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Metal reduction by spores of *Desulfotomaculum reducens*

**Pilar Junier,[1] Manon Frutschi,[1]
Nicholas S. Wigginton,[1] Eleanor J. Schofield,[2]
John R. Bargar[2] and Rizlan Bernier-Latmani[1]***
[1]*Environmental Microbiology Laboratory, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, CH 1015, Switzerland.*
[2]*Stanford Synchrotron Radiation Laboratory, Menlo Park, CA 94025, USA.*

Summary

The bioremediation of uranium-contaminated sites is designed to stimulate the activity of microorganisms able to catalyze the reduction of soluble U(VI) to the less soluble mineral UO_2. U(VI) reduction does not necessarily support growth in previously studied bacteria, but it typically involves viable vegetative cells and the presence of an appropriate electron donor. We characterized U(VI) reduction by the sulfate-reducing bacterium *Desulfotomaculum reducens* strain MI-1 grown fermentatively on pyruvate and observed that spores were capable of U(VI) reduction. Hydrogen gas – a product of pyruvate fermentation – rather than pyruvate, served as the electron donor. The presence of spent growth medium was required for the process, suggesting that an unknown factor produced by the cells was necessary for reduction. Ultrafiltration of the spent medium followed by U(VI) reduction assays revealed that the factor's molecular size was below 3 kDa. Pre-reduced spent medium displayed short-term U(VI) reduction activity, suggesting that the missing factor may be an electron shuttle, but neither anthraquinone-2,6-disulfonic acid nor riboflavin rescued spore activity in fresh medium. Spores of *D. reducens* also reduced Fe(III)-citrate under experimental conditions similar to those for U(VI) reduction. This is the first report of a bacterium able to reduce metals while in a sporulated state and underscores the novel nature of the mechanism of metal reduction by strain MI-1.

*For correspon-dence. E-mail rizlan.bernier-latmani@epfl.ch; Tel. (+41) 21 693 5001; Fax (+41) 21 693 6205.

Introduction

As a result of nuclear fuel and weapon production, U(VI) contamination is a severe problem in many US Department of Energy sites and similar locales worldwide (Riley *et al*., 1992; Abdelouas *et al*., 1999; Meinrath *et al*., 2003). The microbial reduction of U(VI) to the less soluble and less bio-available U(IV), which precipitates as the mineral UO_2, is touted as a potential solution for the immobilization of uranium in the subsurface at contaminated sites. The strategy considered for the direct enzymatic reduction of U(VI) involves the addition of an appropriate electron donor that would stimulate the indigenous microbial community and promote the use of U(VI) as a terminal electron acceptor (Sanford *et al*., 2007; Yabusaki *et al*., 2007; N'Guessan *et al*., 2008).

Species from four genera capable of U(VI) reduction have been studied most extensively: *Shewanella* (Bencheikh-Latmani *et al*., 2005), *Geobacter* (Shelobolina *et al*., 2007), *Desulfovibrio* (Payne *et al*., 2004) and *Anaeromyxobacter* (Wu *et al*., 2006). The former two are genera of dissimilatory metal-reducing bacteria exhibiting a remarkable respiratory versatility and able to couple U(VI) reduction to growth (Lovley *et al*., 1991) whereas the third is a genus of sulfate-reducing bacteria some species of which couple U(VI) reduction to growth (Pietzsch and Babel, 2003). The final genus, *Anaeromyxobacter*, encompasses anaerobic myxobacteria and one of its species was reported to couple growth to U(VI) reduction (Sanford *et al*., 2007). In all four genera, U(VI) reduction also takes place readily under non-growth conditions, but metabolically active cells are needed to catalyse the process (Bencheikh-Latmani *et al*., 2005; Wall and Krumholz, 2006; Marshall *et al*., 2009).

In this study, we characterize U(VI) reduction by the bacterium *Desulfotomaculum reducens* strain MI-1, a sulfate-reducing bacterium isolated from marine sediments contaminated with Cr(VI) (Tebo and Obraztsova, 1998). U(VI) reduction by this organism was deemed interesting and unusual for several reasons. First, strain MI-1 was reported to grow on U(VI) as a sole terminal electron acceptor with butyrate as an electron donor (Tebo and Obraztsova, 1998). Second, several field sites contaminated with U(VI) have been shown to harbour *Desulfotomaculum* and/or its close relative *Desulfosporosinus* either prior to (Chang *et al*., 2001) or during a

bioremediation effort (Anderson *et al*., 2003; Nevin *et al*., 2003; Suzuki *et al*., 2003; Chandler *et al*., 2006), suggesting that these microorganisms may play a role in the environmental U(VI) reduction process. Finally, strain MI-1 differs phylogenetically from other studied U(VI)-reducing organisms because it is a spore-forming *Firmicutes*, whereas *Geobacter*, *Shewanella*, *Desulfovibrio* and *Anaeromyxobacter* are all *Proteobacteria*.

The distinct phylogeny of strain MI-1 raises the possibility that its mechanism of U(VI) reduction is significantly different from those previously described. Therefore, the goal of the study was to characterize the mechanism of U(VI) reduction by strain MI-1. We found that this microorganism exhibited a novel mechanism of U(VI) and Fe(III) reduction that involved its spore form and an unidentified factor excreted by cells.

Results

U(VI) reduction by strain MI-1

The ability to ferment pyruvate is a common trait in the genus *Desulfotomaculum* (Widdel, 1992). *Desulfotomaculum reducens* is no exception as it grew with pyuvate as the sole substrate and produced acetate, H_2 and CO_2 (Fig. 1 and Fig. S1). The presence of U(VI) during fermentative growth on pyruvate had no effect on the temporal change in protein concentration (Fig. 1) or on the transformation of pyruvate to its products (Fig. S1). In addition, little U(VI) reduction took place during active growth because only 8.2 μM U(VI) was reduced between 48 and 180 h, after pyruvate was depleted and growth ceased (Fig. 1). A total of 85 μM U(VI) was reduced after 324 h. Considering that the maximum amount of biomass produced was 18 μg ml^{-1}, we calculated that a maximum of 4.7 μmoles of U(VI) were reduced per mg protein over that time frame.

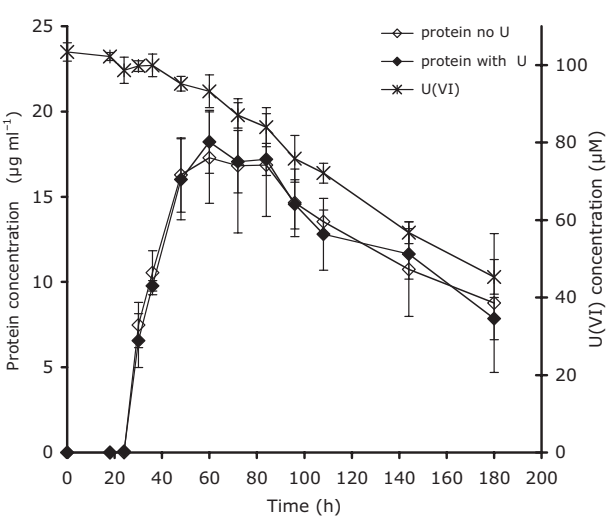

Fig. 1. Pyruvate fermentation by MI-1 in the presence (filled symbols) and absence (open symbols) of U(VI). Results of growth (measured by protein concentration) and U(VI) concentration are displayed. The corresponding pyruvate and acetate data are in Fig. S1. Triplicates were run for all conditions and the error bars represent the standard deviation within the set.

Interestingly, U(VI) reduction proceeded (48–180 h) during a steady decline in the total protein concentration, which occurred independently of the presence of U(VI) (Fig. 1). Microscopic observation of the cultures revealed that 14% of the *D. reducens* cells had sporulated after 108 h. *Desulfotomaculum* spp. are known to sporulate in the absence of an appropriate electron donor and in the presence of acetate (Widdel, 1992). Hereafter, the reader may assume that cultures devoid of pyruvate (typically 48 h old or older) will contain some fraction of spores, unless otherwise indicated. Thus, we attribute the decrease in protein concentration, assayed in centrifuged culture pellets, to sporulation and to the release of soluble protein from disintegrating vegetative cells. The observed temporal relationship suggests that U(VI) reduction could be linked to the formation of spores.

U(VI) reduction by spores of D. reducens

To evaluate the possibility of spore involvement in U(VI) reduction more rigorously, we considered the reduction of U(VI) in the absence of pyruvate by: (i) spores alone (variously pasteurized, unpasteurized or glutaraldehyde-fixed), (ii) vegetative cells alone (growing or pasteurized) and (iii) an abiotic control containing neither vegetative cells nor spores. The results (Table 1) show that U(VI) reduction proceeded when spores (pasteurized or unpasteurized) were present along with both H_2 and spent medium. Spent fermentation medium (Table 1) was the filtrate from a stationary-phase (> 48 h) culture that had converted all of the originally added pyruvate to H_2, acetate and CO_2.

The extent of U(VI) reduction by spores depended on the chemical state of the spores. Glutaraldehyde-fixed spores, which were unable to germinate (data not shown) and are expected to be more extensively modified chemically than pasteurized spores, did not reduce U(VI) (Table 1). In contrast, pasteurized spores were viable (able to germinate) and reduced U(VI), as did untreated spores (Table 1). As pasteurized vegetative cells did not reduce U(VI) (Table 1), the observed activity of pasteurized spores cannot be explained by residual vegetative cell debris that might have remained in the pasteurized spore preparations despite repeated washes to remove them. We therefore conclude that strain MI-1 spores are directly involved in U(VI) reduction.

Vegetative cells grown fermentatively in the presence of U(VI) exhibited some U(VI) reduction, although 100-fold

Table 1. U(VI) reduction by spores and vegetative cells under different conditions in the presence of pyruvate-free spent media amended with or free of H_2, compared with fresh medium amended with acetate and H_2.

	% U(VI) reduced			
	Spent fermentative medium[a]	Fresh WLP medium	N_2-purged spent fermentative medium[a]	
	20 mM H_2 added	20 mM H_2 added	20 mM H_2 added	No H_2
Pasteurized spores[b]	73.5 ± 2.4	3.1 ± 6.4	ND	ND
Unpasteurized spores[b]	83.9 ± 1.0	2.8 ± 4.0	52.3 ± 1.9	4.9 ± 0.4
Glutaraldehyde-fixed spores[b]	0.9 ± 1.3	ND	ND	ND
Pasteurized vegetative cells[c]	1.9 ± 5.6	1.9 ± 5.6	ND	ND
Live vegetative cells[d]	11.2 ± 4.5	ND	ND	ND
Abiotic (no spores or cells)	2.1 ± 5.9	2.5 ± 5.9	4.6 ± 0.4	ND

a. Spent fermentation medium was the filtrate from a stationary-phase culture in which all detectable pyruvate had been removed (fermented) during growth.
b. The spore concentration was 1.6×10^6 spores ml^{-1} and the vegetative cell concentration 2.3×10^7 cells ml^{-1}. Pasteurized and unpasteurized spores were viable (able to germinate) and were expected to have less chemical damage than glutaraldehyde-fixed spores, which were unable to produce viable vegetative cells under germination conditions.
c. Pasteurized vegetative cell preparations contain cell debris comparable to, but in higher concentrations than, the vegetative cell debris found in partially purified, pasteurized spore preparations.
d. Cells grown fermentatively in the presence of U(VI). H_2 was present as a result of pyruvate fermentation.
ND, not determined.

less than that with spores alone (Table 1). Yields were 10^{-8} nmol reduced U(VI) per cell.h versus 10^{-6} nmol reduced U(VI) per spore.h.

As described previously, U(VI) reduction commences after the depletion of pyruvate from the medium. In the absence of pyruvate, H_2, one of the products of fermentation, is presumed to act as the electron donor for U(VI) reduction. A requirement for the presence of H_2 was demonstrated by the lack of U(VI) reduction in the N_2-purged aliquot of spent fermentative growth medium, which was examined both unsupplemented and supplemented with 20 mM H_2 (Table 1). Amendment with H_2 rescues reduction activity, suggesting that it is the electron donor for spore-mediated U(VI) reduction. In addition, the lack of U(VI) reduction in the spent abiotic control (Table 1) confirms that U(VI) reduction is a biological process requiring the presence of spores, rather than a chemical process driven by H_2.

We tested the dependence of the rate of U(VI) reduction on the concentration of spores (Fig. 2) and found a proportional relationship – twice the concentration of spores yields approximately twice the rate – which further confirms the role of spores in U(VI) reduction and suggests an enzymatic process. A comparison of the rate of reduction of U(VI) by *D. reducens* spores and *Shewanella oneidensis* MR-1 cells yields surprisingly similar rates: 10 nmoles h^{-1} per 10^7 spores (this study) and 9.5 nmoles h^{-1} per 10^7 cells (Liu *et al.*, 2002).

We observed the formation of a dark precipitate in all samples after the reduction of U(VI) by spores of strain MI-1. In order to characterize the localization of uranium relative to the spores, a spore preparation incubated in the presence of U(VI), spent growth medium and H_2 was imaged by transmission electron microscopy (TEM). U was the only electron-dense material in the sample and appeared to be located mainly in association with the spore coat (Fig. 3).

We confirmed that the disappearance of U(VI) from solution did indeed stem from U(VI) reduction to U(IV) by characterizing the solid-phase product of U(VI) reduction by X-ray absorption spectroscopy. The X-ray absorption near-edge structure (XANES) spectra of samples containing pyruvate-grown *D. reducens* cultures after 300 h of

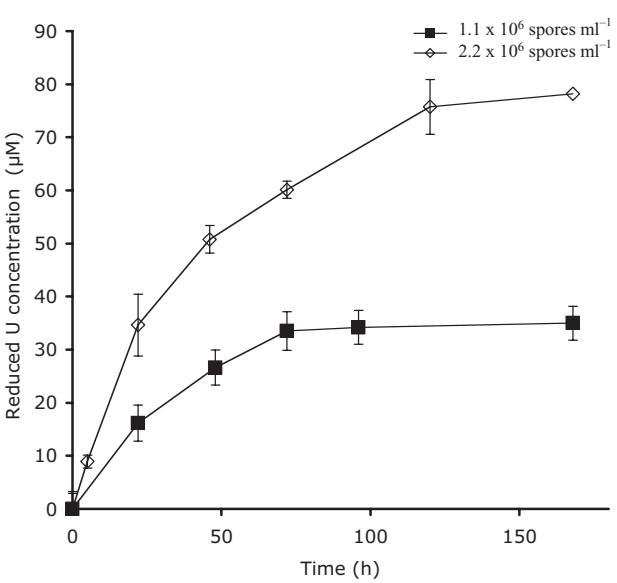

Fig. 2. Concentration of reduced U over time after incubation in spent growth medium, H_2 and different concentration of spores of *D. reducens* strain MI-1.

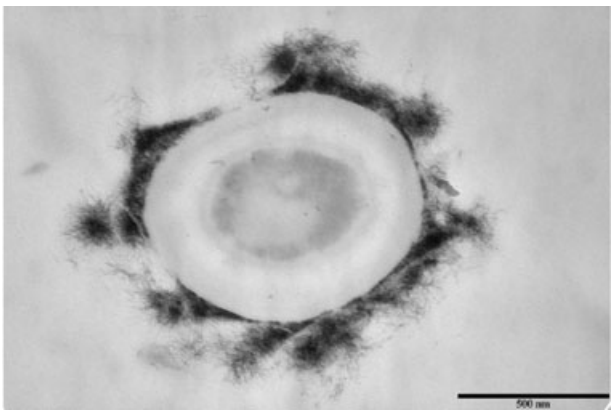

Fig. 3. Transmission electron micrograph of a cell pellet embedded in resin and thin-sectioned showing uranium associated with the coat of a *D. reducens* spore.

exposure to U(VI) (i.e., in which some fraction of the cells had sporulated), as well as pasteurized and unpasteurized spore preparation exposed to U(VI) for 72 h were best fit as a linear combination of U(IV) and U(VI) standards with U(IV) representing 97%, 100% and 90% of the total U for the three samples respectively (Fig. 4).

Dependence of U(VI) reduction on spent growth medium

U(VI) reduction by strain MI-1 spores occurred only in spent growth medium but not in fresh pyruvate-free fermentation medium (Table 1). Spores of strain MI-1 did not reduce U(VI) even when fresh medium was amended with the products of pyruvate fermentation (acetate and H_2, data not shown) or with H_2 alone (Table 1). The requirement for spent growth medium suggests that germinating and/or growing cells release a stimulatory factor that is involved in the U(VI) reduction process.

Because strain MI-1 cultures are typically inoculated from a spore preparation, it is conceivable that the factor could be excreted during the spore germination process or could be excreted by vegetative cells during growth. To evaluate both possibilities, the ability of spent growth medium to support U(VI) reduction by spores was tested for cultures in which germination had or had not occurred (Fig. 5). Spent growth media from two types of cultures were assayed: cultures inoculated from a cell suspension (Fig. 5A) and cultures inoculated from a spore suspension (Fig. 5B). In the former, the absence of spores precluded germination and provided a condition in which only cell growth could account for the presence of the factor. In the case of the culture inoculated from a spore suspension, both germination and subsequent cell growth took place.

Spent growth medium collected at different times from the cell-inoculated culture supported U(VI) reduction by spores at the same rate for all time points. This is evident when the spent medium collected from the same culture at different times is amended with spores and H_2 and the rate of U(VI) reduction is similar regardless of the culture age (Fig. 5A). This suggests that the factor is produced by growing vegetative cells (no germination occurs under these conditions), that its concentration is sufficient to support U(VI) reduction in the cell-inoculated culture for up to 48 h (last time point) and that it is stable enough to remain in the medium for that amount of time.

In contrast, in the spore-inoculated culture, the longer the incubation time of the culture, the slower the rate of U(VI) reduction supported by the spent medium from that culture. The rate of U(VI) reduction decreases as the age of the culture increases (Fig. 5B). Thus, as the spore-inoculated culture ages, the stimulatory factor appears to be consumed.

Comparative microscopic observation of the two cultures (cell- and spore-inoculated) over time indicated that sporulation occurred before 24 h in the culture inoculated from spores (data not shown). In contrast, in the cell-inoculated culture, no sporulation was detected even at 48 h. This result suggests that the factor may be consumed during sporulation, leading to a decrease of the reduction capacity of the corresponding spent growth medium.

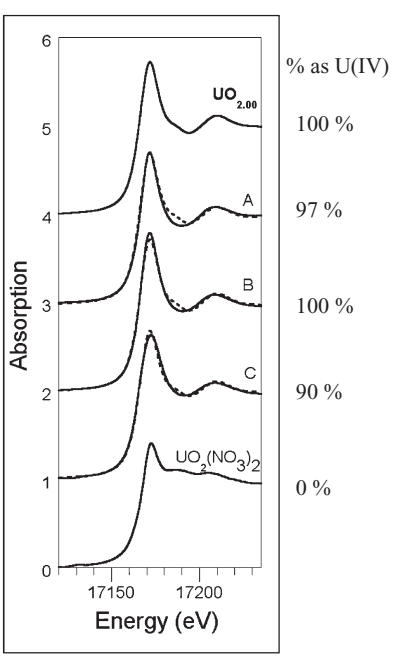

Fig. 4. U L_{III}-edge XANES spectra collected at 77 K. Sample A is a fermentation culture exposed to U(VI) for 300 h. Samples B and C are pasteurized and unpasteurized spores (respectively) exposed to spent fermentation medium for 72 h. Model compounds for U(IV) [$UO_{2.00}$] and U(VI) [$UO_2(NO_3)_2$] are included for comparison. The solid lines represent data and the dotted lines the fit. The fit results are reported along the graph.

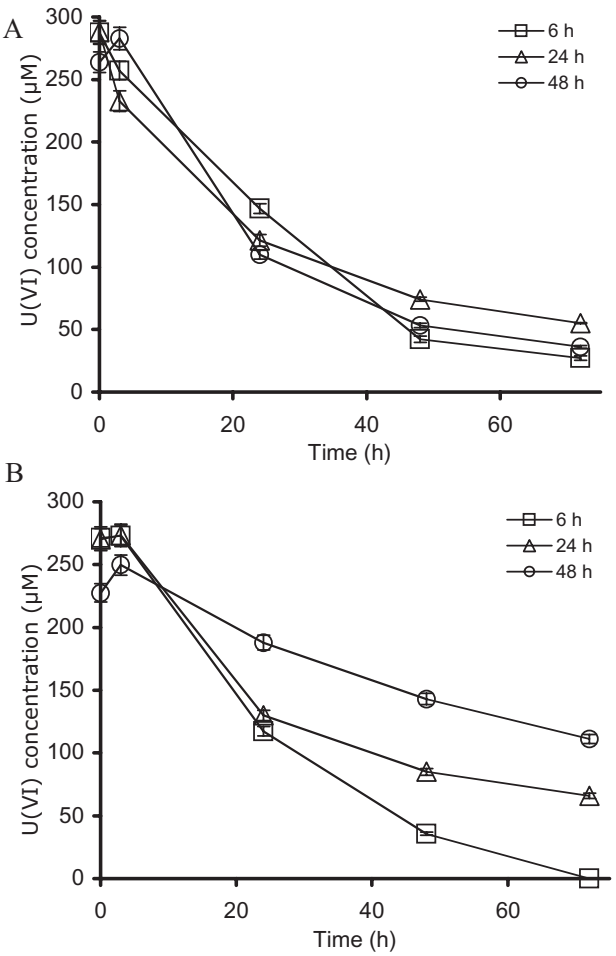

Fig. 5. Reduction of U(VI) by spores of *D. reducens* strain MI-1 over time using spent fermentation medium from cultures inoculated from a cell suspension (A) or cultures inoculated from spores (B). H_2 is provided in all cases.

Additionally, the data in Fig. 5B indicate that the factor is secreted early during growth (6 h), prior to biomass reaching concentrations high enough to be detected by protein or OD_{600} measurements. At 6 h, in the spore-inoculated culture, there is a sufficient concentration of factor to support U(VI) reduction at the usual rate despite the fact that no biomass is detectable by OD_{600} (data not shown). This suggests that very low cell concentrations excrete a sufficient concentration of the factor to sustain U(VI) reduction and that the process likely relies on catalytic amounts of the factor rather than equimolar concentrations with U(VI). We therefore propose that the factor is secreted during cell growth (although we cannot exclude the possibility that spore germination also releases the factor), is consumed during sporulation and is required in small concentrations.

The identity of the stimulatory factor is unknown and currently under investigation. To narrow down the molecular size of the factor, spent fermentative medium was filtered through a 3 kDa nominal molecular weight cut-off centrifugal filter and the filtrate and retentate tested for their ability to support U(VI) reduction by MI-1 spores. The results show that the active compound was < 3 kDa in size because the stimulatory activity was recovered in the flow-through supplemented with spores and H_2, but not when the retained fraction (retentate) was added to fresh medium supplemented with spores and H_2 (Fig. 6).

Dipicolinic acid (DPA or pyridine-2,6-dicarboxylic acid) is a compound secreted by germinating spores (Scott and Ellar, 1978) and consumed by sporulating cells and, as such, was deemed a possible candidate factor. However, it was found not to affect U(VI) reduction when added to fresh medium either in a reduced state (after treatment with Pd and H_2) or in an anaerobic state (after flushing

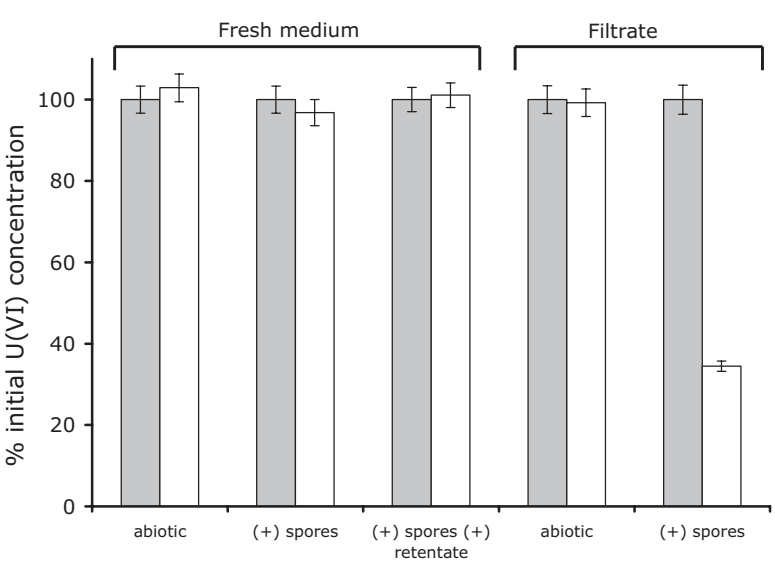

Fig. 6. Reduction of U(VI) by spores of *D. reducens* strain MI-1 in fresh medium supplemented with retentate of spent medium ultrafiltered using 3 kDa molecular weight cut-off membranes and compared with the flow-throw. Initial time in grey and $t = 72$ h in white.

with N_2) (data not shown). No variation of pH was detected after filtration or addition of DPA.

We sought evidence of the involvement of an electron shuttle in spore-mediated uranium reduction by the incubation of U(VI) with pre-reduced spent medium. The ability of the factor to be reduced and transfer electrons to U(VI) was tested by reducing a 3 kDa ultrafiltered spent medium by exposure to Pd and H_2. Subsequently, the reduced, ultrafiltered spent medium was incubated with U(VI) and H_2 in the presence and absence of spores. The pre-reduced ultrafiltered spent medium showed U(VI) reduction in the absence of spores that was initially comparable to that in the presence of spores and non-reduced spent medium (Fig. S2). However, the reduction was not sustained beyond 50 h, which is consistent with the depletion of the reduced factor. This result suggests that a factor present in the spent medium is likely to be an electron shuttle.

Anthraquinone-2,6-disulfonic acid (AQDS) is a humic acid analogue and a known electron shuttle for metal reduction (Hernandez and Newman, 2001), including the reduction of U(VI) (Finneran *et al*., 2002). AQDS and the reduced form of the compound – anthrahydroquinone-2,6-disulfonic acid (AH_2DS) – were tested in order to evaluate the possibility that AQDS could serve as an electron shuttle for U(VI) reduction by spores. AH_2DS was found to reduce U(VI) in fresh medium in the absence of spores but only to a limited extent (~20%) despite being present at a concentration equal to that of U(VI) (Fig. S3). AQDS added to spores suspended in fresh medium containing H_2 did not restore any U(VI) reduction activity (Fig. S3). We attribute the lack of U(VI) reduction to the inability of AQDS to serve as an electron shuttle for the spore and U(VI) system.

Riboflavin, a compound shown to be involved in metal reduction in *Shewanella* (von Canstein *et al*., 2008; Marsili *et al*., 2008) was also tested but did not rescue U(VI) reduction activity when added to fresh medium (containing spores and H_2) in either an oxidized or reduced form (Fig. S3). This result suggests that riboflavin is not an electron shuttle for U(VI) reduction by spores.

Fe(III)-citrate reduction by MI-1 spores

Desulfotomaculum reducens MI-1 has been reported to reduce metals such as Fe(III), Mn(IV) and Cr(VI) (Tebo and Obraztsova, 1998). To test whether MI-1 spores are able to reduce iron, the reduction of chelated iron [Fe(III)-citrate] was assayed under the same experimental conditions used for U(VI) reduction. Immediately after the addition of Fe(III)-citrate to spent growth medium, a change in colour was observed suggesting partial reduction of Fe(III) in the absence of spores. Moreover, in the presence of spores, further Fe(III) reduction was presumed due to a change in medium colour (yellow-orange to clear) after a 48 h incubation and the formation of a dark precipitate. The production of Fe(II) was confirmed quantitatively by recovering the totality of the iron concentration (added to the system as Fe(III)) as measured solid phase Fe(II) by a 0.1 M HCl extraction (data not shown). In addition, we carried out the same experiment with ultra-filtered spent medium pre-reduced with H_2 and Pd. The results (Fig. 7C) show that: (i) the addition of Fe(III) to pre-reduced spent medium in the absence of spores results in the production of ~2 mM Fe(II) and (i) the presence of spores is required for the complete reduction of Fe(III). Concomitantly with the measured production of Fe(II), we observed a change in medium colour with the formation of a dark precipitate (Fig. 7B) and TEM images of whole-mount samples revealed Fe precipitates associated with spores (Fig. 7A). These findings combined support the hypothesis that an electron shuttle is present in the spent medium and involved in metal reduction and that spores are needed for sustained Fe(III) reduction.

Discussion

In this study, we found that *D. reducens* cells incubated with pyruvate and U(VI) grew fermentatively and did not reduce uranium (Fig. 1). This finding would seem to be in contradiction with the previously reported growth of strain MI-1 coupled to butyrate oxidation and U(VI) reduction (Tebo and Obraztsova, 1998). However, this is not the case because we confirmed that, in contrast to limited uranium reduction under the conditions of this study, *D. reducens* vegetative cells carry out substantial reduction of U(VI) with butyrate as an electron donor (data not shown). However, because the focus of this work is on spore-driven reduction, we did not systematically investigate the reasons for these differences in metabolism between electron donors.

Desulfotomaculum reducens shows an unusual strategy for U(VI) reduction distinct from that identified for the four most studied genera: *Shewanella*, *Geobacter, Desulfovibrio* and *Anaeromyxobacter*. In the latter cases, the reduction of U(VI) is catalyzed by live vegetative cells that use *c*-type cytochromes to transfer electrons from an electron donor to U(VI) (for review see Wall and Krumholz, 2006). In contrast, *D. reducens* strain MI-1 is capable of reducing U(VI) when in its sporulated state, provided that H_2 (an electron donor) and an unknown factor (or several factors) found in spent growth medium are present. A similar process is described for Fe(III) reduction.

This is the first report of metal reduction by bacterial spores. The only previously known redox interaction between spores and metals is the catalysis of Mn(II) oxidation by *Bacillus* spores (Francis and Tebo, 2002). The enzyme responsible for Mn oxidation is localized in the

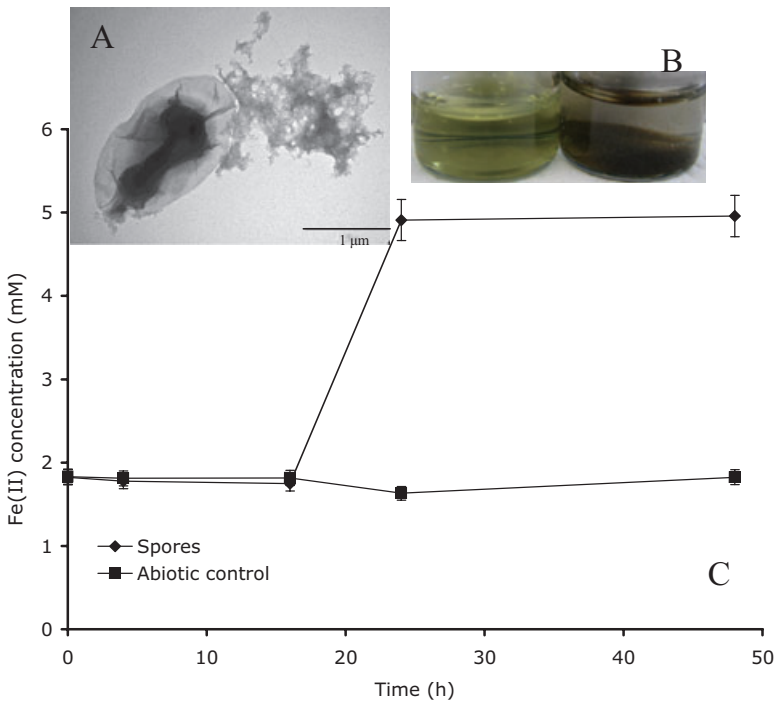

Fig. 7. Fe(III) reduction by *D. reducens* spores.
A. Transmission electron micrograph showing Fe precipitates around spores of *D. reducens*.
B. Evidence of an Fe precipitate formed after 24 h of incubation (picture taken after 48 h of incubation).
C. Iron(III)-citrate reduction by spores of *D. reducens* strain MI-1 in spent medium that underwent ultrafiltration (UF) through a 3 kDa cut-off and that was subjected to a 20 min incubation with H_2 and Pd. Fe(II) measured is 0.1 M HCl-extractable Fe(II) from the Fe precipitate formed during Fe(III) reduction.

exosporium, the outer coating of *Bacillus* sp. SG-1 spores and is part of a protein family, the multicopper oxidases (Francis *et al*., 2002). Thus, Mn(II) serves as an electron donor for the reduction of molecular oxygen, a process catalysed by a spore-associated enzyme.

MI-1 spores could catalyze the reduction of U(VI) with H_2 as an electron donor by a similar mechanism. However, in the case of *D. reducens*, the process requires a factor present in spent medium from fermentative cultures. The identity of this factor is currently unknown. Nonetheless, some information about its properties can be gleaned from the experiments conducted to date. The factor is small: it has a molecular weight of < 3 kDa (Fig. 6), which makes it unlikely to be a protein. In addition, it is secreted by growing cells and consumed by sporulating cells (Fig. 5). Furthermore, results from the pretreatment of spent medium with a reducing agent suggest that the factor is able to directly reduce U(VI). We observe significant U(VI) reduction in the presence of a chemically reduced spent medium and in the absence of spores (Fig. S2). This behaviour is consistent with the factor being an electron shuttle: a small but measurable initial decrease in U(VI) concentration corresponding to the reduction of U(VI) by the reduced factor followed by a constant U(VI) concentration after its depletion. Thus, the stimulatory factor present in *D. reducens* spent medium could be an electron shuttle that is reduced by a spore surface protein receiving electrons from H_2 and that, in turn, reduces U(VI). AQDS, a humic acid analogue and electron shuttle for metal reduction did not restore U(VI) reduction by spores in fresh medium supplemented with H_2 (Fig. S3), suggesting that it does not act as an electron shuttle in this system.

Previous studies have shown that cells can produce electron shuttles to transfer electrons to extracellular electron acceptors. For instance, *S. oneidensis* strain MR-1 is thought to excrete a small (150–300 Da) quinone-containing molecule that transfers electrons to Fe(III) minerals (Newman and Kolter, 2000; Hernandez and Newman, 2001), thereby circumventing the need for direct contact between the bacterium and the solid phase terminal electron acceptor. More recent studies have shown that flavin mononucleotide (450 Da) and riboflavin are excreted by *Shewanella* strains and act as electron shuttles for Fe(III) oxide as well as soluble Fe(III) reduction (von Canstein *et al*., 2008; Marsili *et al*., 2008).

In addition, electron transport molecules are not always involved in shuttling electrons from cells to insoluble electron acceptors; they can also reduce extracellular soluble electron acceptors directly. For example, pyridine-2,6-thiocarboxylic acid (PDTC) is a small (< 500 Da) redox-active compound secreted by the denitrifying bacterium *Pseudomonas stutzeri* strain KC. PDTC is able to reduce carbon tetrachloride when complexed with metals (e.g. Cu^{2+}) (Lee *et al*., 1999). It can also complex and reduce soluble selenite, tellurite and chromate (Zawadzka *et al*., 2006; 2007) and precipitate elemental Se and Te as well as Cr(III).

The redox potential of all of the above [AQDS/AH_2DS $E^0_w = -0.184$ V (Clark, 1972); flavin(ox)/flavin(red)

$E^0_w = -0.185$ to -0.220 V (Clark, 1972); Cu : PDTC complex $E^0_w = -0.512$ V (Cortese *et al*., 2002)) are sufficiently reduced to donate electrons to U(VI). We suggest that, based on its redox potential, a flavin may be a good candidate for an electron shuttle. Indeed, reduced riboflavin was found to reduce U(VI) experimentally (Fig. S3), but re-oxidation of U(IV) was observed subsequently. No effect of riboflavin was observed in a fresh medium amended with spores, suggesting that spore proteins may be unable to catalyze the reduction of the oxidized form of riboflavin.

Based on the observations collected to date, we suggest that the stimulatory factor likely acts as an electron shuttle between spores and U(VI) but that it is not AQDS or riboflavin.

In addition to *D. reducens*, a few Gram-positive spore-forming bacteria have been identified as able to reduce U(VI). Among those, non-SRB bacteria, such as the fermentative bacterium *Clostridium acetobutylicum* (Gao and Francis, 2008) and the thermophile *Carboxydothermus ferrireducens* (Khijniak *et al*., 2005), have been identified. While, to date, the ability of spores to reduce U(VI) is confined to *D. reducens*, this mechanism may not be exclusive to this organism. Field studies of U(VI)-reducing microbial communities increasingly suggest the relevance of Gram-positive bacteria (N'Guessan *et al*., 2008; Madden *et al*., 2009). If spores are also involved in the reduction of U(VI) by other *Firmicutes*, it may become apparent that U(VI) reduction by spores is more widespread than considered and may have a significant impact on uranium biogeochemistry.

In summary, this work shows that *D. reducens* is an organism that exhibits a novel mechanism of metal reduction involving spores. This result is significant both biochemically – the spore protein involved is unlikely to resemble known metal reductases – and environmentally – because strategies for bioremediation may be altered if this pathway is important in the subsurface. The involvement of a presumed electron shuttle in the reduction may also provide opportunity for the study of a hitherto unknown excreted molecule in a Gram-positive bacterium. Thus, understanding the mechanism of metal reduction by spores and identifying the missing factor in spent medium are topics that warrant continued scrutiny.

Experimental procedures

Cultivation conditions

Desulfotomaculum reducens strain MI-1 was kindly provided by Anna Obraztsova. Standard anaerobic conditions were used throughout the study (Balch *et al*., 1979). Widdel low phosphate (WLP) medium, modified from Widdel and Bak (1992), was used for growth experiments. In addition to vitamins and trace minerals, the constituents of WLP were as follows (per litre, all supplied by Acros): NH_4Cl, 0.25 g; $CaCl_2{\cdot}2H_2O$, 0.1 g; $MgCl_2{\cdot}6H_2O$, 0.5 g; NaCl, 5 g; KCl, 0.5 g; and KH_2PO_4, 0.03 g adjusted to pH 7.2. The medium was dispensed in 50 ml volumes into 100 ml glass serum bottles and autoclaved. The following solutions were added from sterile anaerobic stocks (final concentration): yeast extract (Difco), 0.05%; $NaHCO_3$ (Acros), 30 mM; 1,4-piperazinediethane sulfonic acid disodium salt monohydrate (PIPES, Applichem), 20 mM; pyruvic acid (Acros), 10 mM. The final pH of the medium was 7.2 $\pm$ 0.2. For fermentative growth no electron acceptor was added to the medium. For reduction assays, 100 μM U(VI) (as uranyl acetate, Fluka) was added from an anaerobic stock. A sporulated culture (2 ml) was used as an inoculum for 50 ml of medium. Two controls were included in the experiments: one did not contain cells and the other lacked electron donor. All experiments were carried out in biological triplicates. Cultures were incubated at 37°C and sampled using disposable syringes in the anaerobic chamber. The presence or absence of spores was determined by optical microscopy.

Spore preparation and assays

Pasteurized spore suspensions were obtained by centrifuging a 60 h pyruvate-grown culture at 8000 *g* for 15 min and resuspending it in fresh anaerobic medium containing 20 mM acetate and 1 mM sulfate to induce sporulation. The spores were centrifuged (as above), washed three times and resuspended in aerobic MilliQ (18 MΩ) water. They were pasteurized at 80°C for 20 min and washed five times with anaerobic water. Unpasteurized spore preparations were obtained in the same way but omitting the pasteurization step. The final pasteurized or unpasteurized spore preparations were diluted 100-fold in anaerobic MilliQ water and yielded a spore suspension containing $\sim 1.6 \times 10^8$ spores ml^{-1} as determined by direct counting with a Petroff-Hauser counting chamber. Controls with pasteurized cells were prepared using the same procedure but cells from a 48 h fermentation culture (the absence of spores was verified with an optical microscope) were pasteurized directly. Glutaraldehyde-fixed spores were prepared by exposing the unpasteurized spore suspension to 2.5% glutaraldehyde (Sigma) in anaerobic WLP, incubating overnight anaerobically and washing extensively with anaerobic MilliQ before use. The viability of the spores and cells was tested before the U(VI) reduction assays by placing them in fresh medium containing 10 mM pyruvate and 5 mM sodium sulfate (Acros). Microscopic observations and production of sulfide were used to test growth.

U(VI) and Fe(III) reduction assays in spent medium

Spent medium for U(VI) reduction assays was obtained from a pyruvate fermentation culture after the depletion of pyruvate. Cells and spores were removed by centrifugation. The supernatant was filter-sterilized in the anaerobic chamber (5% H_2) and transferred anaerobically to a new sterile container. Aliquots of 5 ml of spent medium were added to sterile Hungate tubes with an atmosphere of 100% hydrogen (26 mM H_2 in the tube) and amended with a final con-

centration of 100 μM anaerobic U(VI) or 5 mM chelated-iron [as Fe(III)-citrate, Sigma]. Pasteurized or unpasteurized spores or pasteurized cells were added to the assays. A no H_2 spent medium control was obtained by purging the medium with N_2 for over 1 h. The experiments were carried out in serum bottles outside the glove box to prevent H_2 contamination.

U(VI) reduction assays in the presence of DPA, AQDS, riboflavin and/or after pre-reduction with Pd

Spent medium from a fermentation culture was prepared as described above. Fresh medium was supplemented with 100 μM of pyridine-2,6-dicarboxylic acid (dipicolinic acid or DPA, Sigma) from an anaerobic stock prepared in MQ water, flushed with nitrogen and sterilized by autoclaving. In addition, AQDS (Sigma) or riboflavin (Sigma) also prepared in anaerobic stocks were added to separate cultures (100 μM each) either untreated or after treatment with Pd and H_2 to pre-reduce the compounds. The treatment consisted of incubation with 10% w/v of palladium pellets (0.5% w/w on 3.2 mm alumina pellets, Sigma) in the presence of hydrogen (100% H_2 in the 25 ml headspace) for 20 min. Palladium was removed by filtration and the filtrate used to amend spent medium.

Spent medium was passed through a 3 kDa molecular weight cut-off filter (Millipore) and exposed to palladium and hydrogen as described above. The 3 kDa filter was pre-treated prior to use with anaerobic MQ water to remove impurities as suggested in the user manual guidelines. Palladium was removed by filtration through a 0.2 μm filter.

For all experiments, 5 ml of the medium (fresh, spent or amended with a compound) was transferred to fresh Hungate tubes supplemented with hydrogen (100% H_2 in the 10 ml headspace) and amended with a final concentration of 100 μM anaerobic U(VI). Unpasteurized spores were added when indicated. Abiotic control from spent medium, fresh medium and fresh medium supplemented with DPA/AQDS/riboflavin were also prepared.

Analytical methods

Protein from a centrifugally pelleted culture was extracted by incubation at 95°C for 10 min in 0.1% Triton X-100, 0.1% SDS, 10 mM EDTA and 1 mM Tris-HCl. After a 100-fold dilution, the protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA). Uranium was analysed by kinetic phosphorescence analysis (KPA-11; Chemcheck Instruments, Richland, WA) after anaerobic filtration (Millipore Millex-GV PVDF 0.2 μm). HCl-extractable Fe(II) was measured with ferrozine as previously described (Lovley and Phillips, 1987). Total iron in the samples was measured using a Perkin-Elmer 2000 Inductively Coupled Plasma Emission Spectrometer in 1% HNO_3. Organic acids were measured using an ion chromatograph (DX-3000, Dionex, Sunnyvale, CA) with an IonPac AS11-HC column. Elution was carried out using a gradient of 0.5–30 mM KOH. Hydrogen was measured using a GC-TCD (Hewlett Packard HP6890-TCD) with a carboxen capillary column and nitrogen as a carrier gas.

XANES

The samples were collected anaerobically and shipped to the Stanford Synchrotron Radiation Laboratory in Menlo Park, CA in an anaerobic serum bottle. The wet sample pellet was loaded into an aluminum sample holder in an anaerobic chamber (95% N_2, 5% H_2). Fluorescence U L-III edge XANES spectra were collected at 77 K at beamline 11-2 using a Si(220) double crystal monochromator, detuned to attenuate harmonic content in the beam. Beam size was set to 1 mm vertical × 3 mm horizontal. Data were background-subtracted and normalized using the software package SIXPACK (Webb, 2005). The standards used were uranyl nitrate [$UO_2(NO_3)_2$] for U(VI) and a pure, crystalline, stoichiometric, chemically produced $UO_{2.00}$ (Conradson *et al.*, 2004) for U(IV). Linear combination fits in SIXPACK using the model compounds given provided the amount of U(VI) and U(IV) present respectively (Table 1) and carried an error of ±10%. Sensitivity to oxidation state is based on the energy position of the X-ray absorption edge [relatively blue shifted for U(VI)] and by the presence or absence of the strong multiple-scattering peak at *c.* 17185 eV, characteristic of the uranyl trans-dioxo cation. The details of data correction and fitting are given in Supporting information.

Electron microscopy

The spore suspension incubated with U(VI) was collected and pelleted by centrifugation at 8000 *g* for 5 min at 10 000 r.p.m. The sample was fixed overnight with 2.5% glutaraldehyde (Sigma). Fixed cells were dehydrated by sequential washes with 25%, 50%, 75%, 90% and 100% ethanol. Washes with 25–75% ethanol were performed twice while the washes with 90–100% ethanol were carried out three times each. The final wash was carried out with dry ethanol from a sealed bottle. The cells were resuspended in N_2-purged LR-White resin (Electron Microscopy Sciences). After a 15 min incubation, the resin was replaced by fresh resin. Up to this point, all the steps were carried out inside the glove box. The polymerization was carried out at 60°C overnight. The sample was thin-sectioned and the sections mounted onto a Cu grid with a formvar film. For the whole-mount sample, a spore suspension incubated with Fe(III)-citrate for 96 h was collected by centrifugation at 7000 *g* for 3 min and washed twice with anaerobic MQ to remove excess salt. The sample was resuspended in 20 μl of anaerobic MQ and mounted onto a Cu grid with a formvar film in the anaerobic chamber. For imaging, the grids were transferred directly into a transmission electron microscope (FEI CM-10) and images were collected using a side-mounted Morada Soft Imaging System camera.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Pyruvate fermentation by MI-1 with and without U(VI). These data correspond to the same experiment shown in Fig. 1.

Fig. S2. U(VI) reduction in the presence and absence of spores and spent medium that underwent ultrafiltration (UF) through a 3 kDa cut-off and that was subjected to 20 min H_2 and Pd incubation. Total concentration of U(VI) reduced for the treatments incubated in the presence of spores.

Fig. S3. Percentage of reduced U(VI) over time after incubation with spores of *D. reducens* strain MI-1 in fresh medium supplemented with AQDS, AHDS or riboflavin (100 μM each) and H_2. Spent medium was used as positive control. FM = fresh medium and SM = spent medium.