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Reduction and Removal of Heptavalent Technetium from Solution by *Escherichia coli*

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Anaerobic, but not aerobic, cultures of *Escherichia coli* accumulated Tc(VII) and reduced it to a black insoluble precipitate. Tc was the predominant element detected when the precipitate was analyzed by proton-induced X-ray emission. Electron microscopy in combination with energy-dispersive X-ray analysis showed that the site of Tc deposition was intracellular. It is proposed that Tc precipitation was a result of enzymatically mediated reduction of Tc(VII) to an insoluble oxide. Formate was an effective electron donor for Tc(VII) reduction which could be replaced by pyruvate, glucose, or glycerol but not by acetate, lactate, succinate, or ethanol. Mutants defective in the synthesis of the transcription factor FNR, in molybdenum cofactor (molybdopterin guanine dinucleotide [MGD]) synthesis, or in formate dehydrogenase H synthesis were all defective in Tc(VII) reduction, implicating a role for the formate hydrogenlyase complex in Tc(VII) reduction. The following observations confirmed that the hydrogenase III (Hyc) component of formate hydrogenlyase is both essential and sufficient for Tc(VII) reduction: (i) dihydrogen could replace formate as an effective electron donor for Tc(VII) reduction by wild-type bacteria and mutants defective in MGD synthesis; (ii) the inability of *fnr* mutants to reduce Tc(VII) can be suppressed phenotypically by growth with 250 μM Ni^{2+} and formate; (iii) Tc(VII) reduction is defective in a *hyc* mutant; (iv) the ability to reduce Tc(VII) was repressed during anaerobic growth in the presence of nitrate, but this repression was counteracted by the addition of formate to the growth medium; (v) H_2, but not formate, was an effective electron donor for a Sel^- mutant which is unable to incorporate selenocysteine into any of the three known formate dehydrogenases of *E. coli*. This appears to be the first report of Hyc functioning as an H_2-oxidizing hydrogenase or as a dissimilatory metal ion reductase in enteric bacteria.

The long-lived β-emitter technetium (^{99}Tc), a fission product of ^{235}uranium, is produced during the generation of nuclear power. In its most stable form, Tc(VII), typified by the pertechnetate ion (TcO_4^-), is highly soluble and mobile in the environment (27). This factor, in combination with a long half-life (2.1×10^5 years) and high biological availability as a sulfate analog (3), makes removal at the source necessary. From a recent study, it was concluded that Tc may be the critical radionuclide in determining the long-term impact of the nuclear fuel cycle (45).

An approach to achieve the removal of Tc(VII) from aqueous solution may be to use metal-reducing microorganisms to reduce the radionuclide to an insoluble oxide (25, 27). For example, TcO, TcO_2, and Tc_2O_5 all form insoluble precipitates at neutral pH (20, 29, 41). Although there has been much speculation that bacteria may be able to reduce Tc enzymatically, few organisms have been shown conclusively to achieve this biotransformation (25).

Anaerobically grown cultures of soil bacteria were shown by Henrot (15) to accumulate Tc(VII). The addition of pure cultures of sulfate-reducing bacteria increased removal by more than an order of magnitude, and it was postulated that Tc precipitation was mediated by microbially produced H_2S with subsequent formation of an insoluble sulfide. Enzymatic reduction and accumulation of Tc(VII) by a *Moraxella* sp. and a *Planococcus* sp. was reported by Pignolet et al. (36), who noted that control cultures containing heat-treated cells, or live cells incubated at 4°C, concentrated significantly less Tc than cells incubated at 21°C. Lloyd and Macaskie (22) have recently used a novel PhosphorImager-based technique to monitor the reduction of Tc(VII) by *Shewanella putrefaciens* and *Geobacter metallireducens*, both of which have documented metal-reducing capabilities (26, 32). Resting cultures of *S. putrefaciens* coupled the oxidation of lactate to the reduction of Tc(VII). Three soluble, reduced Tc species were detected in culture supernatants. Only trace amounts of Tc, however, were removed from solution (22). Soluble, reduced Tc species were also detected in supernatants from resting cultures of *G. metallireducens* supplied with acetate as an electron donor. Appreciable quantities of the radionuclide were removed from solution by this organism, possibly as a low-valence insoluble oxide of Tc.

To date, the enzymes responsible for the reduction of Tc(VII) by these organisms and the chemical factors underlying precipitation of the reduced Tc remain uncharacterized. In the present study, we report that *Escherichia coli* is able to reduce Tc(VII), facilitating removal of the radionuclide from solution. The established role of *E. coli* in fundamental bacteriological studies and the subsequent wealth of information regarding the physiology and genetics of the organism make it an ideal model system in which to identify the enzyme(s) responsible for Tc(VII) reduction. Physiological and genetic analysis of the Tc(VII)-reducing system in *E. coli* and chemical analysis of the reduced Tc precipitate are described.

MATERIALS AND METHODS

Organisms and culture conditions. The *E. coli* K-12 strains used in this study and their sources are listed in Table 1.

Aerobic starter cultures were grown overnight in 250-ml Erlenmeyer flasks containing 100 ml of nutrient broth no. 2 (25 g liter of distilled $water^{-1}$; Oxoid).

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TABLE 1. *E. coli* strains used and their sources

Strain	Genotype	Derivation, source, or reference
DSS301	TG1 *dms*	V. Méjean
FM909	MC4100 *fdhF*	35
FM932	MC4100 Δ(*fdhF-nrf*)	1
HD705	MC4100 *hycE*	38
JCB387	RV Δ*nir*: parental strain	16
JCB303	JCB387 Chl^r	28
JCB351	JCB387 *dsbA*	30
JCB352	JCB387 *nrf*	8
JCB353	JCB387 *nrf*	8
JCB606	JCB387 *dipZ*	7
JCB3513	JCB351 *fnr*	Transduce JCB351 to Tet^r with JCB3871-P1
JCB3871	JCB387 *fnr*	34
JCB3876	JCB387 *fdhF*	8
JCB3877	JCB387 *fdnG*	8
JCB3878	JCB387 Δ(*fdhF-nrf*)	9
JCB3880	JCB387 *fdnG fdhF*	8
JCB3881	JCB387 Δ*sel*	8
JCB3882	JCB387 *fdnG fdhF fdo*	8
JCB9321	FM932 *torC*::Ω	7
JCB9322	FM932 *dms*::Tn*5*	7
JCB9323	FM932 *dms torC*	7
JCB9324	FM932 *dipZ*	7
JCB9325	FM932 *dipZ dms*	7
JRG780	MC4100*frd*	J. Guest
JRG996	MC4100*frd*	J. Guest
LCB628	MC4100 *torC*	V. Méjean
M17s	MC4100, *hycB*::Mud1	A. Böck
MC4100	Parental strain	2
RK4353	Parent of RK strains	V. Stewart
RK4265	RK4353 *narG*	V. Stewart
RK5200	RK4353 *chlA*	V. Stewart
RK5201	RK4353 *chlE*	V. Stewart
RK5206	RK4353 *chlG*	V. Stewart
JW136	HfrH Δ*hya*::*kan* Δ*hyb*::*kan*	A. Böck

Flasks were incubated at 30°C and shaken at 100 rpm on an orbital shaker. A 5% (vol/vol) inoculum from the starter culture was used in all experiments. Oxygen-limited cultures were grown in sealed 2-ml cryopreservation tubes (Sarstedt, Numbrecht, Germany) containing 1.5 ml of nutrient broth no. 2 (Oxoid) supplemented with 0.5% (vol/vol) glycerol and 25 mM sodium fumarate. The tubes were incubated at 30°C. The same medium was used in aerobic cultures (2 ml), which were incubated at 30°C in a 50-ml flask (shaken at 100 rpm). The flasks were sealed with Sealon film (Fuji Film Co.) to prevent evaporation of the medium. Growth of the organism was determined by measurements of the optical density at 600 nm with a Pharmacia LKB Novaspec II spectrophotometer.

Tc(VII) reduction by growing cells. Filter-sterilized solutions of ammonium pertechnetate (NH_4TcO_4: Amersham International, Amersham, Buckinghamshire, United Kingdom) were added aseptically to the growth medium (final concentration, 250 μM [equivalent to 0.5 μCi/ml]) prior to inoculation. Cultures were grown well into the stationary phase (up to 100 h); samples were removed periodically, and Tc in solution and Tc associated with the biomass were measured.

Resting cell assays. Bacteria were grown in nutrient broth supplemented with glycerol and fumarate in cryopreservation tubes for 24 h at 30°C as described above and collected by centrifugation (13,000 rpm for 10 min at ambient temperature in an IEC Centra M centrifuge). The bacterial pellet was washed twice in phosphate-buffered saline (20 mM, pH 7.0; supplemented with 8.5 mM NaCl) and resuspended in the cryopreservation tubes in 1.5 ml of phosphate-buffered saline to a concentration of about 0.5 mg (dry weight) ml^{-1}. Filter-sterilized solutions of ammonium pertechnetate were added to a final concentration of 250 μM. When sodium formate, sodium acetate, sodium pyruvate, glycerol, or ethanol was used as an electron donor, filter-sterilized solutions were added to the Tc-containing cell suspensions (final concentration, 25 mM) and the reaction mix was incubated in the sealed cryopreservation tubes. When hydrogen was supplied as the electron donor for Tc(VII) reduction, the radionuclide-supplemented cell suspension (1.5 ml) was injected, by use of a syringe fitted with a hypodermic needle, into a 12-ml serum bottle (Adelphi Tubes Ltd., Hayward's Heath, Sussex, United Kingdom) sealed with a butyl rubber stopper and the headspace was filled with hydrogen. All samples were incubated at 30°C. Aliquots were taken regularly for measurement of Tc(VII) remaining in solution.

Analysis of precipitates by PIXE. Cell pellets were collected at ambient temperature with a Heraeus Sepatech Biofuge 13 microcentrifuge set at 13,000 rpm for 10 min. The cells were rinsed twice in distilled water, followed by chloroform and methanol (1:1 vol/vol), to extract organic material and then resuspended in distilled water. Samples were then analyzed by proton-induced X-ray emission (PIXE) with the Oxford Scanning Proton Microprobe (12, 17, 46) at the Department of Nuclear Physics, University of Oxford. Elemental maps of 2-mm^2 specimens mounted on hollow aluminum targets within the proton beam were obtained. Samples were dried under nitrogen gas prior to analysis. Matrix major element composition and thickness, which are needed to calculate PIXE corrections, were determined by use of simultaneously determined Rutherford backscattering (RBS) spectra. The intrinsic accuracy of PIXE with the RBS correction was demonstrated by comparison of data obtained by PIXE with that determined by other methods (44).

Electron microscopy. Bacterial pellets were harvested with a microcentrifuge as described above, rinsed twice in distilled water, and sectioned for viewing. Bacterial pellets were fixed for 60 min in 2.5% (wt/vol) aqueous glutaraldehyde, washed once in distilled water, and then fixed for a further 60 min in 1% (wt/vol) aqueous osmium tetroxide. The cells were then dehydrated in progressively more concentrated ethanol solutions (70, 90, 100, 100, and 100% [vol/vol] ethanol; 15 min each step). Two 15-min washes in propylene preceded embedding in epoxy resin under vacuum for 20 min. The resin was then left to polymerize for 24 h at 60°C. Sections (100 to 150 nm thick) were cut from the resin block with a microtome and placed onto a carbon-coated copper grid prior to analyses. Sections were viewed with a JEOL 120CX2 transmission electron microscope (TEM) fitted with a Link ISI energy-dispersive X-ray analysis (EDAX) system (JEOL Ltd., Welwyn Garden City, Hertsfordshire, United Kingdom). The limit of resolution of the EDAX microprobe was approximately 0.1 μm.

Measurement of Tc by PhosphorImager. Tc in solution was assayed by autoradiography with a PhosphorImager (Molecular Dynamics, Sevenoaks, Kent, United Kingdom) as described by Lloyd and Macaskie (22). Samples (150 μl) were removed from the cultures and placed in an Eppendorf tube. A 10-μl sample was placed on 3MM cellulose chromatography paper, and the remaining sample was centrifuged with a Heraeus Sepatech Biofuge 13 microcentrifuge set at 13,000 rpm for 20 min. The culture supernatant (10 μl) was placed adjacent to the noncentrifuged sample on the chromatography paper, and the paper was wrapped in plastic wrap. The radionuclide-impregnated paper was then exposed to a storage phosphor screen (Molecular Dynamics). After 16 h of exposure, the spots of radioactivity were visualized with the PhosphorImager and quantified by densitometry with an Image Quant software package (Molecular Dynamics). The percentage of the initial Tc removed after centrifugation was calculated by dividing the peak height obtained from the sample (prior to centrifugation) by the peak height obtained from the culture supernatant.

RESULTS

Tc(VII) removal by anaerobic cultures of *E. coli*. Anaerobic cultures of *E. coli* JCB387, which is defective only in the NADH-dependent nitrite reductase activity, progressively accumulated Tc(VII) from the growth medium and converted it to a black precipitate (Fig. 1B). In contrast, very little Tc(VII) was removed from solution during aerobic growth, and no black precipitate was formed (Fig. 1A). The slight initial decrease in the Tc(VII) content of the growth medium of aerobic cultures was probably due to adsorption to the bacterial surface. The maximum rate of Tc(VII) removal, which was preceded by a period of growth, occurred between 20 and 70 h after inoculation, following depletion of residual dissolved oxygen. After 96 h of incubation, approximately 90% of the 250 μM Tc(VII) added was removed from solution and the anaerobic cultures contained a black precipitate. It was therefore suggested that enzymatically mediated reduction of the heptavalent form of the radionuclide had facilitated its removal from solution as a black insoluble precipitate and that the enzyme system responsible for Tc(VII) reduction was induced under anaerobic conditions. This hypothesis formed the basis for subsequent studies.

Solid-state analysis of black Tc-containing precipitate. Additions of the radionuclide to the growth medium, to a concentration of 250 μM, had no discernible effect on the growth of the organism. To minimize any potentially deleterious effects of higher concentrations on cell growth and metabolism, the amount of Tc precipitated by the organism was increased

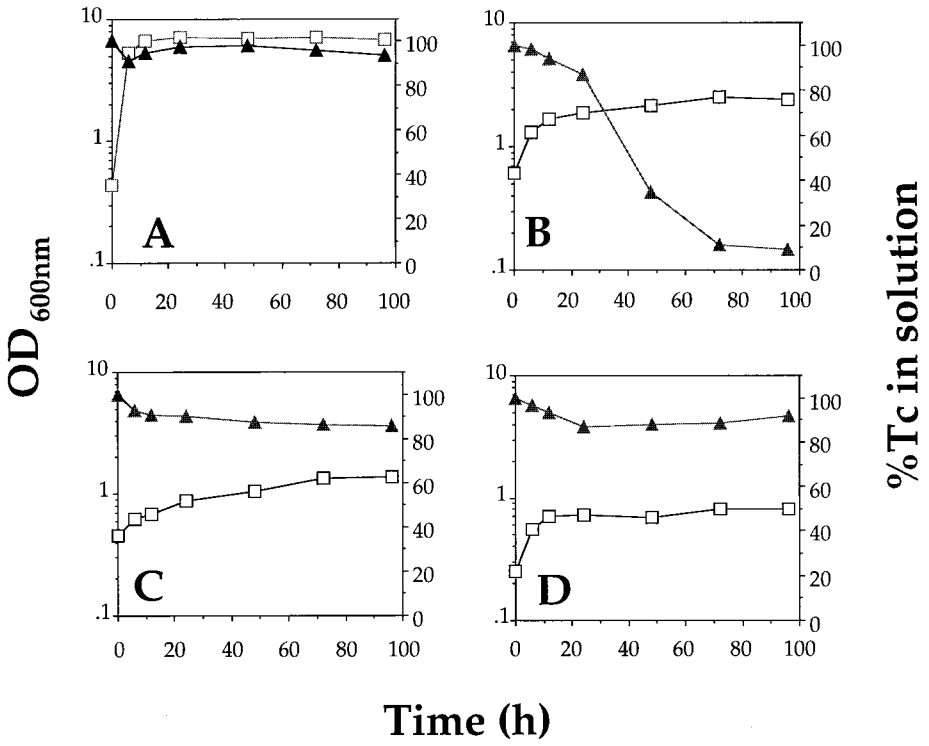

FIG. 1. Growth (squares) and Tc removal (triangles) from solution by various *E. coli* strains. (A and B) Aerobic (A) and anaerobic (B) cultures of the parental strain JCB387; (C) anaerobic culture of the *fnr* mutant strain JCB3871; (D) anaerobic culture of the molybdenum cofactor-deficient strain JCB303. Cells and insoluble, reduced Tc were removed from cultures by centrifugation prior to measurement of the percent Tc remaining in the supernatant with a PhosphorImager. OD_{600}, optical density at 600 nm.

by four sequential 250 μM additions of the radionuclide at 36-h intervals to anaerobic cultures. The bacteria and resulting black precipitate formed were removed by centrifugation after a total of 6 days of incubation. Analysis of the black precipitate (purified by extraction of organic material with chloroform-methanol) by PIXE showed that it contained Tc (Fig. 2). The radionuclide was the most abundant element detected in the sample. The molar ratios of Tc to other elements detected were 1:0.63 (P), 1:0.37 (S), 1:0.05 (Ca), 1:0.04 (K), 1:0.03 (Zn), 1:0.02 (Si), 1:0.02 (Fe), 1:0.004 (Cu), and 1:0.003 (Ni). These elements are normal bacterial constituents (33). Attempts to determine the structure of the purified precipitate by X-ray diffraction were unsuccessful because there was only a single broad peak in the spectrum characteristic of precipitates with a noncrystalline structure (23).

TEM studies: site of Tc deposition. Thin sections of bacteria that had been incubated in the presence Tc(VII) (four 250 μM additions to a final concentration of 1 mM, as described above) were viewed by use of TEM. The site of the deposition of reduced Tc was intracellular. The cytoplasm of bacteria challenged with Tc(VII) was stained electron dense by the radionuclide (Fig. 3A). Control bacteria which had not been challenged with the radionuclide or aerobically incubated, Tc(VII)-supplemented bacteria were unstained (Fig. 3B). Analysis of the sections by TEM in combination with EDAX confirmed that regions of heavy staining in the Tc-challenged cultures contained the radionuclide (Fig. 3C and D). No Tc was detected outside the cell.

Reduction of Tc(VII) requires the transcription factor FNR. Many anaerobically induced enzymes are controlled by the FNR protein which activates the transcription of genes for components of many anaerobically induced electron transfer chains (42). An *fnr* mutant, *E. coli* JCB3871, was therefore tested for the ability to reduce Tc(VII). Even during anaerobic growth, only a very low rate of Tc(VII) reduction was detected and no black precipitate was formed (Fig. 1C). Better growth was obtained when the culture of the FNR mutant was supplemented with glucose, but the pelleted bacteria were pale grey rather than black like the parental strain. These results indicated that the enzyme or enzyme complex required for Tc(VII) reduction is part of the FNR regulon.

Formate is an effective electron donor for Tc reduction. Formate is a major end product of glycolysis during anaerobic growth of *E. coli*. Its formation from pyruvate is catalyzed by the enzyme pyruvate-formate lyase (19). Formate is also a strong reducing agent which can serve as an effective electron donor for several anaerobically induced electron transfer pathways. The abilities of formate, precursors of formate, other fermentation products, and the growth substrates glucose and glycerol to support Tc(VII) removal were therefore compared (Fig. 4). The most effective electron donors tested included formate and its precursor, pyruvate, as well as glucose and glycerol. At most, 20% of the added Tc(VII) was removed by unsupplemented control cultures, and Tc(VII) removal was not stimulated by the addition of acetate, lactate, succinate, or ethanol.

All three known formate dehydrogenases in *E. coli* are molybdoproteins (39, 40). If formate is the physiological electron donor for Tc(VII) reduction during anaerobic growth with glycerol as the primary carbon source, mutants defective in the synthesis of the molybdenum cofactor MGD (molybdopterin guanine dinucleotide) should also be unable to reduce Tc(VII). This was confirmed (Fig. 1D). Unlike the FNR mu-

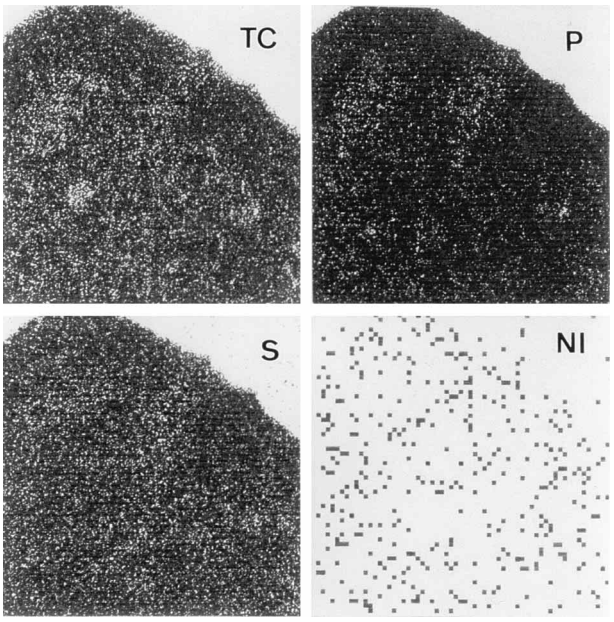

FIG. 2. PIXE analysis of 2-mm^2 area scans of black precipitate from anaerobic *E. coli* JCB387 cultures supplemented with Tc(VII). Elemental maps of the distribution of Tc, P, S, and Ni are shown. RBS corrections were done on selected specimen microareas to give absolute elemental concentration.

tant, which reduced Tc(VII) at a very low but significant rate, no reduction was detected with MGD mutants.

Lack of involvement of anaerobically induced reductases in Tc(VII) reduction. During anaerobic growth, enteric bacteria can reduce several alternative electron acceptors, which include nitrate, nitrite, fumarate, dimethyl sulfoxide (DMSO), and trimethylamine-*N*-oxide (TMAO). Formate is an effective electron donor for many of these enzymes. Three biochemically and genetically independent nitrate reductases and two nitrite and TMAO reductases have been characterized in *E. coli* (reviewed in reference 4). Only the TMAO reductase encoded by the *tor* operon and the minor respiratory nitrate reductase NarZ are expressed independently of the FNR protein. The periplasmic nitrate and nitrite reductases, like the TMAO-inducible *torCAB* operon encoding the major TMAO reductase, require *c*-type cytochromes to be active. All three of the nitrate reductases and both of the TMAO reductases (one of which, DmsA, is also the DMSO reductase) are molybdoproteins. Mutants defective in each of these reductases or in cytochrome *c* synthesis were therefore tested for the ability to reduce Tc(VII) during anaerobic growth (Table 2).

The preferred electron acceptor during anaerobic growth of *E. coli* is nitrate. During nitrate reduction, formate is oxidized predominantly by the formate dehydrogenase complex encoded by the *fdn* operon and nitrate is reduced to nitrite by the respiratory nitrate reductase encoded by the *narGHJI* operon. Both the *fdn* and *narG* operons are part of the FNR regulon, and both encode molybdoproteins (21, 43). Although it has been suggested that nitrate reductases may catalyze the reduction of metal ions in other bacteria (18), strain RK4265, defective in the major nitrate reductase encoded by the *narGHJI* operon, was as active in reducing Tc(VII) as the parental strain, RK4353 (Table 2). During anaerobic growth in the absence of nitrate, fumarate is reduced to succinate by an energy-conserving electron transfer chain terminating in a fumarate reductase encoded by the *frd* operon (6). Two Frd$^-$ strains, JRG780 and JRG996, and strain dss301, which is defective in the DMSO reductase, were all fully competent for Tc(VII) reduction. Mutants defective in the synthesis of either or both of the major nitrite reductases (strains JCB387 and JCB352) were also competent for Tc(VII) reduction (Table 2), as were mutants defective in the recently characterized periplasmic nitrate reductase (strain JCB71201) (data not shown). Although strain LCB628, defective in the synthesis of TMAO reductase due to an insertion mutation in the *torC* gene, reduced little if any Tc(VII), other Tor$^-$ strains which lacked all *c*-type cytochromes were fully competent for Tc(VII) reduction (strains JCB351 and JCB606) (Table 2). In contrast, no detectable Tc(VII) was reduced and bioaccumulated by mutants such as JCB303, RK5200, RK5201, and RK5206, all of which are resistant to chlorate during anaerobic growth and are therefore defective in MGD synthesis.

All three biochemically and genetically independent formate dehydrogenases in *E. coli* contain an essential selenocysteine residue (40). To investigate whether the MGD requirement for Tc(VII) reduction was due to the involvement of one or more of these formate dehydrogenases, a Sel$^-$ strain, JCB3881, was constructed by P1 transduction and tested for Tc(VII) reduction activity. The Sel$^-$ mutant was unable to reduce Tc(VII) (Table 2). Mutants defective in various combinations of the formate dehydrogenases were then tested. The *fdnG* mutant JCB3877, which lacks the nitrate-linked formate dehydrogenase, reduced Tc(VII) as rapidly as the parental strain JCB387, but all *fdhF* mutants (which lack FdhH, the formate dehydrogenase of the formate hydrogenlyase pathway) were also defective in Tc(VII) reduction (Table 2). It is important to note, however, that although strains defective in the *fdhF* gene failed to form a black precipitate even after 112 h, some of the Tc(VII) was always removed. This varied slightly between experiments but was typically in the range of 20 to 28% of the Tc(VII) initially added (Table 2).

Although the involvement of FdhH in Tc(VII) reduction would explain the inability of the chlorate-resistant strains, which lack MGD, to reduce Tc(VII) (Table 2), FNR does not activate transcription of *fdhF*, the structural gene for FdhH (1). This eliminates the possibility that FdhH transfers electrons directly to Tc(VII). The recognized role of FdhH is to couple formate oxidation to H^+ reduction by hydrogenase III during fermentative growth in acidic media (1, 35). This counteracts the acidification of the growth environment and results in the evolution of dihydrogen gas. Hydrogenase III (Hyc) contains Ni^{2+}, which is essential for its catalytic activity: the FNR protein is required for Ni^{2+} uptake by *E. coli* during growth in media containing only low concentrations of Ni^{2+}. It was possible, therefore, that the FNR requirement for Tc(VII) reduction by formate was due to the involvement of an active Hyc complex which, in turn, requires FNR-dependent expression of the *nik* operon (5, 31, 48).

Physiology of pertechnetate reduction by H_2. The studies with strains defective in well-characterized anaerobic functions strongly suggested that Tc(VII) reduction by formate in *E. coli* is catalyzed by the hydrogenase III component of the FHL complex, electrons being transferred from formate via FdhH and Hyc to Tc(VII). Four types of experiment were designed to confirm this proposal.

Synthesis of all of the components of the formate hydrogenlyase complex is repressed during anaerobic growth with nitrate, but nitrate repression can be reversed, at least in part, by the addition of formate (1). We therefore demonstrated that addition of nitrate to the growth medium strongly suppresses Tc(VII) reduction, but this effect is mitigated by formate. Wild-type bacteria, strain JCB387, were pregrown to the late stationary phase with glycerol and fumarate with or without

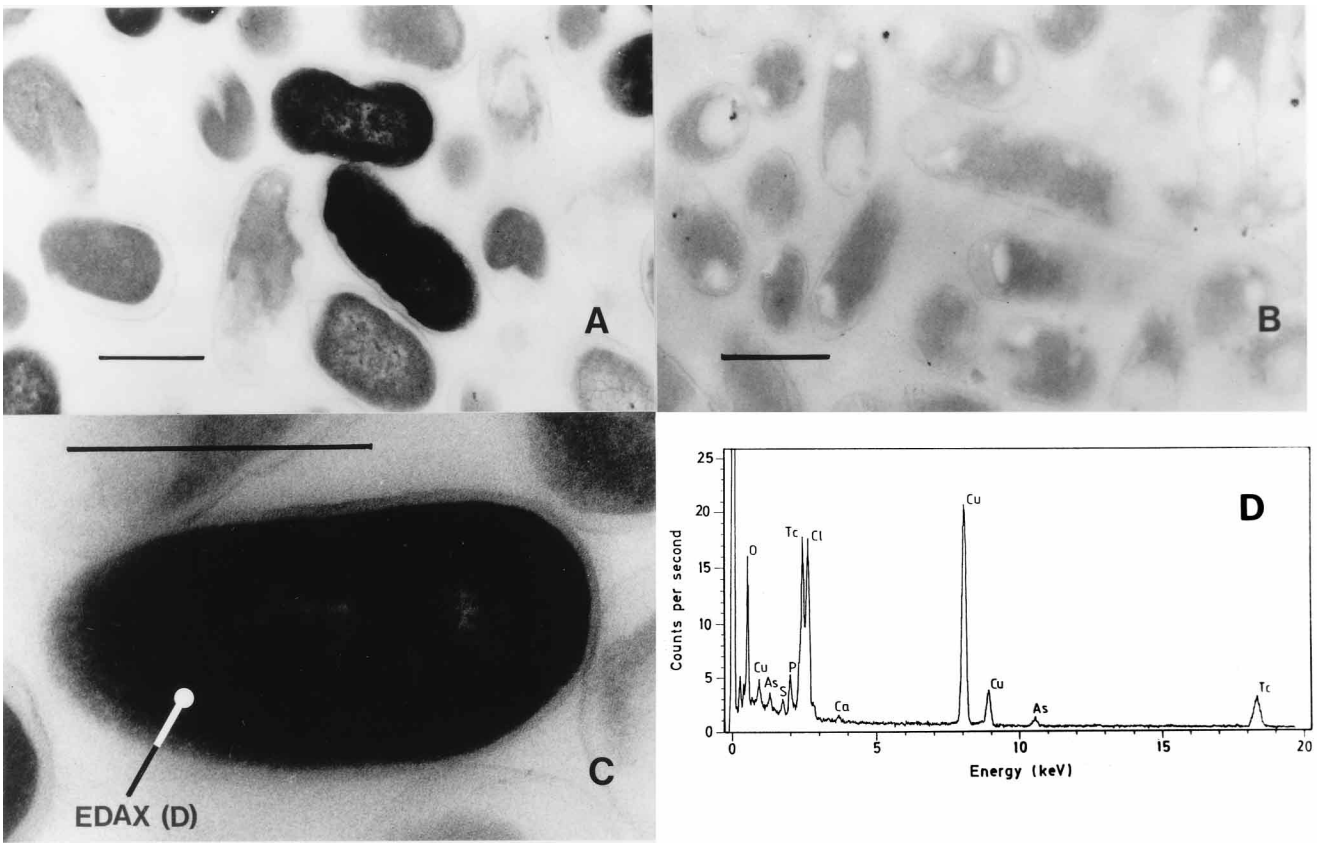

FIG. 3. TEM of thin sections of *E. coli* cells grown under anaerobic conditions in the presence and absence of 1 mM Tc(VII). (A) Cells grown in the presence of the radionuclide were electron dense. (B) Cells which were not challenged with the metal were unstained. (C and D) Solid-state analysis of the sections (C, arrow) by EDAX (D) confirmed that Tc was accumulated within the cell. Cu was from the electron microscope grid. Bars, 1 μm.

supplements of nitrate, formate, or both. The rates of Tc(VII) reduction by resting suspensions of the bacteria in the presence of formate are shown in Fig. 5A. As expected, the presence of nitrate during growth suppressed Tc(VII) reduction and no black precipitate accumulated within the bacteria (Fig. 5A). The addition of formate to nitrate-supplemented cultures substantially reversed nitrate repression of the Tc(VII) reductase activity, which was increased above the background rate of growth in the presence of formate but in the absence of nitrate (Fig. 5A).

It was then established that H_2 was as effective as formate in supporting Tc(VII) reduction by the parental strain JCB387 (data not shown). This raised the possibility that H_2 might also be an effective electron donor for a chlorate-resistant mutant that is defective in MGD synthesis and hence in formate dehydrogenase activity or by the Sel^- strain JCB3881, which is defective in only formate dehydrogenase activity. Both predictions were confirmed by demonstrating that H_2, but not formate, could be used by relevant strains as an electron donor for Tc(VII) reduction (Fig. 5B and C). In contrast, no Tc was reduced by two different mutants defective in Hyc activity, strain M17s, which carries a polar Mud1 insertion in *hycB*, and the *hycE* mutant HD705 (38). A double mutant (JW136) defective in both hydrogenase I and II was, however, able to couple H_2 (and formate) oxidation to Tc(VII) reduction, suggesting that these enzymes play no part in reduction of the radionuclide.

Having established that Hyc can catalyze the oxidation of H_2, at least when coupled to Tc(VII) reduction by a resting bacterial suspension, it was predicted that *fnr* mutants should be competent for Tc(VII) reduction during or after growth in the presence of high concentrations of Ni^{2+} (5). The *fnr* mutant JCB3871 was therefore grown in the presence or absence of 200 μM Ni^{2+} and the rates of Tc(VII) reduction by resting bacterial suspensions were compared. Supplementation with

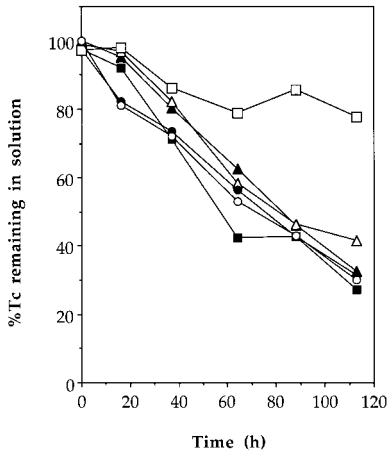

FIG. 4. Reduction and removal of Tc(VII) (250 μM) from solution by resting anaerobic cultures of *E. coli* incubated in phosphate-buffered saline and supplied with 25 mM glucose (△), glycerol (○), formate (●), or pyruvate (■) as an electron donor for metal reduction. No electron donor was added to control cultures (□). Hydrogen (▲) was also supplied in the headspace of the culture bottles. Cells and insoluble reduced Tc were removed from cultures by centrifugation prior to measurement of the Tc remaining in the supernatant with a PhosphorImager.

TABLE 2. Reduction of Tc(VII) by *E. coli* strains defective in the synthesis or activity of anaerobically induced reductases, *c*-type cytochromes, or the MGD cofactor[a]

Strain[b]	Phenotype (defective protein or enzyme activity)	% Tc(VII) removed[c]
JCB387	Nir$^-$ parental strain (NADH-nitrite reductase deficient)	70, 80
JCB3871	Fnr$^-$ (lacks transcription activator for anaerobic growth)	<10, <10
JCB352	Nrf$^-$ (lacks nitrite reduction by formate)	75, 79
JCB351	DsbA$^-$ (Tor$^-$ Nrf$^-$ Nap$^-$; lacks all *c*-type cytochromes)	65, 71
JCB3513	DsbA$^-$ Fnr$^-$	<10, <10
JCB606	DipZ$^-$ (lacks all *c*-type cytochromes)	64, 67
JCB3876	FdhH$^-$ (lacks formate dehydrogenase of FHL)	22, 27
JCB3877	Fdn$^-$ (lacks nitrate-induced formate dehydrogenase)	68, 72
JCB3878	Nrf$^-$ FdhH$^-$	21, 23
JCB3880	FdhH$^-$ Fdn$^-$	17, 22
JCB3881	Sel$^-$ (lacks all formate dehydrogenases)	<10, <10
JCB3882	Lacks all three formate dehydrogenases	11, 24
JCB303	Chl^r (defective in MGD synthesis)	<10, <10
RK4353	Parent of RK strains	63, 57
RK4265	NarG$^-$ (lacks membrane-bound nitrate reductase)	57, 50
RK5200	ChlA$^-$ (defective in MGD synthesis)	<10, <10
RK5201	ChlE$^-$	<10, <10
RK5206	ChlG$^-$	<10, <10

[a] Cells were grown anaerobically in nutrient broth supplemented with 0.5% (vol/vol) glycerol, 25 mM fumarate, and 250 μM Tc(VII).
[b] All of the JCB strains listed lack the major, cytoplasmic, NADH-dependent nitrite reductase (Nir$^-$). All RK strains were derived from RK4353; all others were derived from JCB387.
[c] Values given are averages of results from two separate experiments.

Ni^{2+} alone partially restored Tc(VII) reduction, and the rate was substantially increased by the presence during growth of both Ni^{2+} and formate (Fig. 5D), confirming that the role of FNR in Tc(VII) reduction is a consequence of the indirect requirement for FNR in formate hydrogenlyase synthesis (5, 48). The combined data summarized in Fig. 5 establish that hydrogenase III, i.e., Hyc, from enteric bacteria can oxidize H_2 and is both necessary and sufficient to drive the bioaccumulation and reduction of Tc(VII).

DISCUSSION

Anaerobic cultures of *E. coli* removed Tc(VII) from solution as a black, insoluble precipitate. Tc was the major element detected in the precipitate by PIXE, and the ratio of this element to other major elements precludes precipitation of the radionuclide as a sulfide (TcS_2 or Tc_2S_7) (29). Phosphorus was also detected in the precipitate, but since no insoluble phosphate complexes have been described to date, it is unlikely that the black precipitate was a reduced technetium phosphate. Several low-valence oxides of Tc form black insoluble precipitates under physiological conditions [Tc(V), (IV) and (II)] (22). Recent studies done with X-ray absorption spectroscopy have confirmed, by a characteristic shift in the edge region of the spectrum, that the Tc in the precipitates is indeed reduced from the heptavalent state (23).

By using TEM studies in combination with solid-state EDAX analysis, the site of reduced Tc precipitation was shown to be intracellular, in accordance with the identification of the Tc(VII) reductase as a component of the FHL complex. This complex, the physiological role of which is to counteract acidification, is membrane associated, but the large subunits of Hyc and FdhH are sited in the cytoplasm and are only peripherally associated with the membrane (38). The pertechnetate anion could possibly be transported into the cell by either the sulfate or phosphate uptake system; uptake as a sulfate analog has been demonstrated in soybean plants (3).

Genetic and physiological studies pinpointed the pivotal role of the FHL complex in Tc(VII) reduction. The enzyme system responsible for radionuclide reduction by formate was shown to require the transcription activator FNR in addition to the molybdenum cofactor. Studies using *E. coli* mutants lacking the structural genes for a range of FNR-induced molybdenum cofactor-requiring enzymes, some of which are analogous to metal-reducing enzymes in other organisms, identified the Tc(VII) reductase as a novel metal-reducing enzyme. Karavaiko et al. (18) implicated a broad-specificity nitrate reductase as the enzyme catalyzing Mn(IV) reduction by *Acinetobacter calcoaceticus*. We report that neither the membrane-bound nor the newly discovered periplasmic nitrate reductase (13) was responsible for Tc(VII) reduction by *E. coli*. Type strains of *A.*

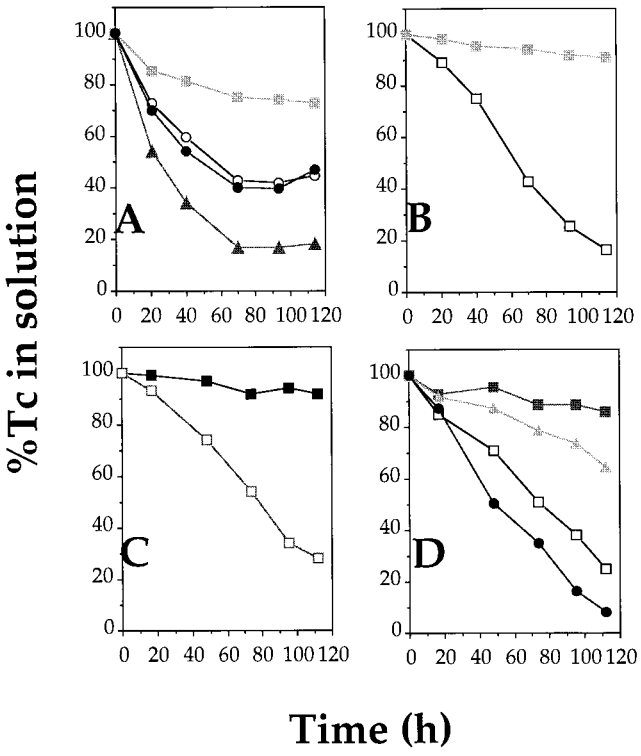

FIG. 5. Identification of Hyc as the Tc(VII)-reducing enzyme in *E. coli*. (A) Effect of formate and nitrate additions to the growth medium on Tc(VII) reductase activity by anaerobic resting cells of the parental strain JCB387. Formate (▲), nitrate (▨), or formate plus nitrate (●) was added to the growth medium to a final concentration of 25 mM. Control cultures (○) were unsupplemented. (B) Reduction and removal of Tc(VII) (250 μM) from resting cultures of the chlorate-resistant *E. coli* mutant strain JCB303, which is unable to synthesize the molybdenum cofactor MGD and hence is deficient in formate dehydrogenase activity. (C) Same as panel B except that the Sel$^-$ mutant JCB3881, which is unable to incorporate selenocysteine into any of the three formate dehydrogenases, was used. (B and C) Tc(VII) reductions by both formate (■) and hydrogen (□) were assayed. (D) Restoration of Tc(VII) reduction by an *fnr* mutant, JCB3871, by supplementation of the medium with Ni^{2+} and formate. Cultures were either unsupplemented (▨) or supplemented with 200 μM Ni^{2+} (▲) or 200 μM Ni^{2+} plus 25 mM formate (●). The parental strain JCB387 was used for the control cultures (□). Cells and insoluble reduced Tc were removed from cultures by centrifugation prior to measurement of the Tc remaining in the supernatant with a PhosphorImager.

calcoaceticus have also been tested in our laboratory and were unable to reduce Tc(VII) (23). The study of DeMoll-Decker and Macy (10) identified the periplasmic nitrite reductase of *Thauera selanitis* as being a selenite reductase. We report that mutants deficient in either of the two known *E. coli* nitrite reductases were, however, able to reduce Tc(VII), establishing that neither of the *E. coli* nitrite reductases contributes significantly to the rate of Tc(VII) reduction. Finally, Lovley and coworkers purified a uranium (chromate) reductase from *Desulfovibrio vulgaris* (25). The enzyme is a *c*-type cytochrome (cytochrome c_3). From our study, we noted that *c*-type cytochromes are not required for Tc(VII) reduction by *E. coli.*

Our results suggest that the reduction of Tc(VII) is catalyzed by the Hyc component of the FHL complex. Particularly interesting is the observation that either the fermentation product formate (long recognized as the source of electrons for H_2 evolution by enteric bacteria [11]) or hydrogen itself can act as the electron donor for Tc(VII) reduction. Since H_2 was an effective electron donor for both growing cultures and resting cell suspensions, this appears to be a physiologically significant activity. If so, it is the first report that Hyc can oxidize H_2, coupling H_2 oxidation to the reduction of an exogenous electron acceptor. Consistent with this suggestion, mutants which could not synthesize the catalytic subunit of Hyc were unable to couple the oxidation of either of these substrates to Tc(VII) reduction. The Mo cofactor MGD was shown to be required only to transfer reducing equivalents from formate via FdhH to Hyc, probably via iron-sulfur electron transfer proteins (38). The requirement for MGD was circumvented by supplying bacteria with hydrogen rather than formate as an electron donor. The FNR requirement was also shown to be indirect and due to a requirement for the Ni^{2+}-processing *nik* gene products to ensure activity of the Ni-containing hydrogenase. The requirement for the FNR protein was circumvented by growing the organism in medium supplemented with Ni^{2+} and formate. Formate was also shown to induce Tc(VII) reductase activity, consistent with the role of the formate-sensitive transcription activator FhlA in FHL synthesis by *E. coli* (37). One unresolved point, however, is why a low rate of formate-dependent Tc(VII) reduction was obtained with mutants defective in the synthesis of all three formate dehydrogenases but not with the Sel^- strain JCB3881 or with chlorate-resistant mutants defective in MGD synthesis.

Following early studies which claimed hydrogenase-mediated reduction of U(VI) by *Micrococcus lactilyticus* (47), *Clostridium pasteurianum* hydrogenase I has recently been identified as a novel selenite reductase (50). Wu and Mandrand (49) have compared 30 sequenced microbial hydrogenases and classified them into six classes according to sequence homology, metal content, and physiological function. On this basis, the clostridial hydrogenase I and *E. coli* Hyc enzymes were allocated to different groups: the clostridial enzyme was classified as an anaerobic, periplasmic Fe-hydrogenase, and the *E. coli* enzyme was classified as a labile, hydrogen-producing Ni-hydrogenase.

Coupling the oxidation of formate or hydrogen to the reduction of Tc(VII) by using resting cells offers a potential bioprocess to reduce and remove the radionuclide from solution. An advantage of the system is that regeneration of a reduced cofactor is not required for continuous use of the biocatalyst. Cofactor regeneration has been cited as a major limitation preventing the widespread use of oxidoreductases in biotransformations (14). A recent study in our laboratory utilized resting *E. coli* immobilized in a membrane bioreactor and challenged with a Tc(VII)-supplemented solution. Bacteria supplied with formate or hydrogen as an electron donor reduced and removed the radionuclide continuously. Cultures were stable for the 2-week period that the immobilized cell reactors were operated (24). The quantity of radionuclide removal was proportional to the logarithm of the residence time in the reactor; at dilution rates up to 0.1 h^{-1}, 80% of the 50 μM Tc(VII) was removed from the feed solution.

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