Biogeochemistry (2009) 94:191–196
DOI 10.1007/s10533-009-9318-8

Impact of the Fe(III)-reducing bacteria *Geobacter sulfurreducens* and *Shewanella oneidensis* on the speciation of plutonium

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Received: 15 October 2008 / Accepted: 27 March 2009 / Published online: 16 April 2009

Abstract Microbial bioreduction of radionuclides has been the subject of much recent interest, in particular as a method for the in situ bioremediation of uranium contaminated sites. However, there have been very few studies investigating the microbially mediated redox transformations of plutonium. The redox chemistry of Pu is complicated, but the dominant environmental oxidation state is insoluble Pu(IV). However, microbial reduction of Pu(IV) to more soluble Pu(III) may enhance migration of Pu in the environment. In this study we investigated the effect of two model metal-reducing bacteria, *Geobacter sulfurreducens* and *Shewanella oneidensis*, on the redox speciation of Pu. Our results show that in all cases, the presence of bacterial cells enhanced removal of Pu from solution. UV/Visible spectra of cells and precipitates formed (dissolved in 1 M HCl), showed that the sorbed and precipitated Pu was mainly Pu(IV), but Pu(III) was also present. The results suggest that the mechanism of interaction between Pu(IV) and the two microorganisms is initial sorption to the cell surface, followed by slow reduction. Although both bacteria could reduce Pu(IV) to Pu(III), there was no increase in the solution concentrations of Pu. This suggests that the potential reduction of sorbed Pu(IV) in sediments that have been stimulated to bioremediate U(VI) may not result in problematic mobilization of Pu(III).

Keywords Plutonium · Microbial reduction · Actinides · Radioactive contamination

Introduction

The bioreduction of radionuclides by anaerobic subsurface microorganisms has been the subject of much recent interest (Lloyd et al. 2005). A wide range of bacteria, including the model subsurface Fe(III)- and Mn(IV)-reducing organisms *Geobacter sulfurreducens* and *Shewanella oneidensis*, can reduce soluble (and therefore environmentally mobile) U(VI) (as UO_2^{2+}) to insoluble U(IV) (as UO_2) (Renshaw et al. 2007). However, studies on the microbial redox transformations of the transuranic elements Np and Pu are much more limited. The most stable environmental oxidation state of Np is +V, as

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NpO_2^+, and in this form it is soluble and relatively mobile. Reduction of Np(V) to less soluble Np(IV) by *S. oneidensis* and a mixed consortium of sulphate-reducing bacteria has been reported, but *G. sulfurreducens* cannot reduce Np(V), suggesting the reductive mechanism in this bacterium is selective for hexavalent over pentavalent actinides (Lloyd et al. 2000; Renshaw et al. 2005; Rittmann et al. 2002). This is important, as *Geobacter*, rather than *Shewanella* species are well known to dominate in subsurface environments where Fe(III) and U(VI) reduction are significant (reviewed in Lloyd 2003). The environmental chemistry of Pu is much more complicated than that of Np. The dominant environmental oxidation state is Pu(IV), which forms a very insoluble hydrous oxide and sorbs strongly to mineral surfaces and humic substances (Lieser 1995; Silva and Nitsche 1995). However, Pu can also exist in the environment in potentially more soluble III and V oxidation states (Renshaw et al. 2007). There have been limited studies into bacterially mediated redox transformations of Pu. *G. metallireducens* and *S. oneidensis* MR1 (Boukhalfa et al. 2007) gave little or no reduction of freshly precipitated amorphous $Pu(OH)_4$ to Pu(III). However, both organisms were able to reduce Pu(IV) when EDTA was also present. With higher oxidation states of Pu, (Panak and Nitsche 2001) observed reduction of Pu(VI) to Pu(V) by *Bacillus sphaericus*.

In this study we investigated the effect of the Fe(III)-reducing (and U(VI)-reducing) organisms *G. sulfurreducens* and *S. oneidensis* on the redox speciation of Pu, using both trace (μM) and higher (mM) concentrations, the latter permitting study of Pu speciation. For *S. oneidensis* the impact of an endogenous secreted electron shuttle was also assessed through the addition of riboflavin, representative of flavins secreted to enhance extracellular metal reduction by *Shewanella* species (von Canstein et al. 2008).

Methodology

Maintenance and growth of organisms

Geobacter sulfurreducens was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) and was grown under strict anaerobic conditions on NBAF medium as described previously (Renshaw et al. 2005). All manipulations were made under an atmosphere of N_2. *S. oneidensis* was obtained from Dianne Newman, (California Institute of Technology, Pasadena, CA, USA) and was grown on *Shewanella* minimal medium (von Canstein et al. 2008) at 30°C under an atmosphere of N_2. For both organisms, washed cell suspensions, harvested at late log phase, were used.

Resting cell experiments with Pu

All manipulations of cells and sampling were carried out under an atmosphere of N_2. Washed cell suspensions of *G. sulfurreducens* or *S. oneidensis* were incubated with Pu in degassed buffer solution with and without electron donor under N_2 at 30°C. Control solutions contained no cells. Initial Pu concentrations were 3 μM (^{239}Pu) and 1 mM (^{242}Pu) and cell concentrations were equivalent to 0.18 mg ml^{-1} (*G. sulfurreducens*) and 0.19 mg ml^{-1} (*S. oneidensis*) protein. The buffers and electron donors used are given in Table 1. For *S. oneidensis*, riboflavin (10 μM) was also added to one set of *S. oneidensis* cell suspensions as an endogenous redox mediator. 3-(N-Morpholino) propanesulfonic acid (MOPS) was used as a buffer with Pu(VI) to prevent precipitation of Pu(VI) carbonate species. A high carbonate concentration (100 mM) was used with ^{239}Pu, which was present only at trace concentrations, in order to keep Pu(IV) in solution, whilst MOPS was required to maintain the pH. As a result of the relatively high

Table 1 Buffers and electron donors used in resting cell experiments

Pu isotope	Oxidation state	Organism	Buffer	Electron donor
239	IV	*G. sulfurreducens*	50 mM MOPS/100 mM $NaHCO_3$, pH 7.2	10 mM acetate
242	IV	*G. sulfurreducens*	30 mM $NaHCO_3$, pH 7.3	10 mM acetate
242	IV	*S. oneidensis*	30 mM $NaHCO_3$, pH 7.0	10 mM lactate
242	VI	*G. sulfurreducens*	50 mM MOPS, pH 6.9	10 mM acetate

carbonate concentration, some cell lysis was observed by ~48 h.

All experiments were conducted in triplicate. ^{239}Pu and ^{242}Pu concentrations were measured by liquid scintillation counting (after Marsden et al. 2006). Solution concentrations in supernatant samples were measured after centrifugation (14,000*g*, for 5 min). Initial total ^{239}Pu concentrations, were measured in a 50 µl aliquot taken immediately after the addition of Pu. For ^{242}Pu experiments, precipitates and cells were collected by centrifugation, dissolved in 1 M HCl and the uv-visible-near infrared spectra recorded as described by Boukhalfa et al. (2007).

Results and discussion

Interaction of Pu(VI) with *Geobacter sulfurreducens*

In all cases there was a rapid loss (~10 min) of Pu from solution, from 1 mM to 272 ± 2 µM (cell-free control), 239 ± 19 µM (cells with acetate) and 215 ± 26 µM (cells without acetate; Fig. 1). In the cell-free control, there was a slow decrease in Pu solution concentration to 32 ± 3 µM by ~24 h, caused by the abiotic reduction of soluble Pu(VI) to insoluble Pu(IV). With washed cell suspensions the concentration of Pu in solution varied over the 24 h period, but in both cases (with acetate and without acetate) the same trend was observed: further rapid loss of Pu from solution in the first hour, to 5 ± 2 µM (with acetate) and 4 ± 3 µM (without acetate)

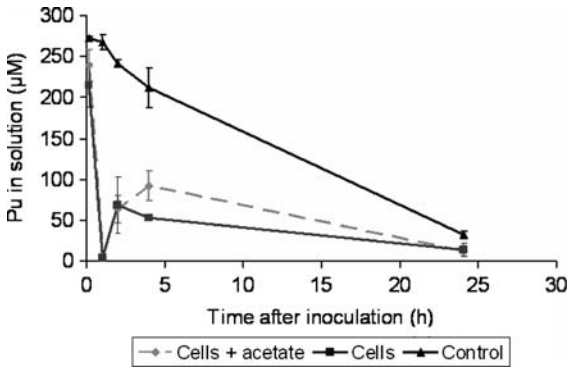

Fig. 1 Effect of *G. sulfurreducens* on millimolar concentrations of ^{242}Pu(VI) with and without acetate present as an electron donor. Error bars show ± one standard deviation ($n = 3$). The initial concentration of Pu(VI) added was 1 mM

followed by an increase in concentration at 2–4 h (92 ± 18 µM at 4 h with acetate; 69 ± 34 µM at 2 h without acetate) then a decrease to final concentrations of 12 ± 6 µM (with acetate) and 14 ± 8 µM (without acetate) at 24 h. The final concentrations were significantly lower than observed in the controls, but there was no difference between the cell suspensions with and without acetate. This somewhat erratic behaviour could be explained by reduction of Pu(VI) to Pu(IV) via Pu(V) and the associated effects on solubility and sorption behaviour. The initial rapid loss of Pu(VI) from solution may reflect sorption of Pu(VI) to the cell surface. The sorbed Pu(VI) is then reduced to the less strongly sorbed Pu(V), giving an increase in solution Pu concentration. Pu(V) disproportionates, forming insoluble Pu(IV) and Pu(VI) (Renshaw et al. 2007). Therefore the concentration of Pu in solution would decrease as Pu(IV) is formed and lost from solution. The same, three-stage mechanism was proposed by Panak and Nitsche (2001) in their study of the interactions of Pu(VI) with *B. sphaericus*. They observed rapid binding of Pu(VI) to the bacterial biomass, followed by slower, one electron reduction of sorbed Pu(VI). The Pu(V) formed was found predominantly in solution, and finally, Pu(IV) was formed. This was attributed to an abiotic process, either disproportionation of Pu(V) or autoreduction of Pu(VI). The formation of Pu(IV) was observed on a much slower timescale (16% Pu(IV) after 1 month) than in this study, although their experiment was conducted under aerobic conditions, using an organism not known to reduce a wide spectrum of metals, whereas the anaerobic conditions and choice of model organism used in this study would accelerate formation of Pu(IV) by abiotic and biotic reactions.

The mechanism proposed is analogous to that for U(VI) reduction by *G. sulfurreducens*: a bacterially mediated one electron reduction from U(VI) to U(V), followed by disproportionation of U(V) forming U(IV) (Renshaw et al. 2005) and is further evidence for the discrimination between An(VI) and An(V) by this organism.

Interaction of Pu(IV) with *Geobacter sulfurreducens*

Initial experiments were undertaken with ^{239}Pu(IV) at tracer concentrations (3 µM; Fig. 2). In cell-free

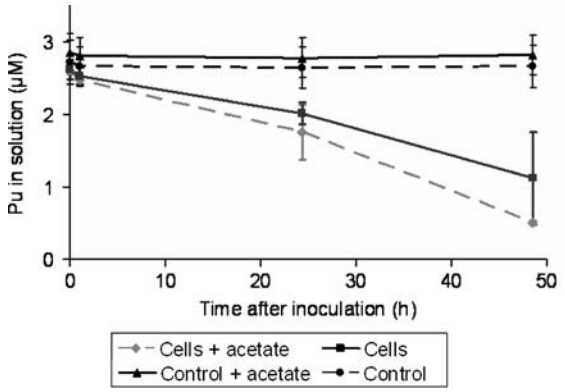

Fig. 2 Effect of *G. sulfurreducens* on micromolar concentrations of ^{239}Pu(IV) with and without acetate present as an electron donor. Error bars show ± one standard deviation ($n = 3$). The initial concentration of Pu(IV) added was 3 μM

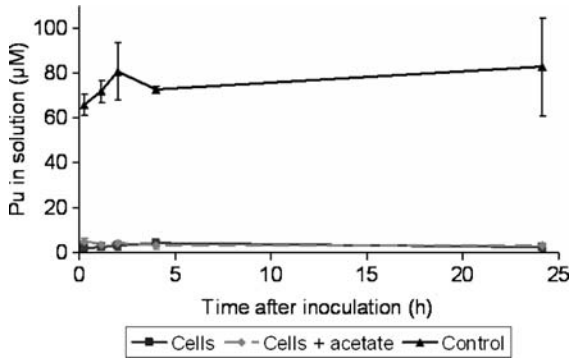

Fig. 3 Effect of *G. sulfurreducens* on millimolar concentrations of ^{242}Pu(IV) with and without acetate present as an electron donor. Error bars show ± one standard deviation ($n = 3$). The initial concentration of Pu(IV) added was 1 mM

controls with and without the electron donor acetate, the concentration of Pu in solution was constant (~2.5–3 μM) over 171 h. With all cell suspensions, there was slow loss of Pu from solution over ~48 h: from 2.61 ± 0.06 to 0.50 ± 0.03 μM for cell suspensions with acetate and 2.62 ± 0.14 to 1.13 ± 0.62 μM for cells without acetate, significantly lower than in the controls. No further changes in Pu solution concentration were observed by 171 h.

In experiments undertaken with 1 mM ^{242}Pu(IV), Pu(IV) precipitated from solution immediately after addition. However, solution Pu concentrations after 15 min were significantly lower for cell suspensions (5.1 ± 1.6 μM with acetate; 2.2 ± 0.6 μM without acetate) compared to the control (65.9 ± 4.8 μM Fig. 3). Over 24 h, there was no further significant change in the Pu solution concentration for all conditions. UV/visible spectra (recorded after 48 h; data not shown) of the precipitates dissolved in 1 M HCl showed the presence of Pu(IV) (diagnostic features at 650–950 and 1,050–1,200 nm) in the control and all cell suspension precipitates; however, the spectra for the cell suspension precipitates also showed the presence of Pu(III) (diagnostic peaks at 600 and 1,025 nm). The proportions of Pu(III) present were 14 and 9% in the cell suspensions with and without acetate, respectively.

At both μM and mM concentrations, the presence of cells increased removal of Pu(IV) from solution. The UV/vis spectra suggest that a relatively small but significant portion of the Pu(IV) added is slowly reduced to Pu(III) by *G. sulfurreducens* and that there may be slightly more Pu(IV) reduced with acetate present as an electron donor. The Pu(IV) reduced without added electron donor was probably reduced via reducing equivalents that accumulated within the biomass prior to cell harvesting. However, despite reduction to Pu(III), there was no increase in the solution Pu concentration with time and the concentrations in the cell suspensions with and without acetate were very similar. Boukhalfa et al. (2007) investigated the reduction of freshly precipitated amorphous $Pu(OH)_4$ by *G. metallireducens* and detected trace amounts of Pu(III) in solution after ~20 h; this was no longer detectable by ~45 h. This difference between the two studies may be a result of the different species investigated (*G. sulfurreducens* in this study and *G. metallireducens* in the study by Boukhalfa et al. 2007), and the different experimental procedures followed. Boukhalfa et al. (2007) measured only the Pu(III) concentration in solution (samples were filtered prior to spectrophotometric analysis of the supernatant), but did not investigate the oxidation state of any cell-associated Pu. It is possible therefore that *G. metallireducens* can also reduce Pu(IV) to Pu(III) but the reduced Pu remains cell-bound. Thus, our results suggest that the removal of Pu(IV) from solution could be through sorption to the cell, followed by or in parallel with slow reduction, probably at the cell surface, with the reduced Pu(III) remaining associated with the biomass. It should be noted that *Geobacter* species are well known to reduce extracellular electron acceptors via outer membrane *c*-type cytochromes (Lloyd 2003).

Interactions of Pu(IV) with *Shewanella oneidensis*

In all cases, Pu was rapidly removed from solution (within 10 min) from an initial solution concentration of 1 mM. However, the solution Pu concentration was significantly lower in the cell suspensions with lactate (72 ± 21 μM), and with lactate and riboflavin (46 ± 19 μM), than in the control (376 ± 12 μM) and in cell suspensions without lactate (250 ± 14 μM; Fig. 4). In controls, the solution concentration of Pu decreased only slightly over 24 h, to 303 ± 9 μM. There was a further decrease in Pu solution concentration within ~1 h for suspensions with lactate (to 22 ± 9 μM), without lactate (72 ± 10 μM) and with lactate and riboflavin (20 ± 7 μM). There was no further change in the Pu solution concentration for suspensions with lactate and with lactate and riboflavin by 24 h. In the cell suspensions without lactate, the concentration decreased further, to 4 ± 1 μM by 24 h so that, by 24 h, the solution Pu concentration in all the cell suspensions were similar. After 24 h, UV/visible spectra of the dissolved precipitates (data not shown), showed the presence of Pu(IV) in the control and cell suspension precipitates but, in precipitates from the cell suspensions, some Pu(III) was also present. The amount of Pu(III) present in the cell suspension without lactate or riboflavin was negligible; for the cell suspensions with lactate and with lactate and riboflavin, the proportions of Pu(III) present were 3 and 17%, respectively. Boukhalfa et al. (2007) also investigated the reduction of Pu(IV) by *S. oneidensis* with lactate as the electron donor. They found that 8% of the Pu(IV) was reduced to Pu(III) after ~24 h; the reduced Pu was in solution, while in our study the amount of Pu reduced is less (3%) and is cell-bound. There are number of possible reasons for the observed differences, including differences in initial Pu(IV) concentrations (1 mM in this study and 0.5 mM in the study of Boukhalfa et al. 2007), contrasting biomass concentrations (and hence Pu:cell ratios) and different growth media. Boukhalfa et al. (2007) also did not investigate the oxidation state of any cell-bound Pu and so it is possible that there was some cell-bound Pu(III), as we found in this study. Therefore it is possible that more than 8% of the Pu was reduced in the study of Boukhalfa et al. (2007) for the reasons given above and some of reduced Pu was cell-bound, but once the sorptive capacity of the cells for Pu was exhausted, Pu(III) was released into solution.

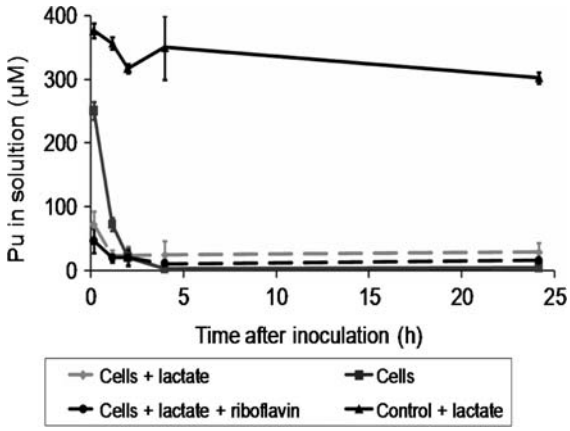

Fig. 4 Effect of *S. oneidensis* on millimolar concentrations of ^{242}Pu(IV) with and without lactate present as an electron donor, and with and without riboflavin present as an endogenous redox mediator, representative of flavins secreted to enhance extracellular metal reduction by *Shewanella* species. Error bars show ± one standard deviation ($n = 3$). The initial concentration of Pu(IV) added was 1 mM

These results confirm that microbial interactions with plutonium are complex and driven by a combination of initial sorption to the cell surface, followed by varying degrees of reduction, shown for Pu(VI) with *G. sulfurreducens* and for Pu(IV) with both Gram-negative metal-reducing bacteria studied here. Enzymatic activity is implicated, consistent with the presence of a battery of low oxidation/reduction potential heme-containing cytochromes traversing the outer cell compartments and terminating on the outer membranes of both these model organisms. Although both organisms have been shown to reduce Pu(IV), the presence of riboflavin, which is secreted by *Shewanella* species, accelerated Pu(IV) reduction and there was a marked difference in the percentage of Pu(IV) reduced by *S. oneidensis* with and without riboflavin present (17 and 3%, respectively). The secretion of riboflavin by *Shewanella* species is, to our knowledge, unique amongst dissimilatory Fe(III)-reducing bacteria and these results support the hypothesis that riboflavin may play a key role in extracellular electron transport in this organism (Marsilli et al. 2008; von Canstein et al. 2008), and imply that this mechanism can play a role in controlling the oxidation state of actinides. Finally,

the lack of impact of microbial reduction on overall Pu solubility in these experiments suggests that the potential reduction of sorbed Pu(IV) in sediments that have been stimulated by electron donor to immobilise U(VI) and Np(V) via microbial reduction to U(IV) and Np(IV), may not necessarily result in problematic mobilization of Pu(III).

Acknowledgments The financial support of the US DOE NABIR/ERS programmes and UK NERC are acknowledged.

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