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Reduction of Uranium by Cytochrome c_3 of *Desulfovibrio vulgaris*

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The mechanism for U(VI) reduction by *Desulfovibrio vulgaris* (Hildenborough) was investigated. The H_2-dependent U(VI) reductase activity in the soluble fraction of the cells was lost when the soluble fraction was passed over a cationic exchange column which extracted cytochrome c_3. Addition of cytochrome c_3 back to the soluble fraction that had been passed over the cationic exchange column restored the U(VI)-reducing capacity. Reduced cytochrome c_3 was oxidized by U(VI), as was a *c*-type cytochrome(s) in whole-cell suspensions. When cytochrome c_3 was combined with hydrogenase, its physiological electron donor, U(VI) was reduced in the presence of H_2. Hydrogenase alone could not reduce U(VI). Rapid U(VI) reduction was followed by a subsequent slow precipitation of the U(IV) mineral uraninite. Cytochrome c_3 reduced U(VI) in a uranium-contaminated surface water and groundwater. Cytochrome c_3 provides the first enzyme model for the reduction and biomineralization of uranium in sedimentary environments. Furthermore, the finding that cytochrome c_3 can catalyze the reductive precipitation of uranium may aid in the development of fixed-enzyme reactors and/or organisms with enhanced U(VI)-reducing capacity for the bioremediation of uranium-contaminated waters and waste streams.

The enzymatic reduction of metals by *Desulfovibrio* species may have an important influence on the geochemistry of sedimentary environments and may be a useful tool for removing metals from contaminated waters and waste streams. For example, *Desulfovibrio* species can reduce the soluble oxidized form of uranium, U(VI), to insoluble U(IV) (17, 20). This reductive precipitation can effectively remove uranium from a variety of uranium-contaminated waters (16) and can be used in conjunction with a soil extraction technique for concentrating uranium from contaminated soils (26). In a similar manner, *Desulfovibrio vulgaris* can reduce highly soluble and toxic Cr(VI) to less soluble, less toxic Cr(III) (18). Precipitation of uranium as the result of enzymatic U(VI) reduction in sedimentary environments by *Desulfovibrio* species and other organisms may be an important global sink for this compound and may lead to the formation of certain uranium ores (14, 17, 19). *Desulfovibrio* species may also play an important role in the enzymatic reduction of Fe(III) in marine and deep subsurface sediments (3, 14).

The enzymatic mechanisms for dissimilatory metal reduction by anaerobic microorganisms are largely unknown. A few microbial enzymes with metals as substrates have been purified. These include the intensively studied mercuric reductase which detoxifies ionic mercury (29) and a recently described Cr(VI) reductase of unknown physiological function (30), as well as Fe(III) reductases that reduce chelated Fe(III) forms as part of the process of iron assimilation (9, 11, 24). However, all of these metal reductases are expressed during aerobic metabolism, and their function has little in common with dissimilatory metal reduction under anoxic conditions.

The potential of using microbial U(VI) reduction for the removal of uranium from contaminated waters (16) led us to further investigate the enzymatic mechanisms for U(VI) reduction with the hope of further optimizing this process. *D. vulgaris* (Hildenborough) was chosen for these studies because (i) U(VI) reductase activity could not be recovered in broken cell extracts of other U(VI)-reducing organisms such as *Geobacter metallireducens* or *Shewanella putrefaciens* (21); (ii) the U(VI) reductase activity in whole cells of *Desulfovibrio* species is very stable and tolerant to air exposure (16, 21); and (iii) of all *Desulfovibrio* species known to reduce U(VI) (20), the biochemistry of *D. vulgaris* (Hildenborough) has been studied most intensively (13). The results presented here indicate that cytochrome c_3 is the U(VI) reductase in *D. vulgaris*.

MATERIALS AND METHODS

Source of organisms and culturing conditions. *D. vulgaris* (Hildenborough) (ATCC 29579) was obtained from the American Type Culture Collection, Rockville, Md. As this is the only *D. vulgaris* strain examined, it is referred to as *D. vulgaris* in the remainder of the text.

D. vulgaris was grown with lactate as the electron donor and sulfate as the electron acceptor in a previously described bicarbonate-buffered medium (17) which contains salts, yeast extract, peptone, trace minerals, and vitamins. The gas phase was N_2-CO_2 (80:20). For studies with whole cells, *D. vulgaris* was cultured in 100-ml volumes in serum bottles (160 ml) capped with thick butyl rubber stoppers. Larger volumes (10 liters) were cultured in glass carboys (12 liters) that were continuously sparged with N_2-CO_2.

U(VI) reduction in soluble protein fractions. All studies reported here were conducted with membrane-free soluble extract because preliminary studies demonstrated that 95% of the H_2-dependent U(VI) reduction capacity was in the soluble fraction. Cells (ca. 10 g) were suspended in 20 ml of 20 mM sodium phosphate buffer (pH 7) and were broken by three passages through a French pressure cell. Unbroken cells were removed by centrifugation (36,000 × *g*, 4°C, 20 min). Membranes were removed by centrifugation (100,000 × *g*, 4°C, 60 min). The soluble fraction was stored under N_2-CO_2 at −70°C prior to use. Preliminary studies demon-

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strated that this storage did not result in loss of U(VI) reductase activity. Protein concentrations were determined by the method of Lowry et al. (22).

The soluble fraction was loaded onto a Waters Protein Pak SP 8HR cationic exchange column (10 by 100 mm) which was eluted with 20 mM sodium phosphate buffer (pH 7) at 1 ml/min. Proteins not retained on the column under these conditions were pooled in one fraction, referred to as the wash fraction. A protein fraction was then eluted with 400 mM NaCl added to the phosphate buffer. This protein was referred to as the eluted fraction. The soluble fraction, the wash fraction, and the eluted fraction were dialyzed overnight against 30 mM bicarbonate buffer (pH 7.6). Aliquots (0.5 ml) of each fraction (soluble, 11.6 mg of protein per ml; wash, 7.3 mg of protein per ml; and eluted, 1.3 mg of protein per ml) were added to sodium bicarbonate buffer (10 ml; 30 mM) under N_2-CO_2 (80:20) in a serum bottle (25 ml). U(VI) was provided as uranyl acetate. H_2 (10 ml) was added when noted. At the times noted, subsamples were removed and analyzed for U(VI) with a Kinetic Phosphorescence Analyzer as described previously (19). Incubations were at 30°C.

Air-oxidized and dithionite-reduced spectra of the eluted fraction were obtained in 20 mM sodium phosphate buffer with a scanning spectrophotometer at room temperature. In order to determine the ability of U(VI) to oxidize the eluted protein, 2 ml of a solution of the eluted protein in phosphate buffer under N_2 was reduced with 0.4 mM (final concentration) sodium dithionite, and then 0.8 mM (final concentration) uranyl acetate was added from a concentrated anaerobic stock.

U(VI) reduction by H_2, hydrogenase, and cytochrome c_3. Additional studies on the reduction of metals by pure cytochrome c_3 were modelled after those of Fauque and coworkers (6) in which the ability of cytochrome c_3 to reduce elemental sulfur was demonstrated. Cytochrome c_3 (0.06 mg) and an excess of hydrogenase were added to bicarbonate buffer (10 ml) containing uranyl acetate, and incubations were run as described above.

Pure periplasmic hydrogenase was used in early experiments. The periplasmic hydrogenase was extracted as described previously (31) and purified on a Waters Protein Pak DEAE column (10 by 100 mm) with 10 mM Tris buffer (pH 8) and a linear NaCl gradient (0 to 300 mM) at 1.5 ml/min. The pure hydrogenase had two subunits of ca. 47 and 10 kDa, as determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, which is consistent with previous descriptions of this enyzme (7, 13).

For studies on the kinetics of U(IV) precipitation and U(VI) reduction in contaminated waters, a hydrogenase-containing fraction was purified from the whole-cell extract after the cytochrome c_3 had been extracted (see above). Purification was on a Waters Protein Pak Q 8HR strong anion-exchange column (10 by 100 mm) with 10 mM Tris buffer (pH 8) and a linear NaCl gradient (0 to 300 mM) at 1.5 ml/min. The hydrogenase fraction contained a combination of the periplasmic hydrogenase and, as described below, another, uncharacterized hydrogenase.

Uranium precipitation. In order to determine whether U(VI) reduction by cytochrome c_3 resulted in the precipitation of U(IV), incubations with H_2, hydrogenases, cytochrome c_3, and uranyl acetate were run as described above. U(VI) and total uranium were determined on aliquots that had been passed through a 0.2-μm-pore-diameter filter (Acrodisc; Gelman) as well as on bulk unfiltered samples. Total uranium was determined by oxidizing the U(IV) in the samples to U(VI) under air (19).

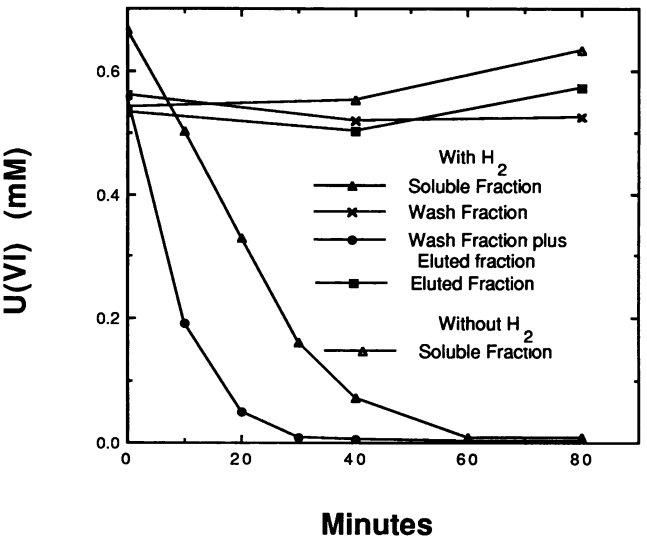

FIG. 1. U(VI) reduction by various protein fractions from *D. vulgaris*. The protein amounts of the various fractions in 10 ml were as follows: soluble fraction, 5.8 mg; protein not initially retained on cationic exchange column (wash fraction), 3.65 mg; protein eluted from cationic exchange column with 400 mM NaCl (eluted fraction), 0.65 mg.

Reduction of uranium in contaminated waters. Uranium-contaminated drainage waters from an inactive uranium mine were provided by Charles Adams of the U.S. Bureau of Mines. Uranium-containing groundwater from the Department of Energy site in Hanford, Wash., was provided by Yuri Gorby of Pacific Northwest Laboratories. Characteristics of these waters were described previously in detail (16). Mine drainage waters I and II had pHs of 7.4 and 4.0 and dissolved U(VI) concentrations of 36 and 125 μM, respectively. The acidic mine drainage water II also had relatively high concentrations of other metals, including copper (4.5 μM), aluminum (4.3 mM), iron (34 μM) manganese (2.7 mM), and zinc (85 μM). The uranium-contaminated groundwater was the one referred to previously as Hanford II (16) and had a pH of 6.2 and contained 0.5 μM U(VI). A unique feature of this water is that it also contained high concentrations of nitrate (4.8 mM). The ability of cytochrome c_3 to reduce the U(VI) in these waters was determined as described above with the exception that the uranium-contaminated water replaced the bicarbonate buffer.

RESULTS AND DISCUSSION

Identification of a U(VI) reductase. When the soluble fraction from *D. vulgaris* was passed over a cationic exchange column, the capacity for H_2-dependent U(VI) reduction was lost (Fig. 1). A protein fraction that was eluted from the cationic exchange column with 400 mM NaCl (eluted fraction) restored H_2-dependent U(VI) reduction when added back to the wash fraction that had not been retained on the column (Fig. 1).

The eluted fraction that was necessary for U(VI) reduction contained a single protein with a molecular weight of ca. 13,000, as determined by SDS-polyacrylamide gel electrophoresis. The protein band stained positively for covalently bound heme (8). The dithionite-reduced protein had absorbance maxima at 552.2, 523.2, and 418.2 nm (Fig. 2). The

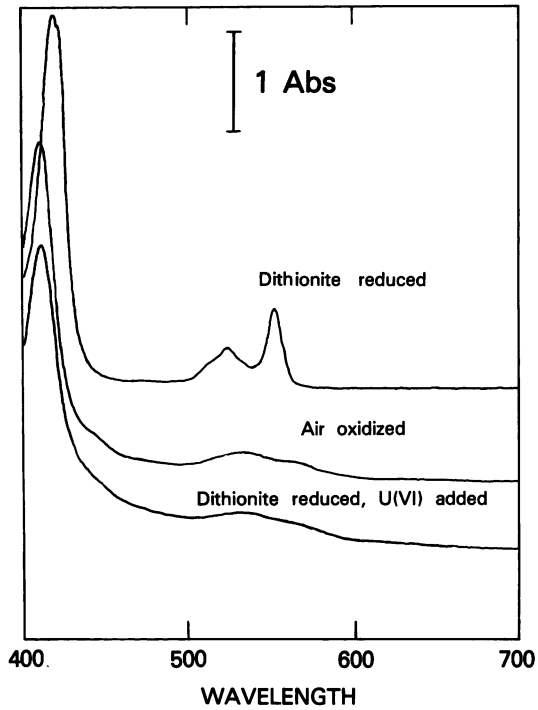

FIG. 2. Spectra of protein responsible for U(VI) reduction in soluble fraction of *D. vulgaris*. Protein concentrations were 0.19 mg/ml for dithionite-reduced and air-oxidized spectra and 0.15 mg/ml for the spectrum in which U(VI) was added. Abs, absorbance.

air-oxidized protein had an absorption maximum at 409.6 nm (Fig. 2). The protein had a *c*-type cytochrome purity index, $(A_{553[reduced]} - A_{570[reduced]})/A_{280[oxidized]}$ (6), of 2.93. These results indicate that the protein retained on the cationic exchange column that was necessary for U(VI) reduction was cytochrome c_3.

In order to determine whether cytochrome c_3 could directly reduce U(VI), U(VI) was added to dithionite-reduced cytochrome c_3. Addition of U(VI) resulted in an immediate change in absorbance from the reduced spectrum to the oxidized spectrum (Fig. 2). When U(VI) was added to whole-cell suspensions of *D. vulgaris* that had been reduced with dithionite, there was a similar spectral change (data not shown). These results demonstrate that cytochrome c_3 can serve as a U(VI) reductase and suggest that cytochrome c_3 is involved in electron transport to U(VI) in whole cells.

Hydrogenase is the physiological electron donor for cytochrome c_3 (13). U(VI) was rapidly reduced when cytochrome c_3 was combined with pure periplasmic hydrogenase from *D. vulgaris* and H_2 (Fig. 3).

Because of our limited capacity for mass culturing cells, it was difficult to generate enough pure periplasmic hydrogenase for further studies on U(VI) reduction by cytochrome c_3. Therefore, subsequent studies were conducted with a hydrogenase preparation that could be obtained from the soluble cell extract after the cytochrome c_3 had been removed with the cation-exchange column. On SDS-polyacrylamide gels, this hydrogenase preparation had major bands which corresponded with the periplasmic hydrogenase as well as another band at ca. 66 kDa. Characterization of the 66-kDa protein has indicated that it is also a hydrogenase, which does not appear to have been reported previously in

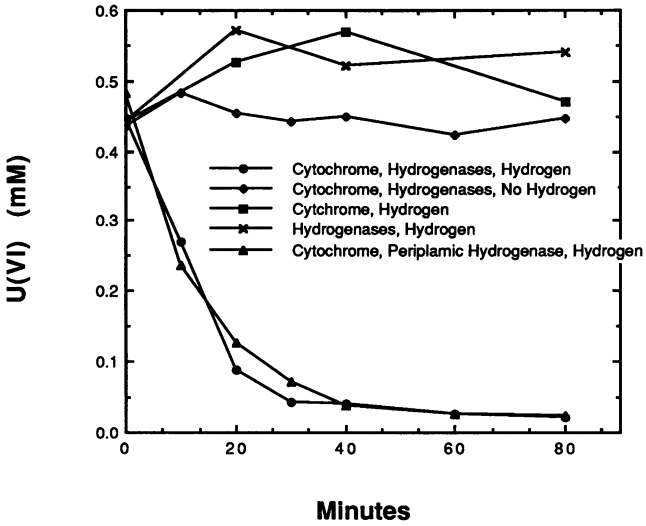

FIG. 3. Reduction of U(VI) by electron transfer from H_2 to U(VI) via hydrogenase and cytochrome c_3. As noted, pure periplasmic hydrogenase or a protein fraction containing two hydrogenases was used.

D. vulgaris (21). Cytochrome c_3 reduced U(VI) at the same rate with the preparation of the two hydrogenases as it did with the pure periplasmic hydrogenase (Fig. 3). The hydrogenases did not reduce U(VI) in the absence of cytochrome c_3 (Fig. 3). In the absence of H_2, there was no reduction of U(VI) by cytochrome c_3 alone or hydrogenase and cytochrome c_3 combined (Fig. 3).

Precipitation of U(VI). With whole cells of U(VI)-reducing microorganisms, the U(VI) that is produced precipitates as uraninite (UO_2) particles too large to pass through a 0.2-μm-pore-size filter within several hours (10, 16). Uraninite (as confirmed by X-ray diffraction analysis) also accumulated during U(VI) reduction with cytochrome c_3 and hydrogenase. However, in the enzyme preparation, uraninite was not removed by filtration for more than 30 h (Fig. 4). The slower precipitation of U(VI) with the enzyme preparation may be due to the lack of cell surfaces to provide nucleation sites for uraninite formation.

Reduction of U(VI) in contaminated waters. In a previous study, whole cells of *D. desulfuricans* reduced U(VI) in all of the uranium-contaminated surface waters and groundwaters evaluated (16). Cytochrome c_3 effectively reduced the U(VI) in the mine drainage water and the groundwater that were near neutral pH (Fig. 5). However, in contrast to previous results with whole cells (16), the purified enzymes did not reduce U(VI) in the pH 4 mine drainage water (data not shown). It has yet to be evaluated whether the inhibition of U(VI) reduction in the acidic mine drainage water was the result of the low pH or the high concentration(s) of one or more of the heavy metals and whether the activity of the hydrogenases or cytochrome c_3, or both, was inhibited.

Physiological implications. Cytochrome c_3 provides the first enzymatic model for anaerobic dissimilatory metal reduction and biomineralization. Although at one time it was considered that the reduction of U(VI) in the presence of sulfate-reducing bacteria was a nonenzymatic process in which sulfide produced from sulfate reduction nonenzymatically reduced U(VI), recent studies have indicated that sulfide does effectively reduce U(VI) and that the U(VI) reduction is enzymatically catalyzed (17, 19). Previous stud-

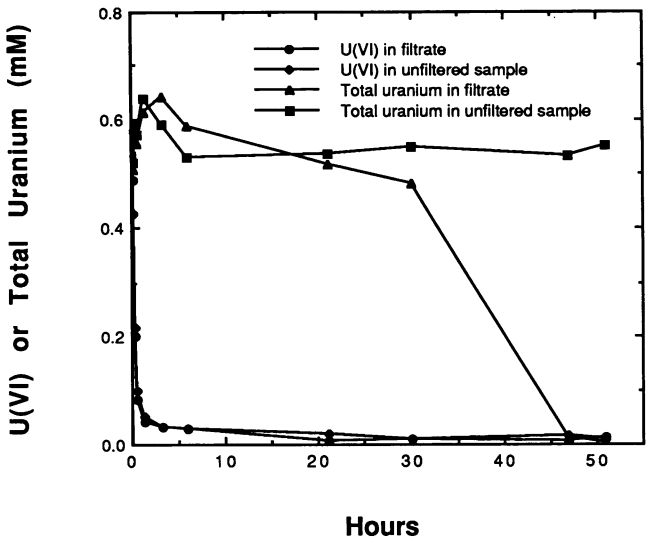

FIG. 4. Kinetics of formation of uraninite precipitate unable to pass through a 0.2-μm-pore-size filter during U(VI) reduction by cytochrome c_3.

ies have suggested that *c*-type cytochromes were involved in metal reduction in other anaerobic dissimilatory metal-reducing microorganisms. For example, Fe(III), which is an electron acceptor for *G. metallireducens* and *Desulfuromonas acetoxidans*, oxidizes the *c*-type cytochrome(s) in these organisms, and U(VI) oxidizes the *c*-type cytochrome(s) in the U(VI) reducer *G. metallireducens* (15, 28). However, it was not determined in either of those studies whether the *c*-type cytochrome(s) reduces the metals directly or is an intermediate in an electron transport chain leading to distinct Fe(III) or U(VI) reductase(s).

The available evidence suggests that cytochrome c_3 is the physiological U(VI) reductase in *D. vulgaris*. Although cytochrome c_3 serves primarily as an intermediary electron carrier for the reduction of various sulfur oxide electron acceptors in *Desulfovibrio* species (13), it also is the direct reductase for S^0 (6, 12) and possibly O_2 (27). It seems unlikely that cytochrome c_3 would be an intermediary electron carrier to another U(VI) reductase in whole cells of *D. vulgaris*, because it can reduce U(VI) directly. The extracellular precipitation of U(VI) during U(VI) reduction in whole cells of *D. desulfuricans* (17) suggests that U(VI) reduction is near the cell surface, which is consistent with the periplasmic location of cytochrome c_3. Cytochrome c_3 must have unique properties other than just low-potential redox centers which permit it to reduce U(VI), as the hydrogenases in *D. vulgaris* which also have low-potential centers (7) do not reduce U(VI).

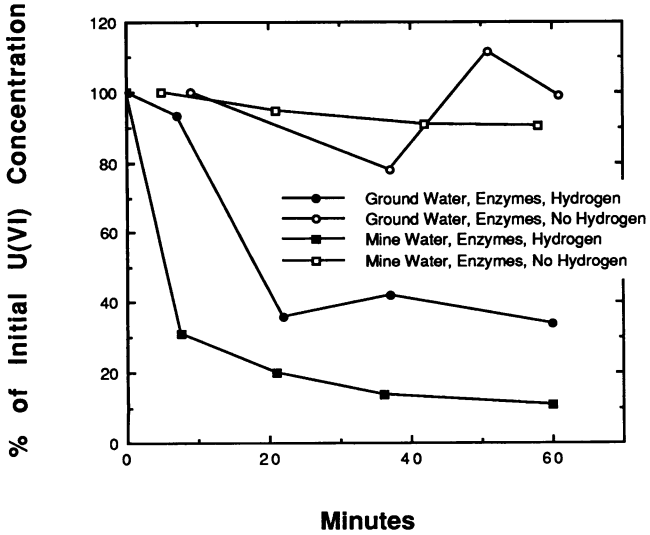

FIG. 5. U(VI) concentrations over time in pH 7.4 mine drainage water and uranium-contaminated groundwater in the presence of hydrogenases and cytochrome c_3, with or without added H_2. The initial concentrations of U(VI) in the mine water and groundwater were 36 and 0.5 μM, respectively.

Only preliminary evidence on the potential physiological significance of U(VI) reduction by cytochrome c_3 is available. All of the *Desulfovibrio* species that have been examined can reduce U(VI), but attempts to grow these organisms with U(VI) as the sole electron acceptor have been unsuccessful (17, 20). U(VI) reduction may be similar to O_2 reduction by *Desulfovibrio* species in which ATP is produced but there is no growth with O_2 as the sole electron acceptor (4, 5). However, the potential for ATP production during U(VI) reduction has not been evaluated. Metal reduction by *Desulfovibrio* species appears to have some physioecological significance in that *D. desulfuricans* can metabolize H_2 down to much lower concentrations with U(VI) or Fe(III) as the electron acceptor than with sulfate (3, 20).

Geochemical implications. Until recently, U(VI) reduction in sedimentary environments was primarily considered to be a nonenzymatic process in which sulfide, H_2, or organics reacted directly with U(VI) to reduce it (14, 19). However, recent laboratory and field studies have suggested that, at the low temperatures and circumneutral pHs of most aquatic sediments and many groundwaters, enzymatically catalyzed U(VI) reduction is the more likely explanation (1, 17, 19). Cytochrome c_3 provides the first enzymatic model for the reduction and biomineralization of uranium in sedimentary environments.

Bioremediation implications. The results presented here demonstrate that cytochrome c_3 can potentially be used for reductively precipitating uranium from contaminated waters. Cytochrome c_3 can also reduce toxic Cr(VI) to the less toxic Cr(III) (18). Cytochrome c_3 is readily mass produced and could be employed in fixed-enzyme reactors. The slow precipitation of uraninite after U(IV) production demonstrates that U(VI) reduction and U(IV) precipitation can be temporally, and thus spatially, separated so that uraninite precipitation does not foul the enzyme preparation in water treatment systems. H_2 coupled with hydrogenase need not be the reductant in such systems, as cytochrome c_3 can be directly reduced electrochemically without the aid of intermediary electron carriers (25). Thus, cytochrome c_3-based electrobioreactors similar to those described previously for removal of nitrate from contaminated waters (23) are potentially feasible.

The gene for cytochrome c_3 from *D. vulgaris* has been cloned and expressed in the closely related *D. desulfuricans* (32) as well as the genetically distant *Rhodobacter sphaeroides* (2). This suggests that the ability to reduce U(VI) and Cr(VI) could be genetically combined with other useful metabolic properties such as the ability to degrade organic contaminants and/or denitrification. This might confer on single organism the ability to remove not only the contami-

nant metals but also other important contaminants such as organic solvents and nitrate that are typically found in wastes bearing these metals. Future studies will evaluate whether engineering an organism to overexpress cytochrome c_3 will enhance the capacity for U(VI) reduction.

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