaluated (Table 1). However, trends of U(VI) reduction from tiron complexes by either bacterium were more similar to

FIG. 6. V_i of U(VI) in test solutions containing organic ligands incubated with *D. desulfuricans* (□) and *S. alga* (■). Ligands initially complexed >98% of the U(VI).

trends observed from the complex with the lowest stability constant (acetate) than from complexes with higher stability constants (multidentate aliphatics). This suggested that reduction of U(VI) from various organic complexes was not primarily related to the respective thermodynamic stability constants. Bolton et al. (5) and Francis et al. (18) have reported that factors such as lability of metal-organic complexes rather than stability constants will dictate the degradation of metal-organic complexes. The functional groups (hydroxyl) involved in U(VI)-tiron complexation and the structural orientation of ternuclear aromatic complexes probably dictated the reduction of U(VI) by microorganisms.

Comparison of U(VI) reduction by *D. desulfuricans* and *S. alga*. A comparison of results indicated that the two bacteria reduced U(VI) very differently from each of the complexes evaluated. *D. desulfuricans* rapidly reduced U(VI) from monodentate aliphatic complexes formed with acetate (Fig. 6). *S. alga* very slowly reduced uranium from this complex. While reduction from multidentate complexes by the sulfate-reducing bacteria was slow, it was rapid in the presence of the iron-reducing bacteria. Rapid reduction of U(VI) by *D. desulfuricans* was also observed in aromatic complexes. Reduction by *S. alga* was slow in these complexes. In addition, a decrease in the amount of organic-U(VI) complex in solution increased the rate of reduction by *D. desulfuricans* and decreased the rate of reduction by *S. alga* (Tables 3 and 4). The vast differences in U(VI) reduction trends observed when the two bacteria were used was due to neither toxicity nor metabolism of organic complexants by either of the bacteria. The results observed may be due to differences in the nature of the enzymes involved in reduction. Sulfate-reducing bacteria have been reported to reduce uranyl ion by using an enzyme (cytochrome c_3) located at the soluble (periplasmic) fraction of the cell (35). This enzyme is not associated with an energy-yielding process leading to growth of the bacteria (34). In contrast, *S. alga* reduced U(VI) by using a multicomponent enzyme system associated with the membrane fraction of the cell (8, 9). The enzyme complex appeared to be involved in phosphorylation-energy-yielding processes of the bacteria (9). Such differences in the location and function of U(VI)-reducing enzymes may have imposed limitations on structural compatibility or transport mechanisms and contributed to the differences in the U(VI) reduction from organic complexes by the two bacteria.

Precipitation of bioreduced uranium. Effective removal of uranium from contaminated water by these microorganisms depends not only on reduction of U(VI) to U(IV) but also on subsequent precipitation of reduced uranium. Precipitation of U(IV), upon microbial reduction, was evaluated by measuring the total dissolved uranium [U(VI) plus U(IV)] in the filtrate

(0.2- μ m-pore-size filter) over time. The filtrate (1 ml) was oxidized by using 2 N HNO₃ to convert U(IV) to U(VI) prior to this analysis (21). The results indicated that precipitation of U(IV) depended on the type and concentration of the organic ligand present in solution (data not shown). Briefly, while >95% of the U(IV) precipitated from solutions containing the highest concentrations of acetate and malonate, less than 20% of the U(IV) precipitated in the presence of oxalate and citrate. At the lower concentrations of oxalate and citrate used, >80% of the bioreduced uranium precipitated from solution. However, little (~10%) of the U(IV) precipitated from tiron solutions, even at the lower concentration utilized. A detailed discussion concerning U(IV) precipitation trends in the presence of organic chelating agents is presented elsewhere (19–22).

Implications for wastewater treatment. Results indicated that reduction of U(VI) was affected by various organic compounds when either of the bacteria was evaluated. It appears that ground and surface waters containing uranium but little or no organic matter can be successfully treated by using *D. desulfuricans*. However, experimental results suggest that these aqueous solutions may need to be amended with strong complexants prior to using *S. alga* for uranium reduction. Mixed-process waste streams from industries and leachates produced from environmental restoration operations generally contain complex organic compounds (37). While *S. alga* can rapidly reduce U(VI) from selected multidentate complexes found in such waste streams, reduction by *D. desulfuricans* may be slow. In addition, precipitation of reduced uranium [U(IV)] will depend upon the type and amount of organic ligands present. Hence, it is essential to evaluate the chemical (organic) composition of waste streams prior to selecting the type of bacteria for uranium reduction. Finally, results suggested that the negative impact on biotreatment due to organic-metal complexation can be greatly alleviated by proper selection of bacteria for treatment.

ACKNOWLEDGMENTS

This project was supported by the U.S. Department of Energy (DE-FC05-92-OR-22056) and by the Waste Management Research and Education Institute at the University of Tennessee, Knoxville.

REFERENCES

- Ahrland, S. 1951. On the complex chemistry of the uranyl ion. IV. The complexity of uranyl acetate. *Acta Chem. Scand.* **5**:199–219.
- Allison, J. D., D. S. Brown, and K. J. Vots Gradac. 1991. MINTEQA2/PRODEFA2A, a geochemical assessment model for environmental systems. Report EPA-600/3-91-021. U.S. Environmental Protection Agency, Athens, Ga.
- Barrio-Lage, G., F. Z. Parsons, and R. S. Nassar. 1987. Kinetics of depletion of trichloroethene. *Environ. Sci. Technol.* **21**:366–369.
- Bergsma, J., and W. N. Konning. 1983. The properties of citrate transport in membrane vesicles from *Bacillus subtilis*. *Eur. J. Biochem.* **131**:151–156.
- Bolton, H., D. C. Girvin, A. E. Plymale, S. D. Harvey, and D. Workman. 1996. Degradation of metal-nitrotriacetate complexes by *Chelatobacter heintzii*. *Environ. Sci. Technol.* **30**:931–938.
- Brina, R., and A. G. Miller. 1992. Direct detection of trace levels of uranium by laser induced kinetic phosphorimeter. *Anal. Chem.* **64**:1415–1418.
- Caccavo, F., Jr., R. P. Blakemore, and D. R. Lovley. 1992. A hydrogen-oxidizing, Fe(III)-reducing microorganism from the Great Bay estuary, New Hampshire. *Appl. Environ. Microbiol.* **58**:3211–3216.
- Caccavo, F., Jr., Y. A. Gorby, and M. J. McInerney. 1995. Characterization of a metal reductase complex in the dissimilatory metal-reducing bacterium *Shewanella alga* strain BrY. Poster presented at the 95th General Meeting of the American Society for Microbiology 1995.
- Caccavo, F., Jr. (Center for Biofilm Engineering, Montana State University.) 1995. Personal communication.
- Coleman, M. L., D. B. Hedrick, D. R. Lovley, D. C. White, and K. Pye. 1993. Reduction of Fe(III) in sediments by sulphate-reducing bacteria. *Nature* **364**:436–438.
- Collern, E., S. Fennegan, and P. Lens. 1995. Anaerobic treatment of sulfate containing waste streams. *Antonie van Leeuwenhoek* **67**:29–46.
- Conover, W. J. 1980. Practical non-parametric statistics. Wiley science in probability and mathematics. Wiley, Ridge, N.J.
- Cox, C. D. 1980. Iron reductases from *Pseudomonas aeruginosa*. *J. Bacteriol.* **80**:2121–2128.
- Emery, T. 1976. Fungal ornithine esterases: relationship to iron transport. *Biochemistry* **15**:2723–2728.
- Firestone, M. K., and J. M. Tiedje. 1975. Biodegradation of metal-nitrotriacetate complexes by a *Pseudomonas* species: mechanism of reaction. *Appl. Microbiol.* **29**:758–764.
- Francis, A. J., C. R. Iden, B. J. Nine, and C. K. Chang. 1980. Characterization of organics in leachates from low level radioactive waste disposal sites. *Nucl. Technol.* **50**:158–163.
- Francis, A. J., and C. J. Dodge. 1993. Influence of complex structure on the biodegradation of iron-citrate complexes. *Appl. Environ. Microbiol.* **59**:109–113.
- Francis, A. J., C. J. Dodge, and J. B. Gillow. 1992. Biodegradation of metal citrate complexes and implications for toxic-metal mobility. *Nature* **356**:140–142.
- Ganesh, R., K. G. Robinson, and G. D. Reed. 1995. Biotransformation of uranium complexed to organic ligands by *Desulfovibrio desulfuricans*, abstr. 57, p. 527–534. In Proceedings of the 50th Purdue Industrial Waste Conference.
- Ganesh, R., K. G. Robinson, G. D. Reed, and M. M. Ghosh. 1995. Reductive precipitation of organically complexed uranium by bacterial strain BrY, abstr. 1, p. 152–155. In Special Symposium on Emerging Technologies in Hazardous Waste Management VII. American Chemical Society, Atlanta, Ga.
- Ganesh, R. 1995. Impact of organic ligands on bioreductive precipitation of hexavalent uranium. Ph.D. thesis. University of Tennessee, Knoxville.
- Ganesh, R., K. G. Robinson, G. D. Reed, and G. S. Saylor. Factors influencing U(VI) reduction from organic complexes by *Shewanella alga*. Submitted for publication.
- Gorby, Y. A., and D. R. Lovley. 1992. Enzymatic uranium precipitation. *Environ. Sci. Technol.* **26**:205–207.
- Goring, A. L., and J. W. Hamaker. 1972. Organic chemicals in the soil environment. Marcel Dekker, Inc., New York, N.Y.
- Gustafson, R. L., C. Richard, and A. E. Martell. 1960. Polymerization of uranyl-tiron chelates. *J. Am. Chem. Soc.* **82**:1526–1534.
- Hungate, R. E. 1969. A roll tube method for cultivation of strict anaerobes. *Methods Microbiol.* **3B**:117–132.
- Huyer, M., and W. J. Page. 1989. Ferric reductase activity in *Azotobacter vinelandii* and its inhibition by Zn²⁺. *J. Bacteriol.* **171**:4031–4037.
- Joshi-Tope, G., and A. J. Francis. 1995. Mechanisms of biodegradation of metal-citrate complexes by *Pseudomonas fluorescens*. *J. Bacteriol.* **177**:8–12.
- Kelly, W. R., M. P. Saliga, M. L. Machesky, and D. L. Freedman. 1996. Biodegradation of BTEX under iron-reducing conditions at batch microcosms, abstr. 3, p. 161–172. In Proceedings of the 69th Annual Water Environment Federation Conference, Water Environment Federation, Dallas, Tex.
- Knight, V., F. Caccavo, S. Wudyka, and R. Blakemore. 1996. Synergistic iron reduction and citrate dissimilation by *Shewanella alga* and *Aeromonas veronii*. *Arch. Microbiol.* **166**:269–274.
- Krieg, N. R., and J. G. Holt. 1984. Bergey's manual of systematic bacteriology, vol. 1, p. 663–680. The Williams & Wilkins Co., Baltimore, Md.
- Lovley, D. R., E. J. P. Phillips, Y. A. Gorby, and E. R. Landa. 1991. Biological reduction of uranium. *Nature* **350**:413–416.
- Lovley, D. R., and E. J. P. Phillips. 1992. Bioremediation of uranium contamination with enzymatic uranium reduction. *Environ. Sci. Technol.* **26**:2228–2234.
- Lovley, D. R., and E. J. P. Phillips. 1992. Reduction of uranium by *Desulfovibrio desulfuricans*. *Environ. Sci. Technol.* **58**:850–856.
- Lovley, D. R., P. K. Wideman, J. C. Woodward, and E. J. P. Phillips. 1993. Reduction of uranium by cytochrome *c*₃ of *Desulfovibrio vulgaris*. *Appl. Environ. Microbiol.* **59**:3572–3576.
- Lovley, D. R., J. C. Woodward, and F. C. Chapelle. 1996. Rapid anaerobic benzene oxidation with a variety of chelated Fe(III) forms. *Appl. Environ. Microbiol.* **62**:288–291.
- Macaskie, L. E. 1991. The application of biotechnology to the treatment of waste produced from the nuclear fuel cycle: biodegradation and bioaccumulation as a means of treating radionuclide containing streams. *Crit. Rev. Biotechnol.* **11**:41–112.
- Madsen, E. L., and M. Alexander. 1985. Effect of chemical speciation on the mineralization of organic compounds by microorganisms. *Appl. Environ. Microbiol.* **50**:342–349.
- Martell, A. E., and R. M. Smith. 1977. Critical stability constants—vol. 3. Other organic ligands. Plenum Press, New York, N.Y.
- Perrin, D. D. 1979. Stability constants for metal ion complexes. Part B—organic ligands. Pergamon Press, Oxford, United Kingdom.
- Phillips, E. J. P., E. R. Landa, and D. R. Lovley. 1995. Remediation of uranium contaminated soils with bicarbonate extraction and microbial

- U(VI) reduction. *J. Ind. Microbiol.* **14**:203–207.
42. **Rajan, S., and A. E. Martell.** 1965. Equilibrium studies on uranyl complexes. III. Interaction of uranyl ion with citric acid. *Inorg. Chem.* **4**:462–469.
43. **Rajan, S., and A. E. Martell.** 1967. Equilibrium studies on uranyl complexes. IV. Reaction with carboxylic acids. *J. Inorg. Nucl. Chem.* **29**:523–529.
44. **Robinson, K. G., R. Ganesh, G. D. Reed, and D. Kucsmas.** 1994. Biologically mediated removal of uranium in a high nitrate/sulfate wastewater, abstr. 1, p. 199–208. *In* Proceedings of the 67th annual Water Environment Federation Conference. Water Environment Federation, Chicago, Ill.
45. **Roden, E. E., and D. R. Lovley.** 1993. Dissimilatory Fe(III) reduction by the marine microorganism *Desulfuromonas acetoxidans*. *Appl. Environ. Microbiol.* **59**:734–742.
46. **Rothstein, A., and C. Larrabee.** 1948. The relationship of cell surface to metabolism. II. The cell surface of yeast as the site of inhibition of glucose metabolism by uranium. *J. Cell. Comp. Physiol.* **32**:247–259.
47. **Rothstein, A., A. Frankel, and C. Larrabee.** 1948. The relationship of cell surface to metabolism. III. Certain characteristics of the uranium complex-
ation with cell surface groups of yeast. *J. Cell Comp. Physiol.* **32**:247–259.
48. **Rothstein, A., R. C. Meier, and L. Hurwitz.** 1951. The relationship of cell surface to metabolism. V. The role of uranium complexing loci of yeast metabolism. *J. Cell. Comp. Physiol.* **37**:57–81.
49. **Sillen, L. G., and A. E. Martell.** 1964. Stability constants of metal ion complexes, 2nd ed. Burlington House, London, United Kingdom.
50. **Suflita, J. M., J. A. Robinson, and J. M. Tiedje.** 1983. Kinetics of microbial dehalogenation on haloaromatic substrates in methanogenic environments. *Appl. Environ. Microbiol.* **45**:1466–1473.
51. **Tuovinan, O. H., and D. P. Kelly.** 1974. Studies on the growth of *Thiobacillus ferrooxidans*. II. Toxicity of uranium to growing culture and tolerance conferred by mutation, other metal cations and EDTA. *Arch. Microbiol.* **95**:153–164.
52. **Van Der Rest, M. E., J. Abbe, D. Molenover, and W. N. Konning.** 1991. Mechanism and energetics of a citrate-transport system of *Klebsiella pneumoniae*. *Eur. J. Biochem.* **195**:71–77.