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Biotransformation of pertechnetate by *Clostridia*

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Summary. *Clostridia* are strict anaerobic, spore-forming, fermentative bacteria commonly present in soils, sediments, and wastes; and, they play a major role in the decomposition of a wide variety of organic compounds. They also are involved in the reduction of iron, manganese, and uranium, thereby affecting their solubility. However, little is known of the ability of *Clostridia* to reduce technetium (Tc). We investigated the reduction and precipitation of pertechnetate by *Clostridium sphenoides* able to metabolize citrate as its sole carbon source, and *Clostridium* sp. capable of fermenting glucose but not citric acid. Both species reduced Tc(VII) to Tc(IV), although *C. sphenoides* did so at a greater rate and extent than *Clostridium* sp. The reduced Tc was predominantly associated with the cell biomass. It also was present in solution complexed with bacterial metabolic products ($MW > 5000$). Adding diethylenetriaminepentaacetic acid (DTPA) to *Clostridium* sp. resulted in the formation of a soluble Tc(IV)-DTPA complex, whereas with *C. sphenoides* only a small amount of Tc was present in solution, indicating that insoluble Tc species were formed. These results suggest that *Clostridia* may play a major role in regulating the mobility of Tc under anaerobic conditions in wastes and subsurface environments.

Introduction

Technetium (Tc) is produced in large quantities by the fission of ^{235}U during nuclear power generation and defense-related activities including nuclear testing and reactor operations [1]. Tc was the first artificial isotope produced. At present there are 17 known isotopes and 6 nuclear isomers with mass numbers from 92 to 107 and half-lives ranging from a few seconds to 2.1×10^5 years [2]. Tc can exist in oxidation states 0, +3, +4, +5, +6, and +7; however, the predominant chemistry concerns only the stable heptavalent pertechnetate ion (TcO_4^-) and the quadrivalent Tc(IV) ion. Their chemical behavior resembles that of rhenium, and, to a lesser extent, manganese. Tc is readily reduced and oxidized. Uncomplexed Tc(III, IV) undergoes hydrolysis and precipitates from solution. Tc(V) and Tc(VI) undergo disproportionation to Tc(IV) and Tc(VII). The soluble pertechnetate ion is precipitated from dilute hydrochloric acid (up to 5 M) by hydrogen sulfide and the insoluble reduced forms (TcS_2, Tc_2S_7) are oxidized to the soluble pertechnetate ion by hydrogen peroxide in alkaline solution [2].

The safe disposal of the Tc-containing wastes and environmental contamination of Tc are major concerns. Microorganisms affect the dissolution or precipitation of Tc by oxidation-reduction reactions and by complexation with organic by-products and macromolecules. Reduction of pertechnetate ion to an insoluble form by chemolithotrophic, aerobic, facultative, and anaerobic sulfate-reducing bacteria [3–7] have been reported. However, little is known of the ability of *Clostridia* to reduce Tc under anaerobic conditions. *Clostridia* are gram-positive, strict anaerobic, spore-forming, fermentative bacteria commonly present in soils, sediments, and wastes; they can metabolize a wide variety of organic compounds. They also are known to reduce iron, manganese and uranium, thereby affecting their solubility [8, 9]. In this communication, we report the reduction and immobilization of soluble pertechnetate (TcO_4^-) to insoluble Tc by *Clostridia* under anaerobic conditions.

Materials and methods

Cultures

Clostridium sphenoides (ATCC 19403), able to metabolize citric acid, was grown in medium containing (per liter): $Na_2Cit_2 \cdot 2H_2O$, 8.2 g; NH_4Cl, 0.5 g; glycerolphosphate, 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 2.8 mg; peptone, 0.1 g; yeast extract, 0.1 g; L-cysteine·HCl, 0.03 g; pH, 6.8. *Clostridium* sp. (ATCC 53464), that can ferment glucose but not citric acid was grown in medium containing the following ingredients (per liter): glucose, 5.0 g; glycerol phosphate, 0.3 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 2.8 mg; peptone, 0.1 g; yeast extract, 0.1 g; pH, 6.8. The media were pre-reduced by boiling while purging them with N_2, then dispensed into serum bottles, sealed with butyl rubber stoppers and autoclaved.

Resting cell suspensions

The appropriate growth medium was inoculated with *Clostridium* sp. or *C. sphenoides* and the cells were grown to late log phase (24 to 48 hours). The cells were harvested by centrifugation at $6000 \times g$ for 20 minutes, washed in a pre-reduced mineral salts medium containing the following (per

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liter): $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.5 g; KCl, 7.4 g; pH, 6.8; and re-suspended in the same medium to give a final optical density of 0.8 at 600 nm. An aliquot was removed for determining dry weight. All operations were performed under N_2 in an anaerobic glove box.

Technetium

^{99m}Tc was obtained as sodium pertechnetate (Syncor International, NY) and calibrated before use with an Atomlab dose calibrator. ^{99m}Tc was determined in bacterial samples by γ-spectroscopy using a Wallac gamma counter. Samples analyzed included Tc standards, Tc containing blank controls, and the bacterial samples. All activity values were corrected for the isotope's 6 hour half-life. Using ^{99m}Tc is advantageous because of its availability, ease of counting, reduction in waste generation, and greater personnel safety compared to the use of beta-emitting isotopes.

Tc reduction

The ability of bacteria to reduce Tc was determined by adding 7 ml of the bacterial cells (OD 0.8) to 10 ml Vacutainer tubes (Becton Dickinson, NJ) and incubating them with the carbon source (100 mg/l) glucose or citric acid in triplicate. ^{99m}Tc was added to give a final concentration of 10 μCi/ml, the tubes were capped with rubber stoppers, and the samples placed in an incubator-shaker at 26 ± 1 °C. Periodically, a set of samples was centrifuged at $3000 \times g$ for 10 minutes, the supernatant was recovered, and filtered through 0.45 μm nylon filter into a liquid scintillation vial. The pH was measured, and aliquots taken for analysis of glucose or citric acid by HPLC using a refractive index detector and UV-vis detector at 210 nm, respectively. Tc adsorbed to the biomass solids and supernatant was analyzed by γ-spectroscopy. Controls without cells were included for each treatment and all operations were performed in a N_2 filled glove box. A counting standard was also included.

Effect of pH on Tc reduction by *Clostridium* sp.

Resting cells of *Clostridium* sp. were washed and suspended in pre-reduced buffered medium containing the following (per liter): $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.5 g; KH_2PO_4, 1 g; K_2HPO_4, 1 g; KCl, 7.4 g; pH to 6.8. The cells then were treated identically to those in the Tc reduction experiments.

Characterization of bioprecipitated Tc

The Tc associated with biomass was determined after treating it with H_2O_2 and analyzing the Tc in solution and in the biomass by γ-spectrometry. Hydrogen peroxide (3 wt. %) was added to the biomass to oxidize the reduced forms of Tc to soluble pertechnetate. The sample was centrifuged and Tc associated with the biomass and supernate was determined. In addition, we assessed the cellular association of Tc with *Clostridium* sp. by measuring the activity of Tc before and after cell lysis. The cells were washed with a pre-reduced mineral salts medium, resuspended in 2 ml of Tris buffer, and incubated with lysozyme (Sigma, St Louis, MO) for 30 minutes at 26 °C. The samples were centrifuged, the supernatant decanted, and the Tc in the supernatant and cell debris was determined.

Tc speciation in solution

The presence of oxidized, reduced, and organic complexes of Tc in the supernatant was determined by gel filtration chromatography [10]. Unfiltered supernate (250 μl) was introduced into a column (35×1 cm) containing Sephadex G-25M gel, which had been conditioned in pre-reduced mineral salts medium. Gel filtration studies have shown that uncomplexed reduced Tc is bound to the Sephadex column whereas Tc(IV) complexed with organic ligands, such as citric acid, DTPA, or microbial by-product elutes from the column before pertechnetate [10].

Results

Effect of ^{99m}Tc on *Clostridia*

The addition of 10 μCi/ml ^{99m}Tc had no effect on the metabolism of citric acid by *C. sphenoides* (Fig. 1). About 89% of the citric acid was metabolized by *C. sphenoides* at the rate of 0.41 mM/h. Citric acid concentration decreased from 0.61 mM to 0.07 mM in 2 hours. During the consumption of citric acid the pH of the medium changed slightly to 6.8 from 7.0 both in the presence and absence of Tc. The degradation products of citric acid were acetic-, butyric-, and lactic-acid. Glucose was completely metabolized by *Clostridium* sp. within 2 hours (data not shown). The final pH of the medium decreased from 6.8 to 4.3. In a similar experiment conducted in phosphate buffered medium the pH remained at 6.8 and there was no difference in the rate or extent of glucose metabolism by the bacterium.

Reduction of Tc

Figs. 2a and 2b show the reduction of Tc by *C. sphenoides* and *Clostridium* sp., respectively. Tc was rapidly removed

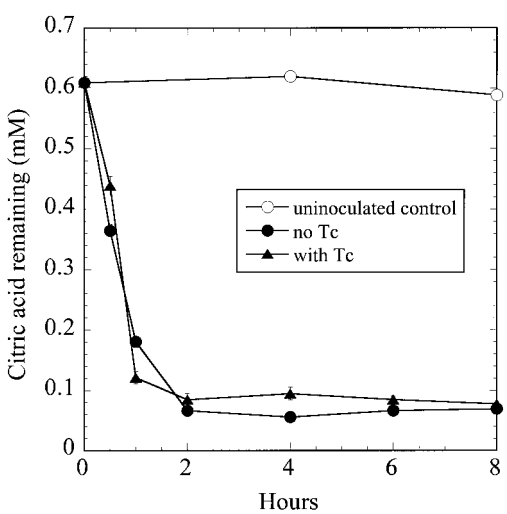

Fig. 1. Effect of addition of ^{99m}Tc (10 μCi/ml) on citric acid metabolism by *Clostridium sphenoides*.

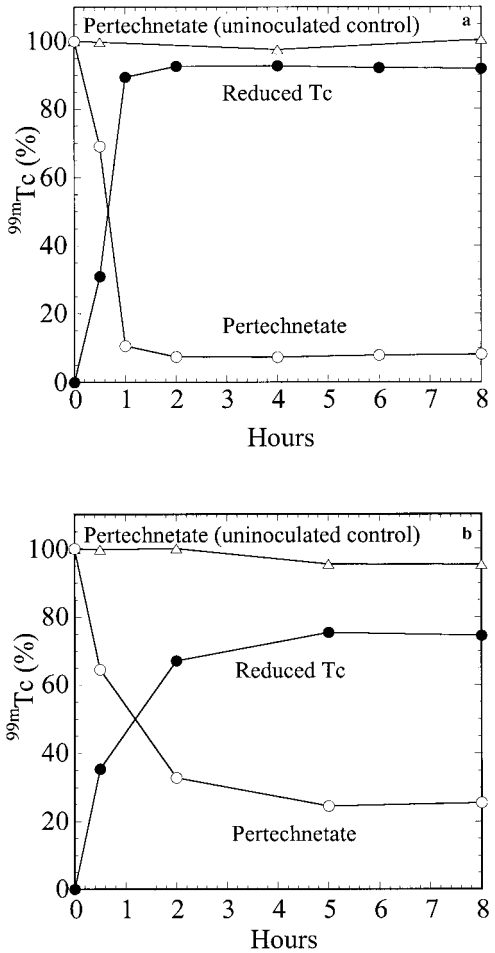

Fig. 2. Reduction of pertechnectate by *C. sphenoides* (**a**), and *Clostridium* sp. (**b**).

from solution by *C. sphenoides* during the first hour, and became associated with the biomass (91%) (Fig. 2a). In contrast, the reduction of Tc by *Clostridium* sp. proceeded at a much slower rate, with a total reduction of ~ 75% of the Tc in 5 hours (Fig. 2b).

Analysis of the cells of *C. sphenoides* (biomass) washed with mineral salts medium at the end of the experiment (8 hours) showed that about 96% of the Tc remained with the biomass (Fig. 3). Adding hydrogen peroxide to the biomass oxidized the reduced Tc to pertechnetate and released 77% of the Tc. However, 21% remained bound to the biomass. The release of Tc from the biomass into solution following oxidation confirms that it was present in reduced form. An experiment in which the cells were incubated under aerobic conditions showed that pertechnetate ion does not significantly adsorb to the cells. The persistent association of a significant amount of Tc with the cells that was not readily oxidized by H_2O_2 indicates that a more recalcitrant Tc species was formed.

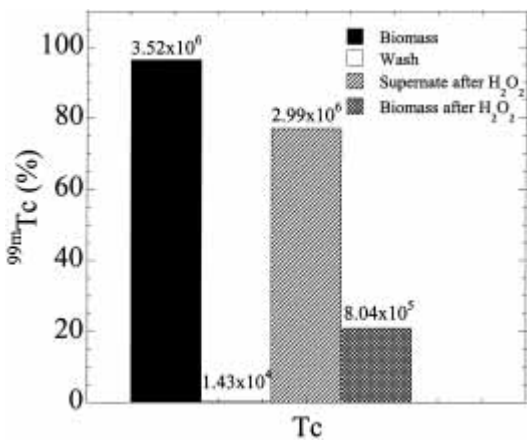

Fig. 3. Effect of hydrogen peroxide on the removal of Tc from the biomass of *C. sphenoides*. Values are in counts per minute (cpm).

Effect of DTPA on Tc reduction by *Clostridia*

Figs. 4a and 4b show the effect of adding DTPA on the fate of Tc in the presence of *Clostridium* sp. The reduction of pertechnetate by the bacterium was accomplished in less than 5 hours. In the absence of DTPA, 75% of the Tc was associated with the biomass, whereas in its presence only

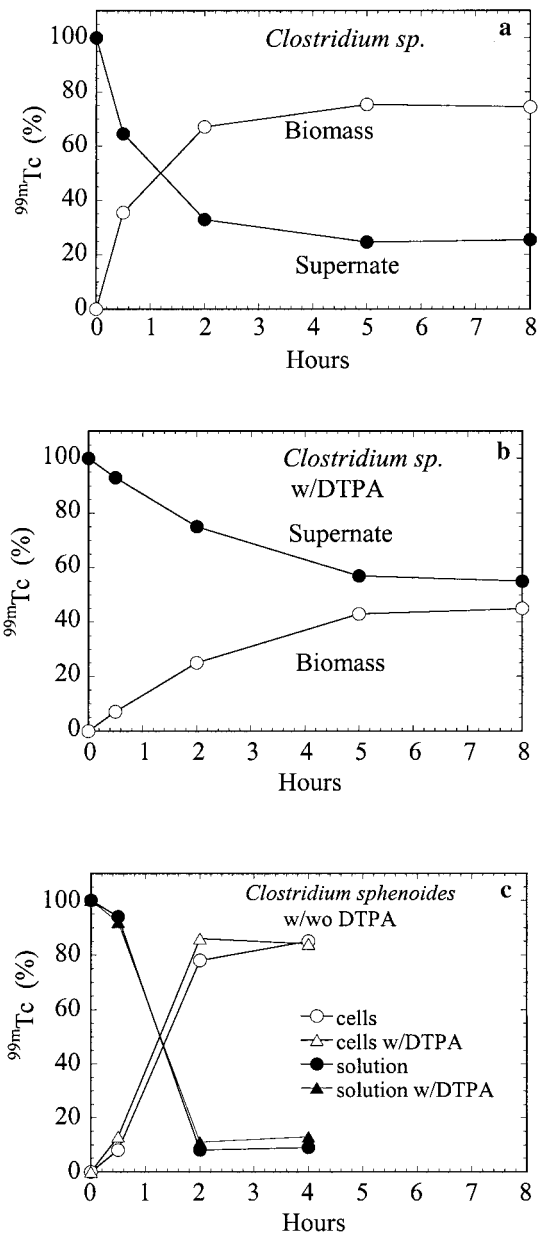

Fig. 4. The effect of DTPA addition on the fate of Tc in the presence of *Clostridium* sp. and *C. sphenoides*.

Table 1. The distribution of Tc-99m in the culture supernatant of *C. sphenoides*.

Time (h)	Treatment	Tc-99m (cpm) Unfiltered	Tc-99m (cpm) Filtrate[a]	Retained by Filter (%)
0.5	no cells	$1.25 \times 10^6 \pm 0$	$1.21 \times 10^6 \pm 0$	3
	cells	$1.22 \times 10^6 \pm 1 \times 10^4$	$1.14 \times 10^6 \pm 0$	7
2	no cells	$1.22 \times 10^6 \pm 1 \times 10^4$	$1.20 \times 10^6 \pm 1 \times 10^3$	2
	cells	$1.65 \times 10^5 \pm 1 \times 10^3$	$9.7 \times 10^4 \pm 6 \times 10^3$	41
4	no cells	$1.21 \times 10^6 \pm 0$	$1.18 \times 10^6 \pm 1 \times 10^4$	2
	cells	$1.82 \times 10^5 \pm 1 \times 10^3$	$1.10 \times 10^5 \pm 1 \times 10^3$	40

a: Samples filtered through a 0.45 μm nylon syringe filter.

45% of Tc was associated with it, indicating that ~ 55% of the reduced Tc was present as a soluble Tc(IV)-DTPA complex. However, due to the complexity of Tc-DTPA chemistry the presence of Tc(III) cannot be ruled out [11]. In contrast, in samples containing *C. sphenoides* about 15% of the Tc was present in solution, regardless of whether DTPA was present or not (Fig. 4c). This suggests that the Tc associated with the *C. sphenoides* biomass either (i) outcompetes DTPA for Tc indicating a stronger affinity for the metal than *Clostridium* sp., or (ii) the rapid reduction (within 2 hours) and subsequent hydrolysis of Tc may have prevented complex formation with DTPA. The data also suggest that there was less citric acid remaining to complex the reduced Tc. Over 50% of the added citric acid was metabolized in the first half hour and up to 90% was metabolized in 2 hours in samples containing citric acid plus DTPA.

Table 1 shows the distribution of Tc in culture supernatant. The cells were removed first by centrifugation and the supernatant was filtered through a 0.45 μm membrane. There was little adsorption of Tc on the filters (3%) in control samples without the cells. However, in the presence of cells a significant amount (~ 40%) of the Tc was retained on the filter at 2 hours and 4 hours. This suggests that a substantial portion of the reduced Tc in the culture supernatant is associated with microbial by-products (*i.e.* proteins, polysaccharides).

Gel filtration chromatography

Fig. 5 shows the elution profile of Tc using gel filtration chromatography of a *Clostridium* sp. sample incubated with and without DTPA. At 0.25 hours most of the soluble Tc

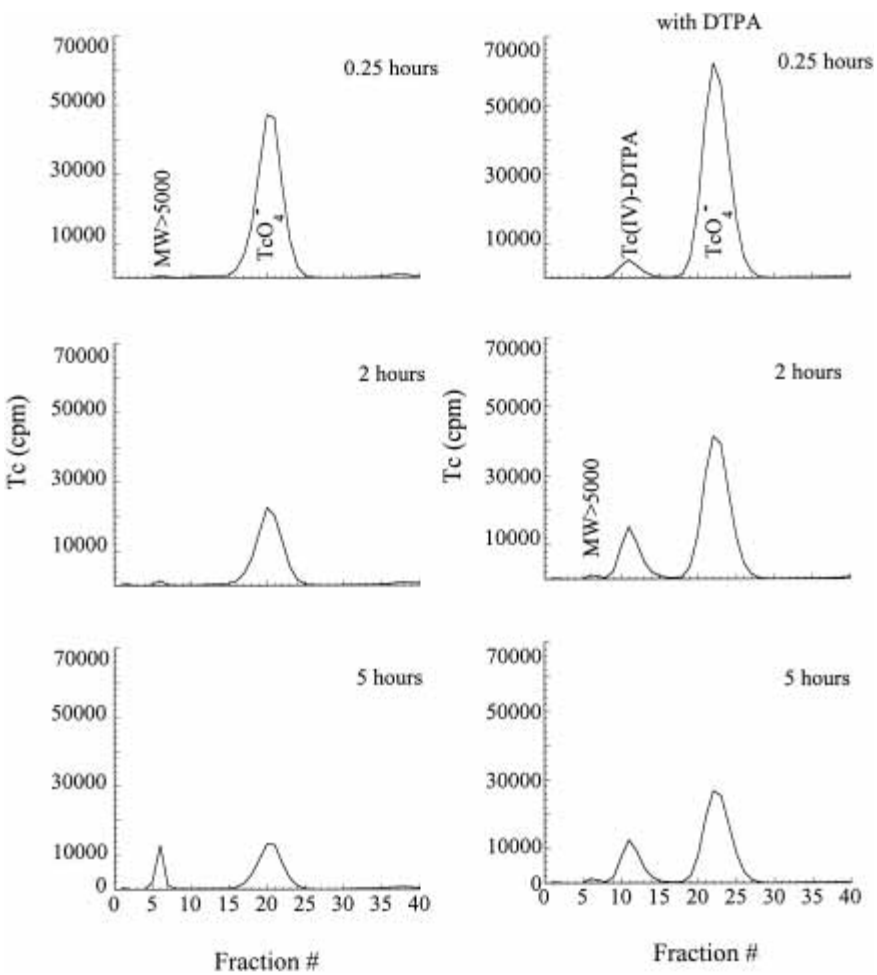

Fig. 5. Comparison of elution profiles of Tc in the presence and absence of DTPA with *Clostridium* sp. Tc(VII) was partially reduced to Tc(IV) by *Clostridium* sp. A portion of the reduced Tc was present in solution complexed with the bacterial metabolic products ($MW > 5000$, fractions 4 to 7). In the presence of DTPA a substantial amount of Tc(IV) was complexed with DTPA (fractions 8 to 15).

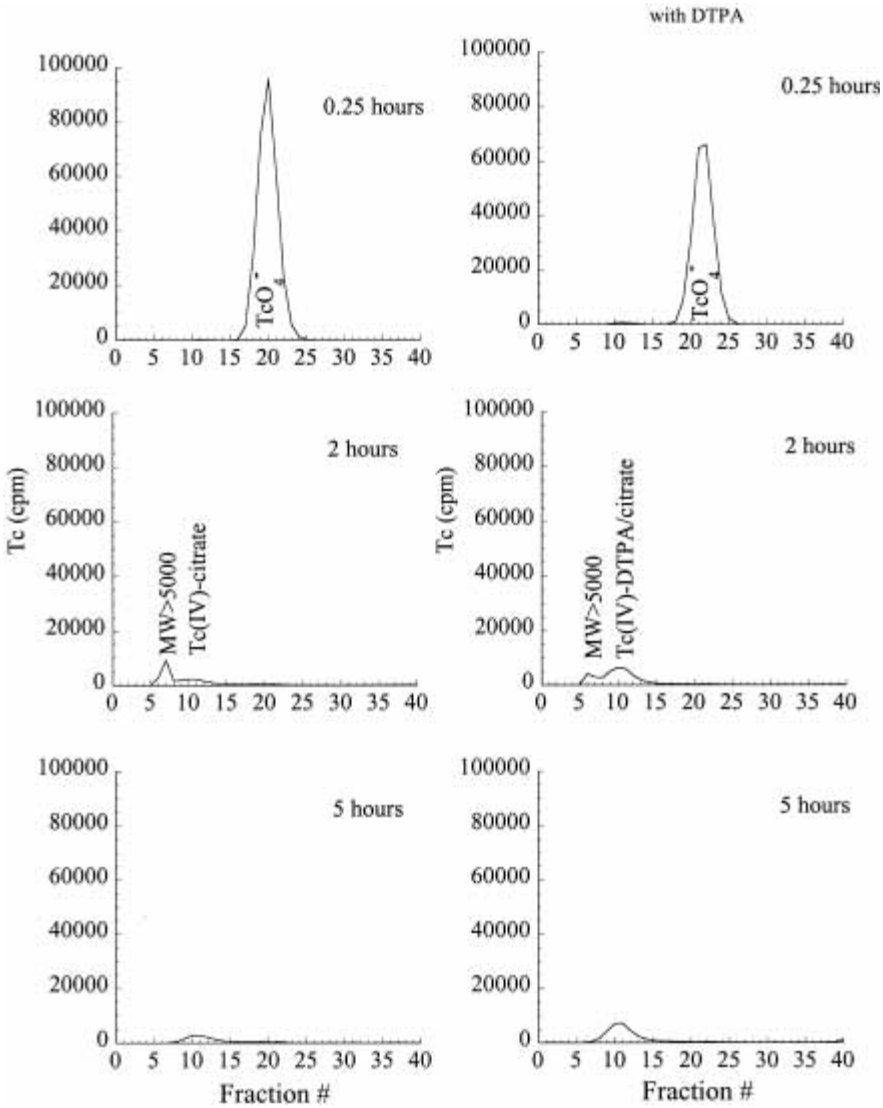

Fig. 6. Comparison of elution profiles of Tc in the presence and absence of DTPA with *C. sphenoides*. All of the added pertechnetate ion was reduced to Tc(IV). A portion of the reduced Tc was present in solution complexed with bacterial metabolic products ($MW > 5000$, fractions 5 to 7) and as the Tc(IV)-citrate complex (fractions 7 to 14). In the presence of DTPA the peak at fractions 7 to 14 was larger than observed for Tc(IV)-citrate alone, indicating the presence of both Tc(IV)-DTPA and Tc(IV)-citrate complexes.

is in the oxidized pertechnetate form (fractions 15–27). After 2 hours and 5 hours of incubation in the absence of DTPA the magnitude of the pertechnetate peak decreased with the concomitant appearance of a peak at fractions 4 to 7 which increased with incubation time. This peak is most probably the reduced Tc complexed with microbial by-product having a molecular weight > 5000 daltons. In the presence of DTPA the predominant peak (fractions 8 to 15) is the Tc(IV)-DTPA complex [10]. The presence of pertechnetate ion in the sample is consistent with its slow rate of reduction by *Clostridium* sp. Analysis of the various fractions confirmed that all of the Tc was recovered and accounted for.

Fig. 6 shows the elution profile of Tc using gel filtration chromatography of a *C. sphenoides* sample incubated with and without DTPA. As with the *Clostridium* sp., the pertechnetate ion (fractions 16 to 25) was detected at 0.25 hours. At 2 hours however, all of the pertechnetate was converted to its reduced form (no peak at fractions 16 to 24), consistent with the rapid rate of reduction observed for this species. The simultaneous appearance of peaks at fractions 5 to 7 and 7 to 14 at 2 hours indicates the association of Tc with microbial by-product ($MW > 5000$) and citrate, respectively. In the DTPA-treated sample, the DTPA peak (fraction 8 to 14) increased at 2 hours and remained at the same level at 5 hours indicating the presence of Tc(IV)-DTPA complex in addition to the Tc(IV)-citrate complex [10]. The peaks at 5 to 7 were not detected at 5 hours. Most of the citric acid was rapidly metabolized by the bacterium leaving little available for complexation with reduced Tc.

Association of Tc with cells

Table 2 shows the cellular association of Tc in *Clostridium* sp. in the presence and absence of DTPA. In samples containing glucose alone 59% of the added Tc was associated with the cells. In contrast, much less Tc (19%) was associated with the cells in samples containing DTPA. Washing the cells removed only a small portion of the Tc (< 1%). Analysis of the lysed cell fractions containing the cell walls and the supernate, showed that 48% of the added Tc was associated with the cell walls or complexed with the cellular macromolecules.

Discussion

Technetium adsorption by soils and sediments has been attributed to microbial activity [12–15]. Landa *et al.* [12]

Table 2. Association of ^{99m}Tc with whole and lysed cell fractions of *Clostridium* sp.

Treatment	Total Tc added	Whole cells[a]	Wash	Lysed fractions	
				Supernate	Cell debris
	Tc-99m (cpm)[b]				
Glucose	$7.45 \times 10^6 \pm 2 \times 10^4$	$4.38 \times 10^6 \pm 5 \times 10^4$	$1.81 \times 10^5 \pm 1 \times 10^4$	$5.46 \times 10^5 \pm 1.4 \times 10^4$	$3.61 \times 10^6 \pm 4.0 \times 10^4$ (99%)[c]
Glucose + DTPA	$7.29 \times 10^6 \pm 2 \times 10^5$	$1.81 \times 10^6 \pm 4 \times 10^4$	$1.92 \times 10^5 \pm 1.8 \times 10^4$	$3.11 \times 10^5 \pm 7.0 \times 10^3$	$1.42 \times 10^6 \pm 5.0 \times 10^4$ (106%)

a: Tc values taken from a separate set of samples at identical time;
b: Counts normalized to total Tc-99m added to treatments;
c: Tc recovery from whole cells (mass balance).

reported eight out of eleven soils tested adsorbed 98% of the pertechnetate within 2 to 5 weeks and that sterilization of the soil eliminated this adsorption. Tagami and Uchida [13] investigated the influence of microbial activity on plant available Tc under aerobic and anaerobic (waterlogged) conditions in soil amended with 0, 0.05 and 0.5% glucose and compared them with sterile soil. They found that Tc was bound to the soil as a result of changes in the redox due to microbial action. Sheppard *et al*. [14] reported negligible sorption of TcO_4^- in aerobic soils, and substantial sorption of reduced Tc in anaerobic environments, especially in the presence of organic matter and suggested that reduced Tc may be transported as a complex with organic ligands. More recent studies by Peretrukhin *et al*. [15] showed that Tc was sorbed on to the sediments of a highly productive lake in Russia by sulfate-reducing bacterial action. Biogenic hydrogen sulfide converts the readily soluble sodium pertechnetate to poorly soluble Tc(VII) and Tc(IV) sulfides.

Technetium is generally considered to be toxic to most microorganisms. However, the effect of Tc on bacteria has not been fully determined. Toxicity is believed to be biochemical rather than due to its radioactivity. The effects of Tc seem to vary with the isotope and type of bacteria investigated. For example, ^{99}Tc at concentrations > 100 mg/l reduced the growth of *Thiobacillus ferrooxidans* [3] Viable counts of *Diplococcus pneumoniae* decreased from a mean of 9.0×10^8 /ml to 6.4×10^2 /ml when the cells were exposed to 4 μCi of ^{99m}Tc [16]. Growth was reduced in *Escherichia coli* by > 500 μg/ml of TcO_4^-; in *Bacillus subtilis* > 100 μg/ml of TcO_4^-; and, in *Rhodospirillum rubrum* < 10 μg/ml of TcO_4^- [17]. Clearly, additional studies are needed to establish the chemical species and its toxicity to microorganisms.

Reduction and precipitation of pertechentate anion is carried out by a wide variety of microorganisms under anaerobic conditions. The predominant chemical species identified include TcO_2, $TcO(OH)_2$, and TcS_2 depending on the type of microorganism involved [4, 6, 7]. For example, the sulfate reducers reduce pertechnetate to slightly soluble Tc(VII) and insoluble TcS_2. *Thiobacillus ferrooxidans* transformed pertechnetate into its pentavalent and tetravalent states [3]. Other organisms are known to generate Tc oxide (TcO_2) and oxyhydroxide species [$TcO(OH)_2$] under anaerobic conditions. In addition, a significant amount of Tc is associated with the cells and with macromolecules as reduced Tc-organic complexes. Pignolet *et al*. [4] observed Tc was bound to high molecular weight cellular constituents in pure and mixed cultures of bacteria isolated from marine sediment. Henrot [7] reported Tc association with bacterial polysaccharides in anaerobically grown bacteria and that the reduction of pertechnetate was metabolically linked and not due to changes in the redox conditions. About 70% of the total Tc was associated with the bacteria and/or precipitated. The remaining Tc in soluble form was associated with organics. Our results indicate that a substantial amount of reduced Tc is associated with bacterial cell biomass and with bacterial macromolecules most probably as an organic complex.

The nature and stability of the Tc-organic complexes is not understood. Moreover, the ability of the bacterium to metabolize Tc-organic complexes such as Tc-DTPA and Tc-citrate is not known. DTPA is poorly biodegraded under aerobic conditions and is recalcitrant under anaerobic conditions [18]. Although the same amount of DTPA was added to *Clostridium* sp. and *C. sphenoides*, the concentration of Tc(IV)-DTPA complex detected in *C. sphenoides* samples was much less than those with *Clostridium* sp. suggesting that not much reduced Tc was available for complexation with DTPA. These results suggest that Tc may be present as insoluble or soluble form or as colloids, depending on the type and extent of bacterial activity in subsurface environments and therefore, the potential exists for the transport of reduced Tc in these forms.

In summary, the rate and extent of reduction of pertechnetate to Tc(IV) by *Clostridia* varied with the species tested. Reduced Tc was predominantly associated with the biomass in a stable form and also present in solution complexed with metabolic product ($MW > 5000$). *Clostridia* are ubiquitous and form spores which are highly resistant to destruction. They can survive on almost any surface for long periods of time and can have a significant impact on Tc mobility and stability in anaerobic environments.

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