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Hydrogenase- and outer membrane *c*-type cytochrome-facilitated reduction of technetium(VII) by *Shewanella oneidensis* MR-1

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Summary

Pertechnetate, $^{99}\text{Tc(VII)O}_4^-$, is a highly mobile radionuclide contaminant at US Department of Energy sites that can be enzymatically reduced by a range of anaerobic and facultatively anaerobic microorganisms, including *Shewanella oneidensis* MR-1, to poorly soluble $\text{Tc(IV)O}_2(\text{s})$. In other microorganisms, Tc(VII)O_4^- reduction is generally considered to be catalysed by hydrogenase. Here, we provide evidence that although the NiFe hydrogenase of MR-1 was involved in the H_2 -driven reduction of Tc(VII)O_4^- [presumably through a direct coupling of H_2 oxidation and Tc(VII) reduction], the deletion of both hydrogenase genes did not completely eliminate the ability of MR-1 to reduce Tc(VII) . With lactate as the electron donor, mutants lacking the outer membrane *c*-type cytochromes MtrC and OmcA or the proteins required for the maturation of *c*-type cytochromes were defective in reducing Tc(VII) to nanoparticulate $\text{TcO}_2 \cdot n\text{H}_2\text{O}(\text{s})$ relative to MR-1 or a NiFe hydrogenase mutant. In addition, reduced MtrC and OmcA were oxidized by Tc(VII)O_4^- , confirming the capacity for direct electron transfer from these OMCs to TcO_4^- . *c*-Type cytochrome-catalysed Tc(VII) reduction could be a potentially important mechanism in environments where organic electron donor concentrations are sufficient to allow this reaction to dominate.

Introduction

At several US Department of Energy (US DOE) sites, $^{99}\text{technetium}$, a by-product of the nuclear fuel cycle, threatens to contaminate ground- and surface-water resources (Riley *et al.*, 1992). Under oxidizing conditions in these environments, ^{99}Tc is present as pertechnetate [Tc(VII)O_4^-], which is not strongly sorbed and considered highly mobile in the subsurface (Wildung *et al.*, 2000). Environmental mobility is a major risk factor for Tc contamination at DOE's Hanford Site, where significant quantities are predicted to migrate in groundwater beneath the site to the Columbia River in the future. One strategy for containing the spread of Tc contamination has been to exploit the decreased solubility of Tc in the (IV) oxidation state. In the absence of strong complexing ligands, $\text{Tc(IV)O}_2(\text{s})$ is poorly soluble (e.g. $< 10^{-8} \text{ mol l}^{-1}$) and hence mobility in the environment is significantly lower. Therefore, the biotic reduction of Tc(VII) to Tc(IV)O_2 by organisms such as *Shewanella*, *Desulfovibrio* and *Geobacter* could substantially limit the mobility of Tc contamination plumes (Lloyd and Macaskie, 1996; Lloyd *et al.*, 1999a; 2000; Wildung *et al.*, 2000).

Shewanella oneidensis MR-1 can reduce a variety of terminal electron acceptors including: O_2 , nitrate, fumarate, dimethylsulfoxide (DMSO), thiosulfate, trimethylamine oxide (TMAO), S^0 , Fe(III), Mn(III, IV), Cr(VI), Co(III), V(V), U(VI) and Tc(VII) via the oxidation of lactate, formate or H_2 and other electron donors (Myers and Nealson, 1988; 1990; Nealson and Saffarini, 1994; Lloyd and Macaskie, 1996; Liu *et al.*, 2002; Carpentier *et al.*, 2003). The annotated genomic sequence (Heidelberg *et al.*, 2002) of *S. oneidensis* MR-1 revealed numerous genes predicted to encode putative redox proteins including cytochromes, hydrogenases, flavins, ferredoxins and Fe-S proteins. Genome comparisons with other sequenced dissimilatory metal-reducing bacteria (DMRB) such as *Geobacter sulfurreducens*, *G. metallireducens* (Lovley *et al.*, 2004) and *Anaeromyxobacter dehalogenans* reveal that these organisms share a remarkable wealth of genes that encode one particular type of electron transfer protein, the multihaem *c*-type cytochrome. While the organisms display no significant sequence homology, the abundance of *c*-type cytochromes appears

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to be a common characteristic of DMRB and is likely linked to their considerable flexibility in electron acceptor utilization.

The diverse electron transport chain of *S. oneidensis* MR-1 features 42 putative *c*-type cytochromes (Meyer *et al.*, 2004), some of which have recently been shown to function as terminal reductases of many substrates and to directly facilitate energy production (Beliaev *et al.*, 2001; Myers and Myers, 2001; Marshall *et al.*, 2006; Shi *et al.*, 2006). In cell fractionation studies using anaerobically grown *S. oneidensis* MR-1, a large percentage of the membrane-bound *c*-type cytochromes were found to be associated with the outer membrane (OM) cell fractions (Myers and Myers, 1992). Importantly, some of these OM *c*-type cytochromes (OMCs) were localized on the outer face of the OM (Myers and Myers, 2003a) directly facilitating contact with both extracellular soluble and insoluble electron acceptors. Mutagenesis studies in *S. oneidensis* MR-1 identified a cluster of metal reduction-specific genes (Beliaev and Saffarini, 1998; Beliaev *et al.*, 2001) including one that encodes a putative decahaem OMC, *mtrC* (locus tag: SO1778) (Beliaev *et al.*, 2001). The gene encoding an additional decahaem OMC with a role in metal reduction, designated *omcA* (SO1779), was subsequently identified and characterized (Myers and Myers, 1998; 2003b). To date, the roles of MtrC and OmcA in electron transfer to metals have been the most extensively studied of the *c*-type cytochromes in *S. oneidensis* MR-1 (Beliaev *et al.*, 2001; Myers and Myers, 2001; 2003a,b; Shi *et al.*, 2006; Xiong *et al.*, 2006; Wigginton *et al.*, 2007). Both MtrC and OmcA have been shown to play a role in U(VI) reduction and subsequent subcellular localization of nanoparticulate uraninite, U(IV)O₂, while *in vitro* assays demonstrated that reduced MtrC was also oxidized directly by U(VI), indicating that MtrC functioned as a terminal reductase of U(VI) (Marshall *et al.*, 2006).

In addition to *c*-type cytochromes, hydrogenases comprise another class of enzymes that play a central role in microbial redox metabolism catalysing the bidirectional reaction of H₂ oxidation with the formation of two protons and two electrons. In some non-DMRB, hydrogenases have been found to function as Tc(VII) reductases by coupling H₂ oxidation and enzymatic Tc(VII) reduction (Lloyd and Macaskie, 1997; De Luca *et al.*, 2001). However, among the DMRB the involvement of hydrogenase in the reduction of Tc(VII) has only been circumstantial based on either amino acid homologies to other hydrogenases (Wildung *et al.*, 2000) or the exclusive requirement for H₂ as the electron donor for direct Tc(VII) reduction (Lloyd *et al.*, 2000). Two major groups of hydrogenases exist, NiFe hydrogenases and Fe-only hydrogenases, which are classified by the metal atoms associated with their active centre (Vignais *et al.*, 2001; Vignais and Colbeau, 2004). The genome of *S. oneidensis* MR-1

encodes both an Fe-only hydrogenase *hydAB* (SO3920 and SO3921 respectively) and a NiFe hydrogenase *hyaAB* (SO2099 and SO2098 respectively), both of which are heterodimers composed of a large and small subunit (Heidelberg *et al.*, 2002). Recently, the roles of these hydrogenases in H₂ metabolism by MR-1 were characterized (Meshulam-Simon *et al.*, 2007). The NiFe hydrogenase was found to be the predominant hydrogenase and functioned bidirectionally, both forming and releasing H₂, while the Fe-only hydrogenase was found to be predominantly involved in H₂ formation. This study also confirmed that these two gene loci encode the only hydrogenases in MR-1. Cross-genome comparisons and sequence alignment revealed conservation of the NiFe hydrogenase (HyaAB) among most sequenced *Shewanella* strains, while few homologues of the Fe-only hydrogenase (HydAB) exist in other *Shewanella* isolates (http://genome.jgi-psf.org/mic_home.html).

The purpose of this study was to determine the mechanism(s) involved in the reduction of Tc(VII)O₄⁻ by *S. oneidensis* MR-1. To date, *c*-type cytochromes have not been shown to have a role in Tc(VII) reduction. The putative role of hydrogenases in the reduction of Tc(VII) has only been predicted using homology to the hydrogenases of other organisms. To better understand the biochemical mechanisms of Tc(VII) reduction in MR-1, we tested several mutants lacking either *c*-type cytochromes or functional hydrogenases and evaluated their ability to reduce Tc(VII) compared with wild-type MR-1 using multiple electron donors. We compared the subcellular localizations of TcO₂ nanoparticles in wild-type MR-1 with a mutant that reduced Tc(VII) at a significantly slower rate. Additionally, we tested purified OMCs for their ability to function as terminal reductases of Tc(VII) in a defined system. Hydrogenase mutants were also characterized for their donor-specific ability to transfer electrons to several other metals.

Results

The role of the NiFe and Fe-only hydrogenases in Tc(VII) reduction

To ascertain if either of the two predicted hydrogenases in *S. oneidensis* MR-1 were involved in H₂ oxidation linked to Tc(VII) reduction, a series of in-frame deletion mutants were constructed. Mutants in genes encoding the large subunit of either the Fe-only hydrogenase, *hydA* (SO3920), or the NiFe hydrogenase, *hyaB* (SO2098), were generated by double homologous recombination (Table 1). A third mutant consisting of deletions of both the *hydA* and *hyaB* genes was also produced (Table 1). The reduction of Tc(VII) in the presence of either H₂ (Fig. 1) or lactate (Fig. 2) as the sole electron donor was evaluated

Table 1. Bacterial strains and plasmids used for this study.

Strains and plasmids	Description	Reference
<i>Shewanella oneidensis</i>		
MR-1	Manganese-reducing strain (Lake Oneida, NY)	Myers and Nealson (1988)
HyaA ⁻	ΔSO3920 (<i>hyaA</i> ⁻) deletion derivative of MR-1.	This work
HyaB ⁻	Also referred to as the Fe-only hydrogenase mutant ΔSO2098 (<i>hyaB</i> ⁻) deletion derivative of MR-1.	This work
CymA ⁻	Also referred to as the NiFe hydrogenase mutant ΔSO4591 (<i>cymA</i> ⁻) deletion derivative of MR-1	This work
HyaA ⁻ /HyaB ⁻	Double-deletion derivative of ΔSO3920 (<i>hyaA</i> ⁻) and ΔSO2098 (<i>hyaB</i> ⁻) within MR-1	This work
MtrC ⁻ /OmcA ⁻	Double-deletion derivative of ΔSO1778 (<i>mtrC</i> ⁻) and ΔSO1779 (<i>omcA</i> ⁻) within MR-1	Marshall <i>et al.</i> (2006)
CcmC ⁻	pMiniHimar RB1 transposon insertion in SO0261 (<i>ccmC</i>) gene of MR-1. Requires kanamycin (50 µg ml ⁻¹) selection.	Bouhenni <i>et al.</i> (2005)
MenD ⁻	Also referred to as BG148 pMiniHimar RB1 transposon insertion in SO4573 (<i>menD</i>) gene of MR-1. Requires kanamycin (50 µg ml ⁻¹) selection	This work
HyaB ⁻ (+pCR4- <i>hyaB</i>)	NiFe hydrogenase mutant (HyaB ⁻) containing the complementation plasmid, pCR4- <i>hyaB</i>	This work
<i>Escherichia coli</i>		
EC100D pir-116	Host used for construction of pDS3.1 clones. F- <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80d <i>lacZ</i> ΔM15 Δ <i>lacX74</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> Δ(<i>ara, leu</i>)7697 <i>galU</i> <i>galK</i> l- <i>rpsL</i> <i>nupG</i> pir-116	Epicentre
B2155 λpir	Host used for mating with MR-1. <i>thrB1004 pro thi strA hsdS lacZ</i> ΔM15 (F9 <i>lacZ</i> ΔM15 <i>lacI</i> ^{tr} <i>traD36</i> <i>proA1</i> <i>proB1</i>) Δ <i>dapA</i> :: <i>erm</i> (Erm ^r) <i>pir</i> ::RP4 [:: <i>kan</i> (Km ^r) from SM10]	Dehio and Meyer (1997)
Top10	Host used for construction of pCR [®] 4-TOPO [®] clones. F- <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> ΔM15 Δ <i>lacX74</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara, leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (<i>StrR</i>) <i>endA1</i> <i>nupG</i>	Invitrogen
Plasmids		
pDS3.1	Suicide vector, Amp ^r , Gm ^r , <i>sacB</i> ⁻	Wan <i>et al.</i> (2004)
pDS3.1:: <i>hyaA</i> ⁻	pDS3.1 containing sequences that flank <i>hyaA</i>	This work
pDS3.1:: <i>hyaB</i> ⁻	pDS3.1 containing sequences that flank <i>hyaB</i>	This work
pDS3.1:: <i>cymA</i> ⁻	pDS3.1 containing sequences that flank <i>cymA</i>	This work
pCR [®] 4-TOPO [®]	Used for complementation of deletions in MR-1 Amp ^r , Kan ^r	Invitrogen
pCR4- <i>hyaB</i>	Used for complementation of the NiFe hydrogenase mutant (HyaB ⁻). pCR [®] 4-TOPO [®] containing native <i>hyaB</i> downstream to <i>lac</i> promoter	This work

Fig. 1. H₂-driven Tc(VII) reduction kinetics by *S. oneidensis* MR-1 and hydrogenase mutant cells. The reduction of 100 µM Tc(VII) was determined for MR-1, single-hydrogenase deletion mutants (HyaA⁻ or HyaB⁻), a double-hydrogenase deletion mutant (HyaA⁻/HyaB⁻) or NiFe hydrogenase mutant cells induced with thiosulfate [HyaB⁻ (thio)] using 10 ml of H₂ as the electron donor. **Fig. 2.** Tc(VII) reduction kinetics by *S. oneidensis* MR-1 and hydrogenase or *c*-type cytochrome mutant cells using lactate as the sole electron donor. The reduction of 100 µM Tc(VII) was determined for MR-1, a deletion of the NiFe hydrogenase (HyaB⁻), a mutant lacking all *c*-type cytochromes (CcmC⁻) and a double-OMC deletion mutant (MtrC⁻/OmcA⁻) using 5 mM sodium lactate as the electron donor.

Fig. 3. H_2 -driven TcO_2 localization in *S. oneidensis* MR-1 and the NiFe hydrogenase mutant cells. Transmission electron microscopy micrographs of thin sections prepared from cell suspensions incubated with 250 μM ammonium pertechnetate and 10 ml of H_2 . The localization of TcO_2 in MR-1 at 24 h (A and B) was compared with NiFe hydrogenase mutant cells ($HyaB^-$) at 48 h (C and D). Locations of the cell membrane (CM), periplasm (P) and outer membrane (OM) are shown.

using MR-1 and the above mutants (Table S1). In the presence of H_2 , MR-1 and the Fe-only hydrogenase mutant ($HyaB^-$) were equally able to completely reduce 100 μM Tc(VII) within 24 h. In contrast, both deletions of the NiFe hydrogenase ($HyaB^-$ or $HyaA^-/HyaB^-$) reduced 13% of the Tc(VII) with H_2 at 24 h and less than 40% at the assay conclusion (96 h). This limited reduction was comparable to the reduction observed by MR-1 cells not supplied an electron donor (Fig. S1). We attributed the partial reduction by both NiFe hydrogenase mutants to residual energy reserves in cells grown in rich medium and not another specific H_2 -driven Tc(VII) reduction mechanism. Compared with H_2 , lactate was a relatively poor electron donor for Tc(VII) reduction (Fig. 2). Wild-type MR-1 cells reduced only 23% and 56% of the initial Tc(VII) after 2 and 5 days respectively. Significantly, although the NiFe hydrogenase mutant was slower to reduce Tc(VII) using lactate as the electron donor, it still reduced 41% of the Tc(VII) during the 5-day assay.

The subcellular localization of TcO_2 in wild-type MR-1 and the NiFe hydrogenase deletion mutant was determined by transmission electron microscopy (TEM) analysis of samples collected after the addition of Tc(VII) and H_2 (Fig. 3). Thin sections of MR-1 revealed that electron-dense TcO_2 nanoparticles accumulated in the cell periplasm and on the outside face of the OM (Fig. 3A and B).

Transmission electron microscopy analysis of the NiFe hydrogenase mutant revealed considerably lower relative abundance of TcO_2 nanoparticles in association with cells than observed in the wild type (Fig. 3C and D). This was consistent with this mutant's impaired capacity to reduce Tc(VII). Similar to the wild type, the NiFe hydrogenase mutant localized TcO_2 nanoparticles on the outside face of the OM. However, TcO_2 nanoparticles were not observed within the periplasm of the NiFe hydrogenase mutant (Fig. 3D). In both mutant and wild-type cells, TcO_2 nanoparticle localization was consistently cell-associated, consistent with enzymatic reduction. The electron-dense material observed in both samples, regardless of subcellular location, consisted of Tc nanoparticles with selected area diffraction (SAED) patterns consistent with those reported for synthetic and biogenic Tc(IV) O_2 (data not shown).

Previous studies of global transcriptome patterns displayed by *S. oneidensis* MR-1 cells grown in the presence of different electron acceptors indicated that *hydAB* (the genes encoding the Fe-only hydrogenase) are upregulated four- to sixfold under thiosulfate-reducing conditions (Beliaev *et al.*, 2005). Therefore, to ensure expression of the Fe-only hydrogenase, we induced cells deficient in functional NiFe hydrogenase ($HyaB^-$) for 3 h with 1 mM sodium thiosulfate prior to anoxic harvesting in preparation for resting-cell reduction assays. During the sample preparation, extreme caution was taken to minimize the cells exposure to O_2 as it has been reported that the Fe-only hydrogenase in other organisms is O_2 -sensitive (Horner *et al.*, 2000; Vignais and Colbeau, 2004; Ghirardi *et al.*, 2005). NiFe hydrogenase mutant cells ($HyaB^-$) induced in this manner reduced ~30% more Tc(VII) by 24 h than the non-induced NiFe hydrogenase mutant cells (Fig. 1) suggesting that the Fe-only hydrogenase can facilitate Tc(VII) reduction. The addition of heat-killed, thiosulfate-induced NiFe hydrogenase mutant cells did not reduce Tc(VII) in our assays (not shown).

The H_2 -driven reduction of Tc(VII) by S. oneidensis MR-1 does not have an absolute requirement for c-type cytochromes or menaquinone

The importance of c-type cytochromes in Tc(VII) reduction was investigated using mutants with deletions of genes previously shown to be involved in electron transport coupled to metal and U(VI) reduction (Myers and Myers, 2000; Beliaev *et al.*, 2001; Bencheikh-Latmani *et al.*, 2005; Marshall *et al.*, 2006) (Fig. 4 and Table S1). Resting-cell reduction assays with Tc(VII) and H_2 demonstrated that in-frame deletion mutants lacking either the cell membrane (CM) tetrahaem cytochrome (*cymA*) or two decahaem OMCs (*mtrC* and *omcA*) genes did not affect reduction compared with wild-type MR-1 cells (Fig. 4A).

Fig. 4. H_2 -driven Tc(VII) reduction kinetics by *S. oneidensis* MR-1, cytochrome mutants and menaquinone deficient cells. A. Tc(VII) reduction kinetics (100 μ M) was determined for MR-1, a single-cytochrome deletion mutant (CymA⁻), a double-outer membrane cytochrome deletion mutant (MtrC⁻/OmcA⁻) and a mutant deficient in the production of menaquinone (MenD⁻). B. The reduction of 100 μ M Tc(VII) was also determined for MR-1 and a mutant lacking all *c*-type cytochromes (CcmC⁻). Ten ml of H_2 was used as the electron donor for all experiments.

To further investigate the involvement of *c*-type cytochromes in Tc(VII) reduction, a previously described *S. oneidensis* MR-1 mutant lacking the ability to covalently incorporate haem into nascent apocytochromes (CcmC⁻) (Bouhenni *et al.*, 2005) was tested. The CcmC⁻ mutant was previously shown to be unable to reduce uranyl(VI) carbonate complexes to uraninite (Marshall *et al.*, 2006). The CcmC⁻ mutant was significantly slower than MR-1 at transferring electrons to Tc(VII) ($P < 0.001$), but did not abolish the ability of cells to quantitatively reduce 100 μ M Tc(VII) to Tc(IV) (Fig. 4B). This indicated that functional *c*-type cytochromes were not an absolute requirement for electron transfer to Tc(VII).

We also investigated the role of intermediary electron shuttling components in Tc(VII) reduction. Menaquinone is

a lipid-soluble component of electron transport chains involved in anaerobic respiration and is an intermediate carrier of electrons to terminal reductase complexes (Saffarini *et al.*, 2002). An interruption of the *menDHCE* operon (MenD⁻) was used to determine the role of menaquinone in H_2 -driven resting-cell Tc(VII) reduction assays. At 24 h, the MenD⁻ mutant cells reduced only 75.4% of the Tc(VII) compared with wild-type cells (Fig. 4A), but by the assay conclusion completely reduced 100 μ M Tc(VII).

OMCs can reduce Tc(VII)

As the NiFe hydrogenase mutant reduced only 15% less Tc(VII) than wild-type MR-1 using lactate as the electron donor, we investigated the lactate-driven Tc(VII) reduction capability of the *c*-type cytochrome mutants, CcmC⁻ and MtrC⁻/OmcA⁻ (Fig. 2 and Table S1). We found that these mutants were deficient, relative to the wild type, in the ability to reduce Tc(VII). In a 5-day resting-cell assay, these mutants reduced only 12% of the available Tc(VII). Under the same conditions, wild-type MR-1 reduced more than fourfold the amount of Tc(VII).

To evaluate whether reduced OMCs can directly transfer electrons to pertechnetate we evaluated whether purified, reduced MtrC or OmcA was oxidized upon exposure to Tc(VII)O₄⁻ *in vitro*. Reactivity did occur and the specific activities of MtrC and OmcA towards Tc were 56.9 ± 9.4 and 4.2 ± 0.6 nmole mg^{-1} min^{-1} respectively. In contrast, no detectable oxidation of either OMC was observed when mixed with the anoxic water alone.

The role of hydrogenase in the reduction of other metals

To determine the role of the *S. oneidensis* MR-1 hydrogenases in the reduction of U(VI), Fe(III), Mn(IV), Co(III) and V(V), we tested wild-type MR-1 and the hydrogenase mutants in resting-cell reduction assays using either H_2 or lactate as the sole electron donor (Fig. S2). When lactate was used as the electron donor, neither the Fe-only nor the NiFe hydrogenase was required for electron transfer. Additionally, a deletion of the Fe-only hydrogenase did not inhibit the cells ability to reduce these metals with H_2 . In contrast, when H_2 was used as the sole electron donor, deletions of the NiFe hydrogenase (either HyaB⁻ or HydA⁻/HyaB⁻) lacked the ability to transfer electrons to these metals. The induction of the Fe-only hydrogenase restored the H_2 -driven ability of NiFe hydrogenase mutant cells to reduce metals (Fig. S2B, F and H), while the complementation of the *hyaB* gene deletion [HyaB⁻ (+pCR4-*hyaB*)] restored the ability of the NiFe hydrogenase mutant to reduce both U(VI) and hydrous ferric oxide (HFO) (Fig. S3).

Discussion

This is the first report of a Tc(VII) reductase activity attributable to the *c*-type cytochromes, specifically the OMCs, MtrC and OmcA. While there has not been direct evidence of the *c*-type cytochrome facilitated Tc(VII) reduction in any DMRB, Wildung and colleagues (2000) first speculated that the OMCs of *Shewanella* would be theoretically capable of reducing TcO_4^- . The slower TcO_4^- reduction kinetics of MR-1 cells lacking all functional *c*-type cytochromes (CcmC⁻) suggested that these proteins play a role in H_2 -driven Tc(VII) reduction. We also demonstrated that MR-1 *c*-type cytochromes (MtrC and OmcA) functioned as reductases of Tc(VII) when coupled to the oxidation of lactate. This also validated our prediction that the bidirectional hydrogenase was not the exclusive Tc(VII) reductase and that an alternative mechanism for electron transfer to Tc(VII) existed in *S. oneidensis* MR-1.

Results from the whole-cell assays were consistent with the finding that purified, reduced OMCs were oxidized by Tc(VII)O_4^- in a defined *in vitro* system. It was not surprising that purified MtrC was at least an order of magnitude faster to transfer electrons to Tc(VII) than that of OmcA. We recently demonstrated that MtrC efficiently transferred electrons to uranyl citrate, while reduced OmcA was not oxidized by U(VI) within the same time frame (Marshall *et al.*, 2006). The electron transfer rates to Tc(VII)O_4^- were at least an order of magnitude slower than the reported electron transfer rates to soluble Fe(III)-NTA (Shi *et al.*, 2006) for either MtrC ($\text{Tc} = 0.069 \pm 0.011 \text{ S}^{-1}$ compared with $\text{Fe} = 2.0 \pm 0.2 \text{ S}^{-1}$) or OmcA ($\text{Tc} = 0.0056 \pm 0.0008 \text{ S}^{-1}$ compared with $\text{Fe} = 1.5 \pm 0.2 \text{ S}^{-1}$), in spite of the fact that the initial concentration of Tc provided was greater than the concentration of Fe(III) in the respective assays. The electrode potential (E°) of the Tc(VII)O_4^- – $\text{Tc(IV)O}_2 \cdot n\text{H}_2\text{O}$ couple is 746 mV at the standard state (Rard *et al.*, 1999) and 154 mV at pH 7.5 after correction for pH effects based on the redox reaction: $\text{Tc(VII)O}_4^- + 4\text{H}^+ + 3\text{e}^- = \text{TcO}_2 \cdot n\text{H}_2\text{O} + 2\text{nH}_2\text{O}$. As a comparison, at circumneutral pH the haems titrate over a range from –35 to –220 mV for OmcA and from –80 to –160 mV for MtrC (D. Richardson, pers. comm.), indicating that it is thermodynamically feasible for either of these two OMCs to reduce Tc(VII) to Tc(IV) over the concentration range used in this study.

Studies of Tc(VII) reduction in other organisms suggested that hydrogenase was involved in anaerobic electron transport to TcO_4^- (Lloyd *et al.*, 1999b; 2000; De Luca *et al.*, 2001). The involvement of *Shewanella* hydrogenases in the reduction of Tc(VII) was first proposed by Wildung and colleagues (2000) because of their significant homology to periplasmic hydrogenases produced by *Desulfovibrio*. To date, the precise biomolecular mecha-

nisms of Tc(VII) reduction by *S. oneidensis* MR-1 have not been identified. In this study, we established that the NiFe hydrogenase of MR-1 was essential for the rapid and complete reduction of Tc(VII) using H_2 as the electron donor. NiFe hydrogenase-deficient cells grown under conditions where the Fe-only hydrogenase was expressed reduced Tc(VII) at near wild-type MR-1 rates. Presumably, these reductions were attributable to the ability of hydrogenases to directly couple H_2 oxidation with Tc(VII) reduction as observed in *Desulfovibrio fructosovorans* (De Luca *et al.*, 2001). Although the electrochemical properties of the MR-1 hydrogenases have not been determined, the [4Fe-4S] clusters of the *Desulfovibrio gigas* NiFe hydrogenase have mid-point potentials of –290 and –340 mV (Teixeira *et al.*, 1989). If the mid-point potentials of the MR-1 and *D. gigas* NiFe hydrogenase were similar, then it would also be thermodynamically plausible for MR-1 hydrogenase to reduce Tc(VII) to Tc(IV). Additional studies to characterize these biochemical properties of the MR-1 hydrogenases are currently in progress.

Our findings suggested that the NiFe hydrogenase was the dominant uptake hydrogenase associated with the H_2 -driven reduction of Tc(VII) or other metals by MR-1 under the specific assay conditions examined herein. This finding was in agreement with another recent report on the functions of the MR-1 hydrogenases (Meshulam-Simon *et al.*, 2007). Hydrogenase, however, was not an absolute requirement for reduction by MR-1 as deletion of hydrogenases did not completely abolish the ability of cells to transfer electrons to Tc(VII) either with H_2 (Fig. 1) or with lactate (Fig. 2). This suggested that MR-1 has another mechanism of electron transfer to Tc(VII) in addition to hydrogenase, but that the kinetics of this alternative mechanism are significantly slower. Experiments investigating the NiFe hydrogenase mutants ability to reduce Tc(VII) coupled to lactate oxidation revealed that this mutant reduced Tc(VII) at a rate nearly equal to the wild-type MR-1. While this result was consistent with the previous finding that electron donor-specific Tc(VII) reduction-deficient mutants of *Shewanella* could be isolated (Payne and DiChristina, 2006), it contrasted with reports that other organisms such as *G. sulfurreducens* and *D. fructosovorans* can only couple Tc(VII) reduction with H_2 oxidation (Lloyd *et al.*, 2000; De Luca *et al.*, 2001).

Studies on the role(s) of *c*-type cytochromes in the reduction of U(VI) have been focused on determining the terminal reductase(s) of U(VI) and hence the role of electron donor type was not considered (Bencheikh-Latmani *et al.*, 2005; Marshall *et al.*, 2006). While lactate oxidation was coupled to *c*-type cytochrome-dependent reduction of U(VI) (Marshall *et al.*, 2006), the reduction of U(VI) by *c*-type cytochromes is not linked to the specific type of electron donor supplied as the addition of H_2 results in a similar *c*-type cytochrome-dependent reduction of

U(VI) (Fig. S4A). Therefore, we conclude that *c*-type cytochrome-facilitated U(VI) reduction is independent of electron donor type even though some differences in electron donor specific-kinetic rates among different dissimilatory metal-reducing *Shewanella* have been reported (Liu *et al.*, 2002). In contrast, the specific rate of H₂-driven Tc(VII) reduction for wild-type MR-1 was approximately tenfold higher than when lactate was provided as an electron donor (Table S1). Similarly, the H₂-driven specific rate of Tc(VII) reduction has been reported to be significantly faster than the formate-driven reduction in *Desulfovibrio desulfuricans* (Lloyd *et al.*, 1999b). These donor-specific rate differences for Tc(VII) reduction may be related to the low potentials of the intermediate Tc valence states [Tc(VI) = −640 mV, and Tc(V) = −600 mV] (Rard *et al.*, 1999) that, once formed, rapidly disproportionate to yield Tc(IV) as a stable product. The low potential, intermediate valence states act as a thermodynamic barrier in the Tc(VII) to Tc(IV) redox reaction. The lower mid-point potential of *D. gigas* NiFe hydrogenases (−290 to −340 mV) as compared with those of either MtrC or OmcA (−35 to −220 mV) indicates that the former proteins should be more able to drive the reduction reaction through the problematic intermediate states. If the MR-1 NiFe hydrogenase possesses a mid-point potential similar to *D. gigas*, then it would also be thermodynamically plausible for MR-1 hydrogenase to drive the reduction reaction in a similar fashion. These differences may explain the relative rates of Tc(VII) reduction via the H₂-hydrogenase and lactate-OMC pathways. In contrast, the electrode potentials for the reaction of U(VI) to U(V) (90 mV) or U(VI) to U(IV) (1870 mV) (Guillaumont *et al.*, 2003) are considerably more thermodynamically favourable for efficient electron transfer by the OMCs.

Under conditions where H₂ and organic compounds are available electron donors, it is likely that both the hydrogenase- and cytochrome-mediated Tc(VII) reduction processes are functioning concurrently. Other researchers have reported the close physical association of hydrogenase and *c*-type cytochrome (Peck, 1993; De Luca *et al.*, 2001). The formate hydrogenlyase complex in *Desulfovibrio* consists of a formate dehydrogenase, linked to a hydrogenase via a cytochrome (Peck, 1993). A small periplasmic *c*-type cytochrome was also associated with the *D. fructosovorans* NiFe hydrogenase *in vitro* Tc(VII) reductase activity (De Luca *et al.*, 2001). They reported that purified hydrogenase reduced Tc(VII) in assays amended with H₂.

Previously, we determined that biogenic UO₂ nanoparticles localized not only within the cell periplasm and at the surface of OM (as seen in Fig. 3), but also with a complex exopolymeric substance (EPS) (Marshall *et al.*, 2006). In the current study, we did not observe the formation of a similar TcO₂-EPS matrix with either the wild-type MR-1 or

NiFe hydrogenase mutant cells. Instead, TcO₂ nanoparticles were localized only to the cell surface, in agreement with previous observations (Lloyd *et al.*, 2000; Wildung *et al.*, 2000). The absence of a TcO₂-EPS matrix could not be attributable to electron donor supplied in our assays as UO₂ nanoparticles were also localized with EPS when H₂ was supplied as the sole electron donor (Fig. S4B). The difference between U and Tc nanoparticle localization may be explained by the periplasmic localization of the NiFe hydrogenase and its role of H⁺ production and Tc(VII) reduction during H₂-driven electron transfer to Tc(VII). In contrast, the redundant network of *c*-type cytochromes involved in reduction of U(VI) are localized not only within the periplasm, but also are found outside of the OM, either attached to the outside face or associated with EPS (Myers and Myers, 2003a; Marshall *et al.*, 2006). Consequently, wild-type MR-1 cells accumulated a large portion of TcO₂ nanoparticles in their periplasmic space whereas UO₂ nanoparticles were more evenly distributed between the periplasm and exterior of the cell. Cells lacking functional NiFe hydrogenase accumulate TcO₂ nanoparticles only at the OM surface, presumably due to the localization of OMCs; however, these cells are slow to reduce Tc(VII) relative to wild-type MR-1. The relatively slow electron transfer rates to Tc(VII) from both MtrC and OmcA (independent of electron donor source) would explain why the NiFe hydrogenase mutant does not accumulate large quantities of TcO₂ at the OM surface during assay conditions where Tc(VII) reduction by OMCs would be the sole reduction pathway. The localization of TcO₂ at the OM surface of the NiFe hydrogenase mutant, in conjunction with the lactate-driven Tc(VII) reduction kinetics for both the CcmC[−] and MtrC[−]/OmcA[−] mutants, suggested that MtrC and OmcA are the principal reductases in the *c*-type cytochrome-mediated Tc(VII) reduction pathway. Understanding the relationship between the electron donor and subcellular fate of TcO₂ nanoparticles could potentially be important in the environment where oxidants such as O₂ or MnO₂ may also be present or where colloidal transport could be facilitated.

This report is the first to systematically investigate the role of hydrogenases and the electron transfer system of *S. oneidensis* MR-1 in the reduction of highly mobile pertechnetate anions. We present a Tc(VII) reductase activity by at least two *c*-type cytochromes. We also conclusively show that the NiFe hydrogenase and, to a lesser degree, the Fe-only hydrogenase play a more direct role in the reduction of Tc(VII) than just the production of H⁺ for subsequent electron transfer reactions. Furthermore, we demonstrate that the source of electron donor may be advantageous for the selection of either the hydrogenase or the *c*-type cytochrome mediated Tc(VII) reduction pathway *in vivo*. Although the kinetics of lactate-driven Tc(VII) reduction may be slower than H₂-driven reduction

Table 2. Primers used in this study.

Primer name	Nucleotide sequence ^a
<i>ΔSO3920 (hyaA⁻)</i>	
3920_F-O	5'-CCCATTAGCCTTTCGTGATG-3'
3920_5-O	5'-ACCGTTCAAGAAATATTCC-3'
3920_5-I	5'- <i>CCCATCCACTAAACTTAAACACGATAGAGTGTACTGCACCC</i> -3'
3920_3-I	5'- <i>TGTTTAAGTTTAGTGGATGGGGAGGTGATGAAC</i> TGCGCTGG-3'
3920_3-O	5'-CCCCCGGGAGCGGTTTAAAAACAATAG-3'
3920_R-O	5'-GGGCGATGAACAGTACCACT-3'
<i>ΔSO2098 (hyaB⁻)</i>	
2098_F-O	5'-CAATAACGGCAGTTTCTTAAC-3'
2098_5-O	5'-AGGATAGAGCTCTGCACGAAGTGGTCAGCAAA-3'
2098_5-I	5'- <i>CCCATCCACTAAACTTAAACACGATAGAGGATGACCATGCTT</i> -3'
2098_3-I	5'- <i>TGTTTAAGTTTAGTGGATGGGCCTTGCATGGCGTGTT</i> CAGT-3'
2098_3-O	5'-AGGATAAAGCTTCCGTGATCCAAGCTGCACTG-3'
2098_R-O	5'-ATGCCCAAGTGAGGTAGTGG-3'
<i>ΔSO4591 (cymA⁻)</i>	
4591_F-O	5'-GCATGACTCATTAAATACCA-3'
4591_5-O	5'-GTCTTGGCAATGAATCATGCA-3'
4591_5-I	5'- <i>GTGTAGCGTTGTGTCTGCCACCAGTTCATTACTCTATCTCCA</i> -3'
4591_3-I	5'- <i>TGGGCAGACACAACGCTACACGGATAAGGTTTAACGCTGCAA</i> -3'
4591_3-O	5'-TGACGACTCACAATCACATGA-3'
4591_R-O	5'-GCGCAAAGTCTCTCACTA-3'
pCR4- <i>hyaB</i> complementation	
HyaB_F	5'-CACCTAACCAAGCCGCTGGAGGAATAAC-3'
HyaB_R	5'-TTATTGACCATTGGGGCTGAC-3'

a. Sequences in italics are added to facilitate fusion of first-round PCR amplicons as described by Link and colleagues (1997).

in the laboratory, the implications of understanding both Tc(VII) reduction mechanisms could potentially be important in the environment where organic electron donor concentrations may be high relative to H₂. Alternatively, the relatively slow lactate-driven Tc(VII) reduction rates may suggest that the *c*-type cytochrome-mediated pathway will not be an important route in environments where H₂ or Fe(II) is high as hydrogenase-mediated or abiotic reduction rates (Fredrickson *et al.*, 2004) may likely be much faster. Together, these findings could significantly influence the future design of *in situ* bio-stimulation/bioremediation strategies designed to exploit the biogenic Tc(VII) reduction and the subsequent formation of Tc(IV)O₂ nanoparticles which would reduce Tc solubility, and hence mobility in the subsurface.

Experimental procedures

Chemicals and media

All chemicals used in this study were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted. Growth media were purchased from BD Diagnostics (Sparks, MD).

Generation of in-frame deletion mutants and complementation vector

Shewanella oneidensis MR-1 mutants lacking selected hydrogenase or *c*-type cytochrome genes were constructed using two-step homologous recombination with a suicide plasmid encoding flanking DNA sequence using a modifica-

tion of previously described methods (Link *et al.*, 1997; Wan *et al.*, 2004). Primer sequences, plasmids and strains used in this study are described in detail in Tables 1 and 2. *Shewanella oneidensis* MR-1 and *Escherichia coli* strains used for mutagenesis were grown using Luria-Bertani (LB) medium at 30°C and 37°C respectively. The plasmid pDS3.1 was purified from *E. coli* using the Qiagen QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and digested with XcmI [New England Biolabs (NEB), Beverly, MA]. *Shewanella oneidensis* chromosomal DNA was isolated using DNAzol (Invitrogen, Carlsbad, CA) and used as the template for polymerase chain reaction (PCR) amplification using Vent DNA polymerase (NEB). Sequences that flank the respective genes targeted for deletion were joined by amplifying each locus with 5-O/5-I and 3-O/3-I primer pairs, annealing them via complementary sequences present in the 5-I and 3-I primers, and then subjected to a second round of PCR using the 5-O and 3-O primers as described (Link *et al.*, 1997). The fusion PCR amplicon was purified using 1% agarose electrophoresis (Sambrook *et al.*, 1989) and ligated into the pDS3.1 plasmid using the FastLink Ligation kit (Epicentre, Madison, WI) prior to transformation into *E. coli* EC100D pir-116 (Epicentre) (Hanahan, 1983) and plating on LB with gentamicin selection (15 µg ml⁻¹). Correct transformants were verified by PCR using the 5-O and 3-O primers and the plasmids were purified and transformed into the *E. coli* β-2155 mating strain by plating on gentamicin (15 µg ml⁻¹) and diaminopimelic acid (100 µg ml⁻¹) selection. Conjugal transfer of plasmid and homologous recombination from *E. coli* mating strain to *S. oneidensis* was performed (de Lorenzo *et al.*, 1990) and primary integrants were selected with gentamicin (7.5 µg ml⁻¹). Polymerase chain reaction screening for homologous recombination of the plasmid and the insertion site of amplicon recombination within the genome were

accomplished using the F-O/3-O or 5-O/R-O primer pairs. Sucrose sensitivity of the primary integrants was verified by plating on LB-NaCl +10% sucrose (Blomfield *et al.*, 1991). The second homologous recombination was selected for by growing the primary integrant for 16–18 h in LB-NaCl broth cultures followed by plating on LB-NaCl +10% sucrose. Colonies sensitive to gentamicin ($7.5 \mu\text{g ml}^{-1}$) were screened for deletion of the gene of interest by PCR using the F-O/R-O primer set and compared with the same fragment amplified from wild-type MR-1. The PCR amplicon generated from the mutants with the F-O/R-O primer set was used as the template for DNA sequencing of the deleted gene(s) and recombination regions (ACGT, Wheeling, IL).

The complementation of the *hyaB* deletion was accomplished by PCR amplification of the *hyaB* coding sequence from *S. oneidensis* chromosomal DNA with the HyaB_F and HyaB_R primer set and cloning into the pCR4-TOPO® vector (Invitrogen) prior to transformation into *E. coli* Top10 (Invitrogen) and plating on LB with kanamycin selection ($50 \mu\text{g ml}^{-1}$) (Table 1). Correct transformants were verified by PCR amplification. The pCR4-*hyaB* plasmid was purified and transferred into NiFe hydrogenase mutant cells [HyaB⁻ (+pCR4-*hyaB*)] by electroporation using previously described protocols (Hanahan, 1983; 1985). Antibiotic selection [kanamycin ($50 \mu\text{g ml}^{-1}$)] was maintained to ensure stability of the pCR4-*hyaB* plasmid in all cultures except for the resting-cell reduction assay tubes. The PCR amplicon generated using the HyaB_F and HyaB_R primer set was used as the template for DNA sequencing of the *hyaB* complementation plasmid.

Tc(VII) reduction assay conditions

The kinetics of aqueous Tc(VII) reduction and localization in wild-type MR-1 and mutant cells were determined in a standard resting-cell assay. Tryptic soy broth without dextrose (TSB-dex) cultures (100 ml) was grown for 16 h (30°C) at 100 r.p.m. and harvested by centrifugation (5000 *g*, 5 min). Cells were washed once in equal volume of 30 mM piperazine-*N,N'*-bis(ethanesulfonic acid) (PIPES) buffer (pH 7.0, 4°C), pelleted, re-suspended in the fresh buffer at a concentration of 2×10^9 cells ml^{-1} and purged for ~10 min with N₂ gas. Tc(VII) reduction assays contained a final concentration of 100 μM ammonium pertechnetate [$\text{NH}_4^{99}\text{Tc(VII)O}_4$] (Perkin-Elmer, Boston, MA) in 30 mM PIPES purged with O₂-free N₂ and sealed with thick butyl rubber stoppers. The electron donors used in all studies were either 10 ml of H₂ or 5 mM sodium lactate. Kinetic studies were initiated by the addition of 1 ml of standardized cells, resulting in a final assay density of 2×10^8 cells ml^{-1} , to the assay tubes followed by horizontal (lactate) or inverted (H₂) incubation at 30°C with slow gyratory shaking (75 r.p.m.). The amount of soluble Tc(VII) remaining in all samples was analysed at multiple time points by liquid scintillation counting of ⁹⁹Tc (0.292 MeV beta) as previously described (Fredrickson *et al.*, 2000; Wildung *et al.*, 2000). Samples using H₂ as the electron donor could be directly counted, whereas samples using lactate as the electron donor required complexation with tetraphenyl arsonium chloride (TPAC) at a 40:1 molar ratio and extracting the TPAC-TcO₄⁻ complex from the soluble phase with chloroform prior to liquid scintillation counting (Tribalat and Beydon, 1953; Wildung *et al.*, 2000).

Expression of the Fe-only hydrogenase (HydAB) was accomplished by induction with thiosulfate. At 13 hours post inoculation at 30°C (100 r.p.m.), a TSB-dex culture (100 ml) of the NiFe hydrogenase mutant (HyaB⁻) was harvested by centrifugation (5000 *g*, 5 min) at room temperature and re-suspended in an equal volume of anaerobic minimal media (Beliaev *et al.*, 2005) containing 1 mM sodium thiosulfate and 20 mM sodium lactate. The medium was purged for 10 min with N₂ and sealed. Cells were grown for an additional 3 h (30°C) at 100 r.p.m. and harvested as described above except that an additional wash step was included to ensure complete removal of thiosulfate prior to the addition of pertechnetate (or other electron acceptor). During harvest and preparation for kinetic assays, cells were kept under N₂ and all solutions used were anoxic (4°C). For all experiments, H₂ (10 ml) was used as the electron donor. To ensure that the thiosulfate in the defined media was completely removed from reduction assays, an aliquot of washed NiFe hydrogenase mutant (HyaB⁻) cells was heat-killed and added to an identical set of assay tubes.

Uranium and metal reduction coupled to lactate or H₂ oxidation

For additional phenotype characterizations, 16 h TSB-dex cultures were grown and washed as described above except that a 30 mM sodium bicarbonate buffer (pH 7.0, 4°C) was substituted for the 30 mM PIPES buffer. Standardized cell suspensions (2×10^9 cells ml^{-1}) were purged for ~10 min with O₂-free mixed gas [N₂:CO₂ (80:20)]. Reduction assays were conducted in 30 mM sodium bicarbonate buffer (pH 7.0) and contained a final concentration of either 250 μM U(VI), as uranyl acetate, 1.0 mM ferric(III)-nitrilotriacetic acid (NTA), 10 mM silica-ferrihydrite (Si-HFO) (Kukkadapu *et al.*, 2004), 1.0 mM cobalt(III)-ethylenediaminetetraacetic acid (EDTA), 10 mM sodium vanadate(V) or 1.0 mM manganese as vermadite [Mn(IV)O₂]. In all assays, either H₂ (10 ml) or sodium lactate (20 mM for tubes containing Si-HFO and 10 mM for all other electron donors) was added the sole electron donor. Assay tubes were purged with mixed gas and sealed prior to the addition of 1 ml of standardized cells followed by horizontal incubation at 30°C with slow gyratory shaking (25 r.p.m.). At multiple time points, samples were collected in an anoxic atmosphere and analysed for specific reduction. In the U(VI) reduction assays, soluble U(VI) remaining in filtrates (< 0.2 μm pore size) from all samples was analysed at multiple time points using a kinetic phosphorescence analyser (KPA-10; Chemchek Instruments, Richland, WA) as previously described (Brina and Miller, 1992). Samples generated in experiments with Si-HFO or Fe(III)-NTA were extracted overnight with 0.5 N HCl before determining the Fe(II) concentration at 562 nm using the ferrozine assay (Fredrickson *et al.*, 1998). The reduction of insoluble brown MnO₂ was monitored by reduction to soluble Mn(II), which is colourless. Co(III) or V(V) reduction was detected by a clearing of the pink colour or the formation of a dark green precipitate in the assay tubes respectively.

Transmission electron microscopy (TEM)

Resting cells were prepared as described above and incubated with 250 μM ammonium pertechnetate and 10 ml of

H₂ for all localization studies. Cells were fixed, embedded and sectioned using anoxic techniques (Marshall *et al.*, 2006). Ultra thin sections were examined at 200 kV using a JEOL 2010 high-resolution TEM equipped with LaB₆ filament with a resolution of 1.9 Å coupled with electron dispersive spectroscopy (EDS) (EDS, Oxford). Images and selected area diffraction (SAED) patterns were digitally collected and analysed using DigitalMicrograph software (Gatan, Pleasanton, CA).

Reductase activity of recombinant cytochromes

The recombinant *c*-type cytochromes, MtrC and OmcA, were expressed and purified as described previously (Shi *et al.*, 2006). Proteins were prepared at a concentration of 5.5 µM (55 µM haem) in buffer containing 50 mM *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid (HEPES) buffer (pH 7.5), 100 mM NaCl, 10% glycerol and 0.5% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and purged with O₂-free N₂ gas for 2 h. A 1.57 mM Tc(VIII) stock in water was prepared and degassed in similar manner. The reaction of recombinant cytochrome (MtrC or OmcA), which was reduced by titrating with sodium dithionite, with Tc(VII) was initiated by the addition of 0.45 ml of cytochrome with 0.05 ml of Tc(VII) stock in an anoxic atmosphere (< 0.1 p.p.m. O₂). The reaction was mixed by gentle inversion and the oxidation of haem immediately monitored using a Cary 500 Scan UV-Vis-NIR spectrophotometer (Varian, Palo Alto, CA) at λ_{540–560} every ~30 s until complete. Similar reaction containing 0.05 ml of anoxic water without Tc(VII) was also monitored. The reaction between reduced cytochrome and Tc(VII) was analysed using protocols detailed by Dobbin and colleagues (1999).

Statistical analysis

Radionuclide and metal reduction curves were compared using non-parametric procedures, specifically the Wilcoxon signed-rank test. These tests were conducted using Systat 10 (SPSS, Chicago, IL) and were considered significant at *P* < 0.01; specific values of *P* are reported where relevant.

Accession numbers

The GenBank (<http://www.ncbi.nlm.nih.gov/GenBank>) accession numbers for the protein sequences described in this article are HydA (gil24375408), HyaB (gil24373658), CymA (gil24376064), MtrC (gil24373344), OmcA (gil24373345), CcmC (gil24371859) and MenD (gil24376046).

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. The effect of TSB-dex pre-growth on the resting-cell Tc(VII) reduction kinetics by *S. oneidensis* MR-1 cells. The reduction of 100 μM Tc(VII) was determined for MR-1 either by using 10 ml of H_2 as the electron donor (MR-1) or by not supplying an electron donor [MR-1 (no donor)].

Fig. S2. The effect of electron donor on metal reduction by *S. oneidensis* MR-1 and hydrogenase mutant cells. The reduction of several metals was determined using either 10 mM sodium lactate (A, C, E and G) or 10 ml of H_2 (B, D, F and H) as the sole electron donor in standard reduction assays. U(VI) reduction kinetics (250 μM) was determined for MR-1, single-hydrogenase deletion mutants (HydA⁻ or HyaB⁻) and a double-hydrogenase deletion mutant (HydA⁻/HyaB⁻) (A and B). The reduction kinetics of 10 mM HFO (C and D) or 1.0 mM Fe(III)-NTA (E and F) was determined by quantifying the 0.5 N HCl extractable Fe(II) in solution at all time points. The NiFe hydrogenase deletion mutant pre-induced with thiosulfate incubation [HyaB⁻ (thio)] was also tested for reduction of U(VI) or Fe(III)-NTA using H_2 as the electron donor (B and F). The reduction of 1.0 mM Co(III), 1.0 mM Mn(IV) or 10 mM V(V) was determined visually for MR-1 (lanes 1), HydA⁻ (lanes 2), HyaB⁻ (lanes 3), HydA⁻/HyaB⁻ (lanes 4), HyaB⁻ (thio) (lane 5) and heat-killed MR-1 cells (lanes 6) (G and H). Reduction was recorded as colour change from pink to clear [Co(III) reduction], clearing of the black precipitate [Mn(IV) reduction] or the appearance of a dark green precipitate [V(V) reduction] relative to non-reducing, heat-killed MR-1 cells. No reduction was observed in our assays when heat-killed, thiosulfate-induced NiFe hydrogenase mutant cells were assayed (data not shown). Images illustrate the representative data from multiple experiments.

Fig. S3. The complementation of the H_2 -driven metal reduction-deficient phenotype of the NiFe hydrogenase mutant cells. The reduction of 250 μM U(VI) or 10 μM HFO was determined for NiFe hydrogenase mutant cells containing the complementation plasmid [HyaB⁻ (+pCR4-hyaB)] and wild-type MR-1 cells using 10 ml of H_2 as the electron donor.

Fig. S4. The H_2 -driven U(VI) reduction kinetics and UO_2 nanoparticle localization by *S. oneidensis* MR-1. The reduction of 250 μM U(VI) was determined for MR-1 and a mutant lacking all c-type cytochromes (CcmC⁻). The U(VI) reduction kinetics was determined using 10 ml of H_2 as the sole electron donor.

A. Whole mount TEM micrograph prepared from cell suspension of *S. oneidensis* MR-1 incubated with 250 μM uranyl acetate and 10 ml of H_2 for 24 h.

B. The localization of the UO_2 -EPS is shown in association with intact cells.

Table S1. Electron donor-dependant specific rates of Tc(VII) reduction.

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Supporting Information

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Table S1. Electron donor-dependant specific rates of Tc(VII) reduction.

A **B** **C** **D** **E** **F** **G** **H**

A **B**

A **B**

Table S1.**Electron donor-dependant specific rates of Tc(VII) reduction.**

Strain	Specific rate ^{ab}	
	H ₂	Lactate
MR-1	39.56 ± 1.40	4.17 ± 0.60
HydA ⁻	38.08 ± 2.55	nd
HyaB ⁻	5.24 ± 1.51	2.58 ± 0.78
HydA ⁻ /HyaB ⁻	5.48 ± 1.73	nd
MtrC ⁻ /OmcA ⁻	42.72 ± 3.56	0.76 ± 0.80

^aSpecific rate is reported in umole Tc(VII) reduced g total protein⁻¹ h⁻¹ at 30°C.

^bInitial specific rate was determined for the first 9 hours of Tc(VII) reduction with H₂ or the first 48 hours of Tc(VII) reduction with lactate.

Data represents the mean and standard deviation from representative experiments (*n*=4).

nd, not determined