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A Novel PhosphorImager-Based Technique for Monitoring the Microbial Reduction of Technetium

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A novel PhosphorImager-based technique which can be used to quantify low concentrations of radionuclides is described. The technique offers several benefits, combining very high sensitivity with containment of the radioisotope in the solid state, thus minimizing disposal procedures. In this study, it was used in conjunction with paper chromatography to quantify different oxidation states of ^{99}Tc in solution. The technique was used to evaluate the potential of anaerobic cultures of *Shewanella putrefaciens* and *Geobacter metallireducens* (bacteria with known metal-reducing capabilities) to reduce highly soluble Tc(VII) to insoluble lower-valence species, facilitating its removal from solution. Both organisms reduced Tc(VII), but profiles of Tc species produced in culture supernatants were strain specific. *S. putrefaciens* produced Tc(V), Tc(IV), and one unidentified species, but no Tc was removed from solution. *G. metallireducens* removed 70% of the 250 μM Tc added in solution, with trace amounts of Tc(V) and the unidentified species detected in culture supernatants. Possible uses for these organisms in the bioremediation of Tc-contaminated waters are discussed, and other uses of the PhosphorImager technique are highlighted.

Fission of ^{235}U during the production of nuclear power results in the generation of substantial quantities of the β-particle emitter technetium (^{99}Tc). This element can exist in valences +7 to −1 (9). The most stable state is Tc(VII), typified by the highly soluble pertechnetate ion (TcO_4^-), which is the predominant species in aerated soil (19), seawater (1), and some groundwaters (5). In this form, Tc shows poor ligand-complexing capabilities and is therefore highly mobile in the environment. Its biological activity is high, however: it acts as a sulfate analog (2), and assimilation of TcO_4^- by plants facilitates entry into the food chain (3, 22). This factor, in combination with the long half-life of the radionuclide (2.1×10^5 years), makes removal at the source necessary.

One biotechnological route to the removal of Tc from solution could utilize metal-reducing microorganisms to reduce Tc(VII) to a less soluble oxidation state, overcoming the chemical constraints imposed by the solution properties of the heptavalent anionic species and facilitating its removal (12, 15). For example, several low-valence oxides of Tc are insoluble (7, 9) and Tc(V) is readily removed from solution by biochemicals rich in sulfhydryl groups, for example, cysteine. It has been postulated that the sulfhydryl groups further reduce Tc(V) to an insoluble low-valence oxide (11).

Many recent studies have shown that bacteria from a variety of taxonomic groups can reduce high-valence metals including Mn(IV), U(VI), Cr(VI), Se(VI), Mo(VI), and V(V) (11). Although there has been speculation that bacterial reduction of Tc(VII) may occur in the environment, there is currently little evidence that microbes can reduce Tc enzymically (12). Although it has been established that the solubility of Tc is greatly reduced in anaerobic soil samples, presumably as a result of Tc(VII) reduction (19), there have been, to date, only a few studies to elucidate the role of the soil microflora in Tc reduction and removal from solution (12). Henrot (6) showed that anaerobically (but not aerobically) grown mixed cultures of soil bacteria accumulated Tc and that addition of the sulfate-reducing bacteria *Desulfovibrio vulgaris* and *Desulfovibrio gigas* increased Tc removal by more than an order of magnitude. It was postulated, but not conclusively shown, that Tc reduction and removal were mediated by microbially generated H_2S. Pignolet et al. (18) accordingly considered that microbially derived H_2S played a major role in Tc reduction and precipitation by anaerobic cultures containing mixed populations of microorganisms isolated from a marine sediment. In contrast to indirect (chemical) mechanisms of reduction, the authors suggested that Tc reduction and removal by oxygen-limited cultures of *Moraxella* sp. and *Planococcus* sp. may be enzymatically catalyzed. Cells incubated at 21°C removed significantly more Tc from solution than those incubated at 4°C, and negligible Tc was concentrated by heat-treated (tyndallized) cultures.

A major problem which has limited the success of screening programs to identify other microorganisms capable of reducing Tc and prevented detailed studies on the biochemistry of such organisms has been the lack of a simple and quantitative method for determining the valence of Tc in solution. In this communication, a novel technique which was used to quantify Tc species in supernatants from spent bacterial culture media is described. This method uses a PhosphorImager, a piece of equipment used in molecular biology to digitize spatially separated areas of radioisotope, to detect and quantify Tc species following their separation by paper chromatography. The technique was used to evaluate the potential of *Shewanella putrefaciens* and *Geobacter metallireducens* [both of which can couple anaerobic growth to the reduction of U(VI), Mn(IV), Fe(III), or nitrate (12, 16)] for biochemical reduction of Tc(VII), facilitating removal of the latter from solution.

MATERIALS AND METHODS

Organisms and culture conditions. *S. putrefaciens* (ATCC 8071) and *G. metallireducens* (formerly GS15) were obtained from D. R. Lovley, U.S. Geological Survey, Reston, Va. The organisms were grown under an anaerobic atmosphere consisting of N_2 and CO_2 (80:20) in the defined medium described by Lovley and Phillips (14). The N_2-CO_2 gas mix was passed through an oxygen trap (Phase Separations Ltd., Deeside, Clwyd, United Kingdom). Growth medium contained 100 mM sodium lactate or 80 mM sodium acetate as the electron donor for *S. putrefaciens* and *G. metallireducens*, respectively. Ferric citrate (50 mM) was

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added as the electron acceptor in both sets of cultures. All manipulations of the organisms were done under N_2-CO_2.

Medium was dispensed (10-ml aliquots) into 12-ml serum bottles (Adelphi Tubes Ltd., Hayward's Heath, Sussex, United Kingdom) and sealed with butyl rubber stoppers. Dissolved oxygen was displaced from the medium by bubbling N_2-CO_2 through the liquid for 10 min. A 10% (vol/vol) inoculum of a culture (pregrown for 2 days in the same medium) was added to the bottle after autoclaving (15 min at 121°C). After 2 days of static culture at 30°C, the stationary-phase cells were centrifuged in the bottles at 5,000 rpm for 20 min with an MSE Super Minor benchtop centrifuge, washed three times with carbonate buffer (2.5 g of $NaHCO_3$, 1.5 g of NaH_2PO_4, 0.1 g of KCl per liter of distilled water, deaerated with an 80/20 ratio of N_2-CO_2; approximately pH 7.0 after deaeration), and resuspended in the same buffer supplemented with 100 mM sodium lactate or 80 mM sodium acetate as appropriate as the electron donor. Aliquots (2 ml) of the buffer containing cells and electron donor were then transferred under N_2-CO_2 to 12-ml serum bottles which were sealed, and ammonium pertechnetate (NH_4TcO_4; Amersham International, Amersham, Buckinghamshire, United Kingdom) was added to 0.25 mM in lieu of ferric citrate. Static cultures were incubated at 30°C. Microbial protein concentration in the cultures was measured with a bicinchoninic acid assay kit (Sigma) by the method of Smith et al. (21).

Measurement of Tc: construction of a calibration curve. Tc in solution was assayed by autoradiography with a PhosphorImager (Molecular Dynamics, Sevenoaks, Kent, United Kingdom). This technique is routinely used to detect and quantify radioactive labels used in cellular and molecular biological studies. A storage phosphor screen, highly sensitive to β particles, γ rays, and X rays, is used to trap energy from the radioactive decay. The trapped energy is stable until scanned with an 88-μm laser beam (with a PhosphorImager) which releases the stored energy as blue light. The resulting emission is collected by a fiber optic bundle connected to a photomultiplier and digitized. Scanning and data analysis are managed through the Molecular Dynamics software package, ImageQuant.

A calibration curve was prepared by placing samples of various concentrations (1.0, 0.75, 0.5, 0.25, 0.1, 0.075, 0.050, 0.025, and 0.010 mM) in sequential spots (10 μl) on a strip of Whatman 3MM cellulose chromatography paper which was then wrapped in clingfilm. The clingfilm-wrapped sample was exposed to a storage phosphor screen (Molecular Dynamics) for 16 h, and the spots of radioactivity were visualized and quantified with the PhosphorImager. A densitometer scan of the resulting image was made with an ImageQuant software package (Molecular Dynamics) run on a Dell 486/ME personal computer. Image density and subsequent peak area values from the scan were used to construct a standard curve. For comparison, Tc was also quantified in parallel with a Packard 2500 TR Liquid Scintillation Analyzer. A 10-μl sample was added to a vial, and 10 ml of Optiphase HiSafe II scintillation fluid was added (LKB Scintillation Products). Disintegration counts per minute were recorded between 20 and 250 keV for 20 min.

Chromatographic separation of Tc species in solution. Different valences of Tc were separated by paper chromatography. Samples (10 μl) were spotted onto Whatman 3MM chromatography paper and separated with 0.1 M HCl as a mobile phase (20). Papers were acid washed in 2 M HCl prior to use. Air-dried chromatographs were wrapped in clingfilm, and different species of Tc were visualized and quantified with the PhosphorImager as described above. Tc(VII) gave an R_f of 0.7. A Tc(V) standard was prepared by reducing Tc(VII) with $SnCl_2$. The Tc(V) exhibited an R_f of 0.0 when separated chromatographically (20). Freshly prepared $SnCl_2$ solution (15 g in 25 ml of concentrated HCl [4.2 μl]) was added per 100-μl sample as a reductant.

Measurement of total Tc and Tc species identification in culture supernatants. Samples (150 μl) were removed from the cultures periodically and assayed for total Tc in solution. Samples were centrifuged prior to analysis with a Heraeus Sepatech Biofuge 13 Microfuge set at 13,000 rpm for 20 min. A total of 10 μl of culture supernatant was then placed on 3MM cellulose chromatography paper and wrapped in clingfilm. The wrapped filter paper impregnated with sample was exposed to the PhosphorImager screen for 16 h, prior to visualization of the image and quantification with the PhosphorImager. In addition, Tc species in solution were also separated in duplicate samples by paper chromatography, and the component species were quantified with the PhosphorImager.

Reproducibility of data. Protein determinations, PhosphorImager analyses, and scintillation counts were in triplicate; the experimental error was within 5% of the mean throughout.

RESULTS

Quantification of Tc in solution with a PhosphorImager. The PhosphorImager technique was used to quantify accurately the concentration of Tc in solution over several orders of magnitude. The method was sensitive to Tc (a soft β-particle-emitting radionuclide) in the range of 10 μM to 1 mM (Fig. 1a). The limit of detection was approximately 1 μM, although this could be decreased further if the exposure time was increased or if samples of Tc-containing solution were repeatedly applied. Figure 1b shows a densitometer scan of Fig. 1a. The image intensity of each sample (and the corresponding peak area of the image derived from that sample) was proportional to the concentration of Tc applied. A calibration curve was constructed to allow quantification of Tc in the test solution (Fig. 1c). In addition, there was complete agreement between results obtained by the PhosphorImager technique and those obtained by scintillation counting (Fig. 2). A linear relationship (correlation coefficient = 1.00) was recorded between data sets obtained by the two methods.

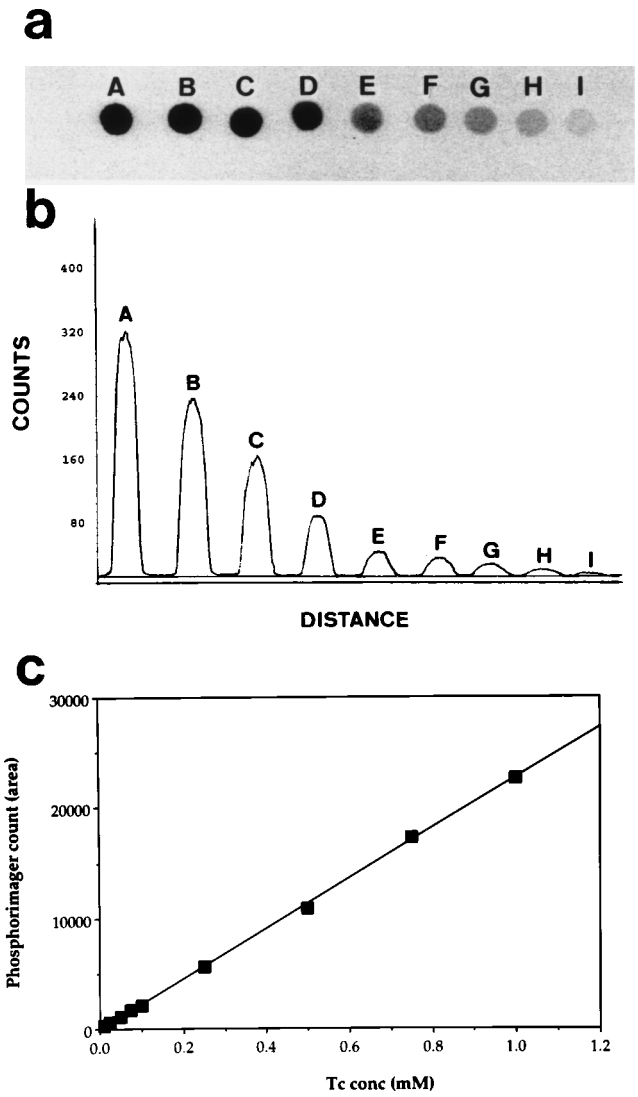

FIG. 1. Construction of a calibration curve for Tc with a PhosphorImager. Ten-microliter samples (10 μM to 1 mM) were spotted onto chromatography paper, air dried, and wrapped in plastic film before exposure to the PhosphorImager screen for 16 h (a). The concentrations of Tc applied were as follows: A, 1 mM; B, 0.75 mM; C, 0.5 mM; D, 0.25 mM; E, 0.1 mM; F, 0.075 mM; G, 0.050 mM; H, 0.025 mM, I, 0.010 mM. A densitometer scan of the image was obtained (b), and the peak area values from the scan were used to construct a calibration curve to allow quantification of the radionuclide in solution (C).

Tc species in solution, separated chromatographically, were also quantified with the PhosphorImager. Tc(VII) and Tc(V) [prepared by reducing Tc(VII) with acidified $SnCl_2$ solution] were separated on cellulose paper by the method of Shukla (20). R_f values of 0.7 and 0.0 were obtained for Tc(VII) and

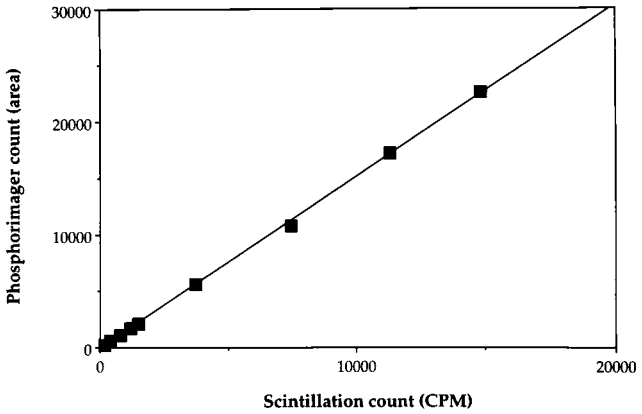

FIG. 2. Correlation of results (peak area) obtained with the PhosphorImager technique with data obtained with a scintillation counter (counts per minute).

Tc(V), respectively, when 0.1 M HCl was used as a mobile phase (Fig. 3). These R_f values were in accordance with published results (20). Image analysis of Fig. 3 by the method shown in Fig. 1 allows accurate measurement of the quantity of Tc(V) and Tc(VII) in solution. The amounts of radioactivity in the Tc(VII) and Tc(V) spots in chromatographs were also checked with a scintillation counter and were within 5% of the results obtained with the PhosphorImager. Regions of the chromatographs with high activity were cut out and analyzed, and results were corrected to account for 13% of the activity quenched by the filter paper added with the sample.

Tc reduction by *S. putrefaciens*. After 22 h of incubation under anaerobic conditions, approximately 65% of the 0.25 mM Tc present had been reduced by *S. putrefaciens* to Tc(V), Tc(IV), and one unidentified species [R_f for Tc(V) = 0.0 and for Tc(IV) = 0.9 (20); unidentified species R_f = 1.0] (Fig. 4a). Tc(VII) was not reduced by cultures of the organism grown aerobically in nutrient broth (Oxoid) or in control experiments containing dead (autoclaved) cells, in which the concentration of Tc(VII) remained constant (data not shown). The stability of the dissolved nascent Tc species exposed to air was assessed by vigorously shaking filter-sterilized samples (1 ml) in 25-ml Erlenmeyer flasks. No change in Tc species formation was recorded over 24 h.

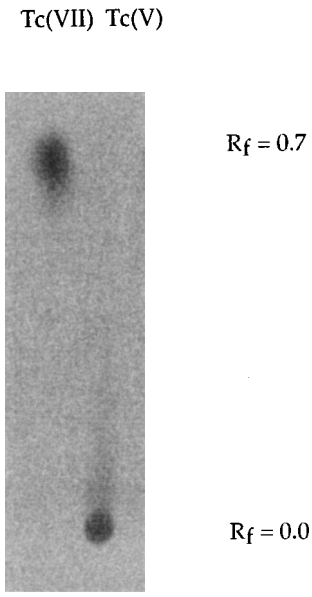

FIG. 3. Migration of Tc(VII) (left) and Tc(V) (right) on Whatman 3MM cellulose paper with 0.1 M HCl as the mobile phase. Tc(VII) and Tc(V) gave R_f values of 0.7 and 0.0, respectively. Tc species were visualized with the PhosphorImager as described in the text.

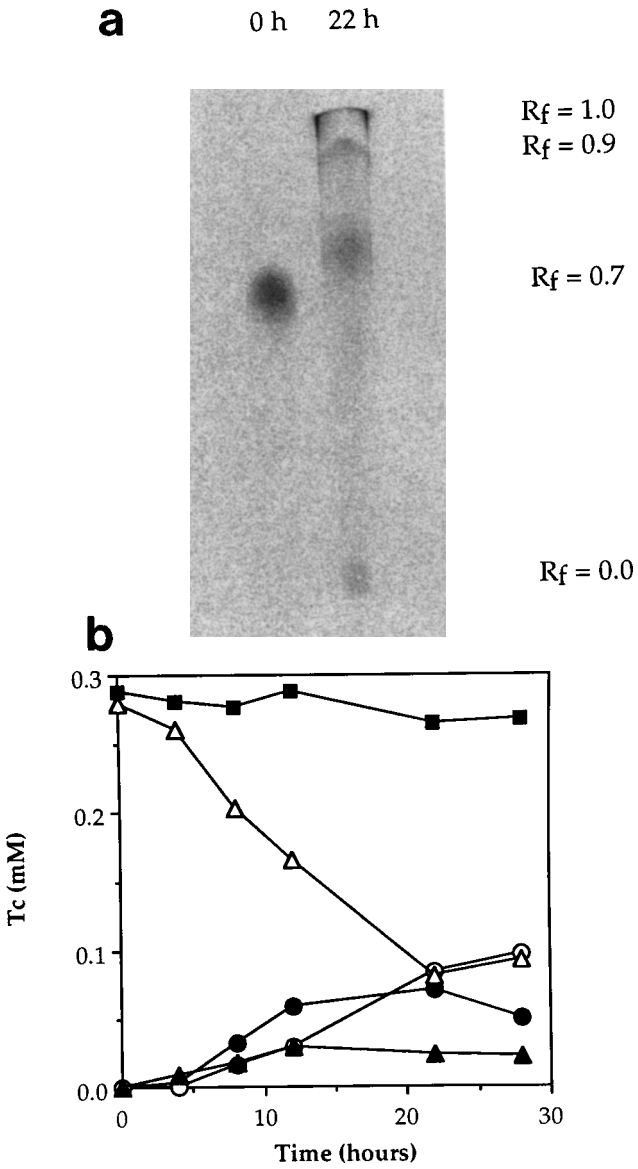

FIG. 4. Tc reduction by anaerobic cultures of *S. putrefaciens*. After 22 h of incubation, Tc(IV) (R_f = 0.9), Tc(V) (R_f = 0.0), and an unidentified Tc species (R_f = 1.0) were detected in culture supernatants in addition to Tc(VII) (R_f = 0.7), which was added at the start of the experiment (a). The concentrations of Tc(VII) (△), Tc(IV) (●), Tc(V) (▲), and the unidentified Tc species (○) were quantified with the PhosphorImager at regular intervals over a 28-h period (b). Total Tc in solution was also determined (■).

The kinetics of Tc reduction were monitored over a 28-h period (Fig. 4b). Assuming a cell concentration of 0.31 mg (dry weight) of cells ml^{-1} (0.17 mg of protein ml^{-1}; biomass contains 55% [dry weight] protein [17]), the rate of Tc reduction was calculated to be 28 nmol of Tc mg of biomass (dry weight)$^{-1}$ h^{-1} over the first 22 h of the experiment. At the end of the experiment, approximately 33% remained as Tc(VII),

8% remained as Tc(V), 17% remained as Tc(IV), and 35% remained as the unidentified Tc species. Although much of the Tc(VII) was reduced by anaerobic cultures of *S. putrefaciens*, only 7% of the Tc was removed from solution by the organism. The concentration ratio (becquerels of Tc per g [dry weight] to becquerels of Tc ml of spent medium^{-1}) was 65. Similar results were obtained with the dead nonreducing control cultures (concentration ratio was 49), and this bioaccumulation probably represented nonspecific biosorption onto the biomass.

Tc reduction by *G. metallireducens*. PhosphorImager analysis of chromatographs obtained from supernatants from anaerobic cultures of *G. metallireducens* showed that Tc(VII) was metabolized to Tc species with R_f values of 0.0 [Tc(V)] and 1.0 (unidentified) (Fig. 5a). No reduction was noted in control experiments containing dead (autoclaved) cells. Low concentrations of the two reduced Tc species were detected in the medium (Fig. 5b). Approximately 22% remained as Tc(VII), 4% remained as Tc(V), and 4% remained as the unidentified Tc species. A total of 70% of the Tc added was removed from solution. The maximum specific rate of Tc reduction was slightly lower than that recorded in cultures of *S. putrefaciens* (22 nmol of Tc mg of biomass [dry weight]$^{-1}$ h^{-1}), assuming a biomass concentration of 0.69 mg (dry weight) of cells ml^{-1}. The rate of reduction dropped significantly after 8 h. The final concentration ratio was 3,382 for the live cells compared with 58 for the dead cells; the latter is comparable to the biosorption seen with *S. putrefaciens* (above).

DISCUSSION

A PhosphorImager-based technique was used to detect and quantify Tc in solution by autoradiography. The technique offers several advantages compared with other liquid-based methods used to quantify radionuclides in solution (e.g., scintillation counting). The samples to be analyzed are impregnated on filter paper and sealed in plastic laboratory film, allowing safe handling of the radionuclides and simplifying disposal of the waste. The technique is also more sensitive than X-ray film autoradiography. Johnston et al. (8) determined that a storage phosphor screen is approximately 250 times more sensitive to β-particles from ^{32}P than X-ray film. In addition, the PhosphorImager-based technique offers a linear dynamic range over 5 (compared with 3) orders of magnitude. The limit of detection is dependent on the type of radiation emitted by the isotope. This technique has been routinely used in our laboratory to quantify α-particle, β-particle, and γ-ray-emitting radionuclides at millimolar, micromolar, and picomolar concentrations, respectively. Samples of α-particle-emitting isotopes (e.g., ^{238}U, ^{232}Th, ^{237}Np, and ^{239}Pu) enclosed in plastic film are detected, probably via the small amount of penetrating γ-radiation emitted by the radionuclides, since the penetrative capacity of the α-particles is small. In practice, the usefulness of this technique for α-particle emitters is limited, since a relatively concentrated solution is required; indeed, at millimolar concentrations chemical assays are available. For routine analyses of α-particle-emitting transuranic elements, it has been found convenient to spike the common α-particle-emitting isotopes ^{237}Np and ^{239}Pu with appropriate γ-ray- and β-particle-emitting isotopes of the elements for evaluation of microbial activity against these actinides (10a).

A major advantage of the PhosphorImager technique is that it can be used to quantify radionuclides present in samples in which regions of activity are spatially separated. As such, it can be used to quantify different oxidation states of radionuclides separated chromatographically, as applied in this study for analysis of a mixed-valence solution of Tc. A similar technique has also been developed in our laboratory for separating actinide(VI) and -(IV) and lanthanide(III) species (23).

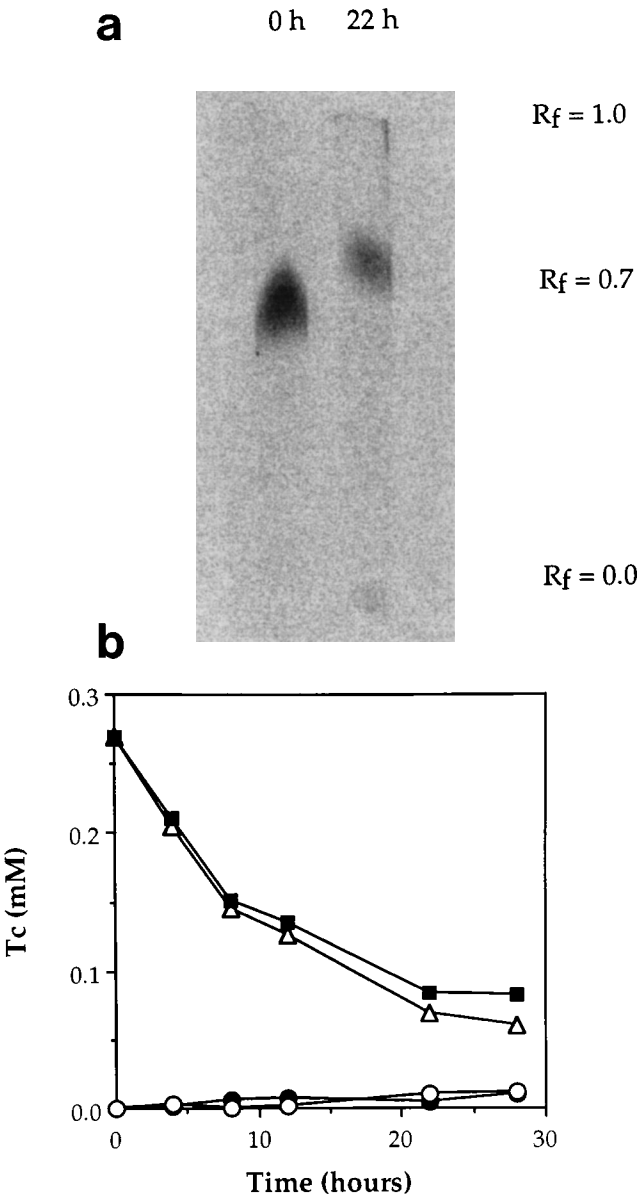

FIG. 5. Tc reduction by anaerobic cultures of *G. metallireducens*. After 22 h of incubation, Tc(V) (R_f = 0) and an unidentified Tc species (R_f = 1.0) were detected in culture supernatants in addition to Tc(VII) (R_f = 0.7), which was added at the start of the experiment (a). The concentrations of Tc(VII) (△), Tc(V) (○), and the unidentified Tc species (●) were quantified with the PhosphorImager at regular intervals over a 28-h period (b). Total Tc in solution was also determined (■).

Anaerobic cultures of *S. putrefaciens* and *G. metallireducens* were tested for the ability to reduce Tc(VII) by the PhosphorImager technique described here. Anaerobic cultures of both organisms, pregrown on ferric iron (supplied as an electron acceptor), were able to reduce Tc(VII) in lieu of Fe(III). Aerobic cultures of the facultative anaerobe *S. putrefaciens* were unable to reduce Tc. *G. metallireducens* is obligately anaerobic, precluding this test.

Although both organisms were able to reduce Tc, the profile of nascent Tc species was different in the two cultures. *S.*

putrefaciens reduced Tc(VII) to Tc(IV), Tc(V), and an unidentified Tc species. Although little Tc was removed by *S. putrefaciens*, coimmobilization with sulfhydryl-rich biochemicals may result in further reduction of Tc to low-valence insoluble species (15). In this context, the use of immobilized metallothionein has been demonstrated for the removal of Cd from solution (4). This material is not yet commercially available (3a) but would repay investigation against Tc (15).

G. metallireducens also reduced Tc and removed 70% of the presented radionuclide from solution. A total of 8% of the residual Tc was detected as Tc(V) and an unidentified species. The mode of removal may have been due to the formation of the insoluble hydrolysis product of Tc(V) (Tc_2O_5) (20) or further reduction of Tc(V) or Tc(IV) to an alternative insoluble oxide. A further bioreduction could be achieved via a redox active enzyme or by a cysteine-mediated reaction. It is unlikely that nonspecific binding of the pertechnetate anion to the cell biomass was responsible for Tc removal. Removal was sustained over a 22-h period, but biosorption by physiochemical processes would have reached equilibrium rapidly. A recent study has shown that biosorption of Tc to the biomass of over 30 microbial isolates accounted for less than 10% of the removal of the radionuclide from solution (10a). The concentrations of biomass and Tc used in the present study were similar to those used in the previous study. The mode of Tc removal from solution by *G. metallireducens* awaits further investigation.

The finding that *G. metallireducens* can reduce and remove Tc has important implications regarding other microbes that may be able to perform this transformation. The related sulfate-reducing bacteria have the ability to remove Tc from solution, but it was previously thought that this was achieved indirectly, with microbially derived H_2S acting as the reductant (6). Lovley (12) has, however, argued that the recent observation that sulfate-reducing bacteria (*D. vulgaris* and *Desulfovibrio desulfuricans*) can reduce U(VI), Cr(VI), and Fe(III) makes it likely that these organisms may be able to reduce Tc(VII) enzymatically. Indeed, the uranium-reducing enzyme of *D. vulgaris* has been identified as cytochrome c_3 (12). 16S rRNA phylogenetic analysis has shown that the gram-negative sulfate- and sulfur-reducing bacteria are the closest known relatives to *G. metallireducens* in the delta proteobacteria (13). The observation that *G. metallireducens* can reduce Tc supports the hypothesis that sulfate-reducing bacteria may be able to achieve this biotransformation enzymatically. If Tc-reducing activity is widespread in environmental isolates, it may have implications for Tc mobility in anaerobic sediments.

In conclusion, by a novel PhosphorImager-based technique, two culture type bacteria with documented metal-reducing capabilities have been shown to be able to reduce the artificial element Tc. This technique is currently being used to screen new microorganisms for the ability to reduce and remove this and other environmentally important radionuclides from solution. The ultimate aim of this work is to harness such organisms to treat Tc-contaminated waste and groundwaters in bioremediation programs.

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