Reduction of Technetium(VII) by Desulfovibrio fructosovorans Is Mediated by the Nickel-Iron Hydrogenase

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Resting cells of the sulfate-reducing bacterium Desulfovibrio fructosovorans grown in the absence of sulfate had a very high Tc(VII)-reducing activity, which led to the formation of an insoluble black precipitate. The involvement of a periplasmic hydrogenase in Tc(VII) reduction was indicated (i) by the requirement for hydrogen as an electron donor; (ii) by the tolerance of this activity to oxygen, and (iii) by the inhibition of this activity by Cu(II). Moreover, a mutant carrying a deletion in the nickel-iron hydrogenase operon showed a dramatic decrease in the rate of Tc(VII) reduction. The restoration of Tc(VII) reduction by complementation of this mutation with nickel-iron hydrogenase genes demonstrated the specific involvement of the periplasmic nickel-iron hydrogenase in the mechanism in vivo. The Tc(VII)-reducing activity was also observed with cell extracts in the presence of hydrogen. Under these conditions, Tc(VII) was reduced enzymatically to soluble Tc(V) or precipitated to an insoluble black precipitate, depending on the chemical nature of the buffer used. The purified nickel-iron hydrogenase performed Tc(VII) reduction and precipitation at high rates. These series of genetic and biochemical approaches demonstrated that the periplasmic nickel-iron hydrogenase of sulfate-reducing bacteria functions as a Tc(VII) reductase. The role of cytochrome c₃ in the mechanism is also discussed.

Technetium (99Tc) is a fission product of 235U formed during the generation of nuclear power. The solubility and mobility of Tc are highly dependent upon its redox state. Under oxic conditions, Tc is present in its most stable form, the pertechnetate anion [Tc(VII)O₄]⁻. This form, which is highly soluble and mobile in the environment, can enter the food chain as a sulfate analogue (2, 20, 34). These properties, coupled with its long half-life (2.13 × 10⁷ years), make contamination by Tc one of the major factors in the long-term impact of the nuclear fuel cycle. One approach to remove 99Tc from aqueous solution is to use the periplasmic reductant Tc(VII) into the insoluble, low-valence form Tc(IV). This can be achieved by abiotic (5) or biotic (19, 20) processes.

Abiotic reduction involves electron transfer between Fe(II)-containing minerals and Tc(VII) (3). The most efficient mineral appears to be magnetite, particularly when it is anodically polarized (5). Biotic precipitation of pertechnetate, probably its reduction into a low-valence, insoluble Tc oxide, has been reported for several species of bacteria during the past few years. These include species such as Geobacter metallireducens (14), Geobacter sulfurreducens (17), Escherichia coli (13), Desulfovibrio desulfuricans (16), Shewanella putrefaciens (33), and Deinococcus radiodurans (9). Both indirect (chemical) and direct (enzymatic) reduction processes have been observed, depending on the bacterial growth conditions. Chemical processes have been clearly demonstrated in the case of the metal-reducing bacterium G. sulfurreducens (15, 17). Cultures of D. desulfuricans supplied with sulfate and lactate as electron acceptor and donor, respectively, precipitated Tc extracellularly as an insoluble sulfide. In this case, the Tc sulfide results from the chemical reaction between H₂S, formed during reduction of sulfate, and TcO₄⁻ (19). A chemical reduction of Tc(VII) by the Fe(II) is also observed during reduction of Fe(II) by G. sulfurreducens (17). In addition, enzymatic reduction of Tc(VII) has been reported for different bacterial species. There are several lines of evidence indicating that this process involves hydrogenase, an enzyme which reversibly catalyzes the splitting of molecular hydrogen into protons and electrons. Indeed, hydrogen is an effective electron donor for Tc(VII) reduction for E. coli, D. desulfuricans, and S. putrefaciens (13, 15, 16, 33). Similarly, in the case of G. sulfurreducens, Tc(VII) reduction has an exclusive requirement for hydrogen as the electron donor, unlike the reduction of Fe(III), which can be coupled to the oxidation of different organic electron donors (17). The most convincing evidence has come from the work of Lloyd et al. (13), who showed that mutants of E. coli defective in the synthesis of transcription factor FNR, of molybdenum cofactor, or of formate dehydrogenase H were unable to reduce Tc(VII), indicating a role for the formate-hydrogenlyase complex in the reduction.

Desulfovibrio fructosovorans (24) is a sulfate-reducing bacterium amenable to molecular biological study. Three different hydrogenases have been identified in this bacterium; [NiFe] and [Fe] hydrogenases (1, 10, 27), localized in the periplasm, and a heterotetrrameric NADP-reducing [Fe] hydrogenase, localized in the cytoplasm (4, 21, 22). In the present work, we have combined physiological, genetic, and biochemical ap-
approaches to determine, at the molecular level, the exact roles of these different hydrogenases in Tc(VII) reduction and precipitation.

**MATERIALS AND METHODS**

**Growth of organisms and preparation of extracts.** The wild-type strain *D. fructosovorans* DSM 3804 (24), *D. fructosovorans* strain MR400 carrying a deletion in the nickel-iron hydrogenase operon (26), or strain MR400 complemented with the nickel-iron hydrogenase genes (28) was grown for 72 h at 37°C in stopped 100-ml bottles supplemented, when required, with kanamycin and gentamicin (50 µg/ml) in a minimal medium defined by Widdel and Pfennig (32). For Tc(VII) reduction assay, the strains were subcultured three times in a medium containing 20 mM fructose as an electron donor and 20 mM fumarate as an electron acceptor.

*D. fructosovorans* cells grown to an optical density of 600 nm of 1.0 (20 g [wet weight]) were collected by centrifugation at 2,000 × g, washed twice with Tris-HCl (10 mM, pH 7.6), and stored at −80°C before use. Unless otherwise noted, all operations were performed under air at 4°C. Freshly thawed cells were passed twice in a French pressure cell at 1,000 lb/in² pressure in the presence of a few crystals of DNase. Cell debris were removed by centrifugation at 4,000 × g for 30 min, and the supernatant (crude extract) was then centrifuged at 120,000 × g for 1 h. The resulting soluble fraction was used for purification and for Tc(VII) reduction.

**Tc(VII) reduction by resting cell suspensions or purified proteins.** Bacteria grown for 72 h at 37°C were transferred anaerobically in a centrifuge tube stoppered with a rubber septum (Suba seal no. 37; Aldrich) and washed four times in 50 mM Tris-HCl buffer (pH 8.0). The bacterial pellet was resuspended anaerobically to either either Tris-HCl (20 mM, pH 8.0 or 8.5), MES (N-morpholinoethanesulfonic acid) (20 mM, pH 5.5), MOPS (morpholinopropanesulfonic acid) (20 mM, pH 6.5 or 7.5), or citrate-sodium phosphate buffer (20 mM, pH 4.5) to a concentration of about 0.5 mg of cells (dry weight) per ml. Aliquots (1.9 ml) of the washed cell suspension were transferred under nitrogen to 10-ml serum bottles sealed with butyl rubber stoppers. Electron donors (fructose, fumarate, lactate, pyruvate, or formate) were added from concentrated stock solutions to a final concentration of 10 mM. For these experiments, all of the bottles were depleted of oxygen by three cycles of vacuum-nitrogen and then flushed under nitrogen for 10 min. When hydrogen was supplied as an electron donor for metal reduction, the gas was flushed into the headspace of the bottles for 20 min with resting cell suspensions or for 180 min with soluble extracts or purified proteins in order to activate the nickel-iron hydrogenase, as described by Fernandez et al. (6) and Hatchikian et al. (10). A solution (100 mM, pH 7.6), and eluted at 320 mM NaCl. The eluted fraction was then concentrated in a 50-ml ultrafiltration cell with a PM 30 membrane (Amicon) and filtered through a Sephacryl S-200 high-resolution column (Pharmacia). Finally, hydrogenase was loaded on a hydroxylapatite (Bio-Rad) column and eluted at 180 mM potassium phosphate (pH 7.6). The hydrogenase was judged to be homogeneous by the following criteria: (i) native polyacrylamide gel electrophoresis giving a single band of protein which catalyzed the hydrogen-dependent reduction of methyl viologen, (ii) the presence of two single bands at 29 and 60 kDa after sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and (iii) an absorbance ratio (A$_{400}$/A$_{380}$) equal to 0.26.

**Analytical procedures.** Protein concentrations were measured with a bichoninic acid assay kit (Pierce) by the method of Smith et al. (30), using bovine serum albumin as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (11). The hydrogen uptake activity was visualized in the gel after native 7.5% polyacrylamide gel electrophoresis as previously described (4). UV-visible spectra of the pure nickel-iron hydrogenase and the cytochrome $c_5$ were recorded on a Varian spectrophotometer.

**RESULTS**

**Microbial reduction of technetium: physiology and kinetic parameters.** In order to study exclusively the enzymatic contribution to Tc(VII) reduction by sulfate-reducing bacteria, chemical Tc(VII) reduction was prevented by growing *D. fructosovorans* in a mineral medium in the absence of sulfate. Cells grown under these conditions had a greyish color with no black precipitate of iron sulfide. These conditions were preferred to those used by Lloyd et al. (15) for *D. desulfuricans*, which did not completely abolish the formation of iron sulfide in the case of *D. fructosovorans* (data not shown).

After 2 h of incubation under hydrogen, approximately 92% of the Tc(VII) (1 mM) present was reduced by a resting cell suspension of *D. fructosovorans* in an insoluble black precipitate (Fig. 1A), as observed in the case of *S. putrefaciens* (33). On the other hand, only a minor reduction (13%) occurred after 24 h when hydrogen was replaced by nitrogen (Fig. 1A and Table 1). Tc(VII) reduction occurred between pH 5.5 and 8.0 (Fig. 1B) and between 10 and 40°C (data not shown). The highest rate was observed at pH 5.5 and between 30 and 40°C, where an apparent $K_m$ of 2 mM and a maximal velocity of 7 mmol of Tc(VII) reduced per g (dry weight) of bacteria per h were determined using a Lineweaver-Burk plot.

Several compounds, such as fructose, lactate, pyruvate, fumarate, and formate, which are efficient electron donors for sulfate reduction in *D. fructosovorans* (24) were tested for Tc(VII) reduction (Table 1). Only hydrogen appeared to be an efficient electron donor for Tc(VII) reduction (Table 1), suggesting the involvement of a hydrogenase in this process.

**Inhibitors and genetic determinants.** The Tc(VII) reduction activity of *D. fructosovorans* was not inhibited by exposure of the cells to air, which indicated oxygen tolerance of the enzyme (Fig. 2). Moreover, this activity was irreversibly inhibited by a 10-min preincubation of the cells with 0.5 mM CuCl$_2$, a specific...
inhibitor of the periplasmic hydrogen uptake activity in vivo (8) (Fig. 2). These results excluded a possible role of NADP-reducing hydrogenase in the reduction mechanism, as this enzyme is cytoplasmic and oxygen sensitive (4, 22). To investigate the role of the [NiFe] hydrogenase, which is periplasmic and oxygen tolerant and is the major hydrogenase produced by D. fructosovorans (10), Tc(VII) reduction by strain MR400, which carries a specific deletion of the structural genes of this hydrogenase (26), was studied. This deletion strain showed a dramatic decrease in the rate of reduction (Fig. 2): only 20% of the Tc(VII) was reduced in 3 h, although iron-only hydrogenase activity still represented about 16% of the wild-type level in both the hydrogen uptake and deuterium-hydrogen exchange activities (data not shown). Moreover, the complementation of strain MR400 by the nickel-iron hydrogenase genes carried on a multicopy plasmid (28) restored hydrogenase activity (hydrogen uptake and deuterium exchange) (data not shown) and Tc(VII) reductase activity (Fig. 2) to wild-type levels. These results demonstrated the essential role of the nickel-iron hydrogenase in the in vivo reduction of Tc(VII) by the sulfate-reducing bacterium D. fructosovorans.

Reduction and precipitation of Tc(VII) by soluble extracts and purified proteins. In order to provide biochemical evidence of the involvement of the [NiFe] hydrogenase and possibly other electron carriers in the biological reduction of Tc(VII), this activity was tested in different fractions: (i) crude extract (soluble and membranes proteins) and (ii) soluble fraction (supernatant from centrifugation at 120,000 g) suspended in MOPS (20 mM, pH 6.5). In both cases, 85% of the Tc(VII) was reduced and precipitated as shown by the appearance of brownish particles after 18 h of incubation in the presence of hydrogen. No reduction or precipitation occurred when hydrogen was omitted. This experiment demonstrates that reduction of Tc(VII) is performed in vitro by soluble proteins. This further shows that the precipitation of reduced forms of Tc

![FIG. 1. (A) Tc(VII) reduction and precipitation by resting cells of D. fructosovorans supplied with hydrogen (closed circles) as an electron donor. Control cultures with nitrogen (open circles) contained no added electron donor. The cells were incubated for 24 h at 23°C and pH 8.0. (B) Effect of pH on Tc(VII) reduction by D. fructosovorans. Hydrogen was supplied as the electron donor, and the cells were incubated at room temperature (23°C) and pH 4.5 (open circles), 5.5 (closed triangles), 6.5 (open squares), 7.5 (open triangles), 8.0 (closed circles), or 8.5 (inverted closed triangles).](http://aem.asm.org/)
and pH 8.0 (20 mM Tris-HCl) with hydrogen as an electron donor and a high concentration (3.9 M) did not precipitate or reduce Tc(VII) with hydrogen. In contrast, Tc(VII) was reduced in less than 1 h (Table 2). The high reducing activity observed under such conditions may be related to the reactivation of the hydrogenase in the presence of cytochrome c₃.

Reduction of Tc(VII) at the expense of molecular hydrogen is a good candidate for the removal of this radionuclide from solution in a bioremediation process.

The Tc(VII)-reducing activity of D. fructosovorans requires the presence of hydrogen as an electron donor, whereas organic electron donors such as lactate, pyruvate, fumarate, and formate are inefficient (Table 1). The essential role of the nickel-iron hydrogenase in the process of reduction of Tc(VII) was further proved by genetic and biochemical studies. This role is supported by (i) in vivo and in vitro inhibition of the activity by Cu(II), (ii) oxygen tolerance of the activity, (iii) the dramatic decrease of Tc(VII) reduction in a mutant lacking [NiFe] hydrogenase structural genes, and (iv) demonstration of direct reduction by purified [NiFe] hydrogenase. This is the first report which demonstrates the reduction and removal of Tc(VII) from solution with purified nickel-iron hydrogenase. Reduction of Tc(VII) at the expense of molecular hydrogen is the most efficient and most widespread mechanism in the bacteria tested so far. Even though alternative electron donors can be used by several species, such as E. coli (13), S. putrefaciens (14, 33), and D. desulfuricans (16), the oxidation of these organic substrates often leads to the production of hydrogen or formate. Therefore, hydrogenase (alone or in a formate-hydrogenase complex) probably reactivates the inactivated hydrogenase in the presence of its physiological electron acceptor, cytochrome c₃ (6).

**DISCUSSION**

In the present work, we report that D. fructosovorans reduces Tc(VII) and removes it efficiently from solution. The reduction process occurs at wide ranges of temperature (10 to 40°C) and pH (5.5 to 8.0). The optimum pH (around pH 5.5) probably reflects the best affinity of hydrogenase for TcO₄⁻. Reduction of Tc(VII) with increasing Tc concentrations gave an apparent $K_m$ of 2 mM, which is slightly higher than but consistent with the $K_m$ of 0.5 mM determined by Lloyd et al. (18) with E. coli and D. desulfuricans supplied with formate as an electron donor. At a high concentration of TcO₄⁻ (6 mM), D. fructosovorans exhibits the highest rate of reduction described so far, i.e., 7 mmol of Tc reduced per g (dry weight) of bacteria per h. The corresponding values for E. coli and D. desulfuricans were 12.5 and 800 μmol of Tc reduced per g (dry weight) of bacteria per h, respectively (18). The high efficiency of the Tc(VII)-reducing activity of D. fructosovorans within wide ranges of pH and temperature makes this bacterium a good candidate for the removal of this radionuclide from solution in a bioremediation process.

To identify the proteins and enzymes involved in Tc(VII) reduction in vitro, we have tested the involvement of the purified [NiFe] hydrogenase and its physiological electron acceptor (12, 25), cytochrome c₃, and Tc(VII) reduction by soluble fractions of D. fructosovorans at pH 8.0. Soluble proteins were incubated at 23°C and pH 8.0 (20 mM Tris-HCl) with hydrogen as an electron donor and 0.5 mM Tc(VII). Tc(VII), $R_f$ = 0.7; Tc(V), $R_f$ = 0.

![FIG. 3. Chromatograms of Tc(VII) reduction by soluble fractions of D. fructosovorans at pH 8.0. Soluble proteins were incubated at 23°C and pH 8.0 (20 mM Tris-HCl) with hydrogen as an electron donor and 0.5 mM Tc(VII). Tc(VII), $R_f$ = 0.7; Tc(V), $R_f$ = 0.](http://aem.asm.org/)

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**TABLE 2. Tc(VII) reduction and precipitation by soluble proteins of D. fructosovorans**

<table>
<thead>
<tr>
<th>Protein(s) tested</th>
<th>Conc</th>
<th>% Tc remaining in solution after (h):</th>
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<tbody>
<tr>
<td></td>
<td>mg/ml</td>
<td>μM</td>
</tr>
<tr>
<td>Soluble fraction</td>
<td>0.35</td>
<td>3.9</td>
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<tr>
<td>[NiFe] hydrogenase</td>
<td>0.35</td>
<td>0.4</td>
</tr>
<tr>
<td>[NiFe] hydrogenase</td>
<td>0.037</td>
<td>0.4</td>
</tr>
<tr>
<td>[NiFe] hydrogenase + 0.5 mM CuCl₂</td>
<td>0.037</td>
<td>0.4</td>
</tr>
<tr>
<td>Cytochrome c₃</td>
<td>0.006</td>
<td>0.4</td>
</tr>
<tr>
<td>Cytochrome c₃ + [NiFe] hydrogenase</td>
<td>0.006 + 0.037</td>
<td>0.4 + 0.4</td>
</tr>
</tbody>
</table>

*Proteins were incubated at 30°C and pH 6.5 (20 mM MOPS) with hydrogen as an electron donor and 0.5 mM Tc(VII) as an electron acceptor.


drogenase complex) appears to be the major component involved in enzymatic Tc(VII) reduction.

In addition to the ability to reduce Tc(VII), selenite- and chromate-reducing activities have been reported for the [Fe] hydrogenase of _Clostridium pasteurianum_ (35) and the [Fe] hydrogenase of sulfate-reducing bacteria (23), respectively. Metal reductase activity of hydrogenases therefore appears to be a widespread property.

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