Mechanisms for the reduction of radionuclides and other metal contaminants in *Geobacter sulfurreducens*

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Introduction

- Many dissimilatory metal-reducing microorganisms can reduce the key radioactive contaminants, U and Tc from soluble high oxidation state forms to insoluble, low oxidation states forms (1).
- Little is known about the enzymatic mechanisms for these bioreductions.
- An understanding of these mechanisms is needed if these microorganisms are to be exploited in bioremediation technologies effectively and modeled accurately.

G. sulfurreducens has been chosen for study because

- It is an important component of subsurface biota (2).
- It's entire genome sequence is available (2).
- A genetic system for this organism is available (3).

Aims & Objectives

- To characterise the mechanisms of U(VI) and Tc(VII) reduction in *G. sulfurreducens*.
- To confirm the identity of the genes encoding the relevant reductases in this organism.
- To determine the range of other radionuclides (Np, Pu) and key metal pollutants (Cr, Hg, Co) reduced by *G. sulfurreducens*.
- To identify the roles of the U(VI) and Tc(VII) reductases in the reduction of these other key pollutants.

Methodology

Resting Cell Experiments

- Cells grown in NBAF medium (*G.sulfurreducens*) or ferric citrate medium (*Shewanella putrefaciens*) were harvested at late log phase, washed twice with buffer and resuspended in buffer.
- Cells were incubated at 30°C under anaerobic conditions, in buffer with the metal ion of interest and electron donor as appropriate (see Table below).

	Conc	Buffer	e- donor
TcO_4^-	250□M	30mM $NaHCO_3$ pH7	Acetate, H_2
CrO_4^{2-}	100□M	30mM $NaHCO_3$ pH7	
UO_2^{2+}	5mM	30mM $NaHCO_3$ pH7	Acetate
NpO_2^+	1mM[1] 0.5, 0.1mM[2]	20mM MOPS pH7	Acetate
Pu(IV)	3□M	50mM MOPS/100mM $NaHCO_3$ pH7.7	Acetate

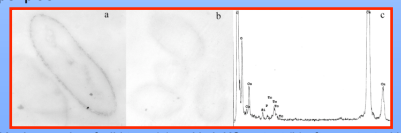
[1]*G. sulfurreducens*; [2]*S. putrefaciens*

Growth Experiments

- *G. sulfurreducens* was grown in NBAF medium with up to 0.1 mM NpO_2^{2+}, or 0.1mM Hg^{2+}.

Technetium

- *G.,sulfurreducens* can couple oxidation of H_2 to the reduction of Tc(VII) to insoluble $Tc^{IV}O_2$ (4).
- Studies involving a *hyb* Knockout mutant (supplied by Drs Coppi & Lovley) showed that Hyb, a periplasmic NiFe hydrogenase is a key enzyme in Tc(VII) reduction.
- Electron microscopy confirms that Tc is precipitated in the periplasm.



TEM micrographs of wild type (a) and hyb KO mutant (b) after contact with Tc(VII). Wild type cells have a dark precipitate (Tc(IV)) around the periphery of the cell. Bar = 0.5 □m. EDAX spectrum (c) of the periphery of a wild type cell, confirmed the presence of Tc. TEM by Dr S. Glasauer.

Chromium

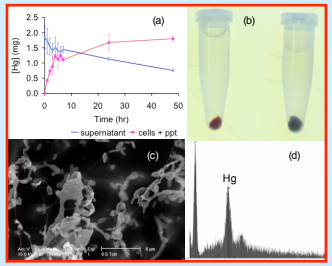
- Strains used: wild type strain (with no e^- donor or acetate) and a mutant with cytochrome c_7 gene (*ppcA*) deleted (supplied by Drs Leang & Lovley, UMASS).
- *G. sulfurreducens* can couple oxidation of acetate to the reductive precipitation of Cr(VI).
- Loss of cytochrome c_7 causes a decrease in Cr(VI) reduction.
- Cytochrome c_7 is involved in the Cr(VI) reductive mechanism but is not critical to it.

	Specific rate of reduction nmol Cr (mg [dry weight]of biomass)$^{-1}$ h^{-1}
Wild type, no e^- donor	0.8
Wild type, acetate	35.8
C_7 KO mutant	23.5

- EPR studies showed that a Cr(V) intermediate is formed, associated with the cells.

Mercury

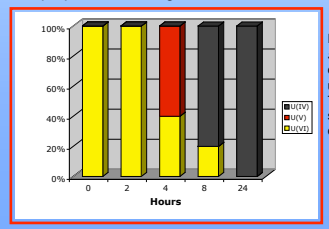
- Hg (II) is removed from solution by *G. sulfurreducens*
- However, growth is inhibited by Hg(II).



(a) Loss of Hg from solution & increase in cell-associated & precipitated Hg
(b) Cell pellets from cultures with (right) & without (left) Hg
(c) ESEM micrograph of cells & precipitate from a culture with Hg
(d) EDAX spectrum of precipitate

Uranium

- We have previously shown U(VI) is reduced *via* a mechanism involving periplasmic cytochrome c_7 (PpcA) (5).
- PpcA has been expressed in *E. coli*, but there was no U(VI) reduction activity □ PpcA not part of a functional electron transfer chain in *E. coli*.
- *In vitro* studies have shown that U(VI) can be reduced by PpcA (coupled to a NiFe hydrogenase from *Desulfovibrio vulgaris*).
- XAS studies demonstrated that $\{U^{VI}O_2\}^{2+}$ is reduced *via* a one-electron step to an unstable $\{U^{V}O_2\}^{+}$ intermediate, that disproportionates to give insoluble UO_2.

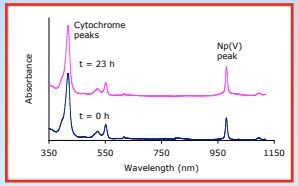


Reduction of U(VI) by *G. sulfurreducens* & the detection of U(V) as an unstable intermediate. The 3 uranium oxidation states were distinguished by EXAFS

Neptunium

Geobacter sulfurreducens

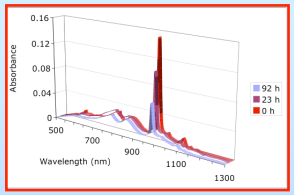
- Growth was observed at Np concentrations from 0 to 0.1 mM.
- However, at the higher concentration, ~ 50 % of Np was lost in the control (no cells) and ~ 70% in the cultures, due to precipitation and biosorption.
- UV/vis spectra showed no evidence for reduction of Np(V)
- Resting cells were unable to reduce Np(V) at 1mM.
- *In vitro* studies showed that Np(V) was not reduced by the reduced form of cytochrome c_7 (coupled to a NiFe hydrogenase from *Desulfovibrio vulgaris*)



UV/vis spectra of Np(V) incubated with cytochrome c_7. The spectra show only Np(V) is present and cytochrome $c_7$remained reduced throughout.

Shewanella putrefaciens

- Np(V) (up to 0.5 mM) was reduced by *S. putrefaciens*, with lactate as the electron donor.
- With H_2, as the electron donor, Np(V) was reduced at 0.1 mM concentration, but not 0.5 mM.

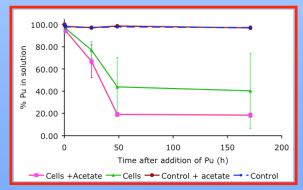


UV/vis spectra of supernatant samples from cell suspensions of *S. putrefaciens* with 0.5 mM Np(V) & 100 mM lactate. The spectra show a decrease in the Np(V) peak at 980 nm with time.

- *S. putrefaciens* can couple reduction of Np(V) to oxidation of lactate and H_2, but reduction is dependent on Np concentration and electron donor.

Plutonium

- Preliminary expts with Pu239 show that *G. sulfurreducens* can remove Pu(IV) from solution.
- The mechanism of removal is not known.



Removal of Pu(IV) from solution by *G. sulfurreducens*

- Insoluble Pu(IV) was not resolubilzed by *G. sulfurreducens*

Enzyme Purification

- Hyb, a periplasmic NiFe hydrogenase involved in Tc(VII) reduction, has been partially purified.
- Initial *in vitro* studies suggest Hyb is unable to couple oxidation of H_2 to U(VI) reduction.
- 3 other hydrogenases have been identified: one in the membrane fraction and two in the soluble fraction (one NADH-reducing & one Ni-reducing).
- A periplasmic 12 heme cytochrome containing 4 c_7 domains has also been purified and characterized from the soluble fraction.

Summary

- Tc(VII) is reduced via periplasmic NiFe hydrogenase Hyb.
- U(VI) and Cr(VI) are reduced *via* an alternative cytochrome c_7 dependent pathway, with Cr(V) and U(V) intermediates formed.
- Reduction of U(V) is by disproportionation.
- Hg(II) is removed from solution by *G. sulfurreducens*
- Np(V) is not reduced by *G. sulfurreducens*, suggesting a surprising degree of specificity for key actinide species in this organism.
- *G. sulfurreducens* can remove Pu(IV) from solution

Future Work

- Investigate the effect of *G. sulfurreducens* on the redox chemistry of Pu, using UV/vis spectroscopy to monitor oxidation states.
- Check full range of metals/radionuclides reduced by *G. sulfurreducens* (including Np(VI), Co(III)-EDTA).
- Characterize the end products of Cr(VI) and Hg(II) reduction.
- Determine the roles of cytochrome c_7 and Hyb in metal reduction *in vitro* and *in vivo* using (partially) purified proteins and deletion mutants.
- Investigate the structural basis of U(VI) reduction by cytochrome c_7 , using mutated derivatives of the enzyme *in vitro*.
- Determine the physiological role of the 12 heme cytochrome (with Drs M. Bruschi & D.R. Lovley).
- Assess role of other proteins required for Fe(III) reduction, in the reduction of actinides (with Dr. D.R. Lovley).

Publications

Renshaw *et al.* Bioreduction of uranium: environmental implications of a pentavalent intermediate. *Env. Sci. Technol.* (in revision)
Lloyd & Renshaw. Microbial transformations of radionuclides. *Curr. Opin. Biotechnol.* (submitted)
Renshaw *et al.* Enzymatic reduction of Tc(VII)is catalyzed by a periplasmic NiFe hydrogenase in *Geobacter sulfurreducens*. *Appl. Environ. Microbiol.* (submitted)
Lloyd *et al.* Biotransformation of actinides: Microbial reduction of actinides and fission products. *J. Nucl. Radiochem. Res.* (in press)
Lloyd & Renshaw. Microbial transformations of radionuclides: fundamental mechanisms and biogeochemical implications. *Metal Ions in Biological Systems* **42** (in press)
Renshaw *et al.* Reductive precipitation of the nuclear fuel cycle contaminant Tc(VII) by *Geobacter sulfurreducens*. *Proc. Eur. Symp. Environ. Biotechnol.* 2004, 251-254
Lloyd *et al.* (2003) Biochemical and genetic characterization of PpcA, a periplasmic c-type cytochrome in *Geobacter sulfurreducens*. *Biochem. J.* **369**: 153-161

References

1. Lloyd *et al.* (2002) *Geomicrobiol. J.* **19**: 103
2. Methe *et al.* (2003) *Science* **302**: 1967
3. Coppi *et al.* (2001) *Appl. Environ. Microbiol.* **67**: 3180
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5. Lloyd *et al.* (2003) *Biochem. J.* **369**: 153

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