Characterization of extracellular minerals produced during dissimilatory Fe(III) and U(VI) reduction at 100 °C by *Pyrobaculum islandicum*

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ABSTRACT

In order to gain insight into the significance of biotic metal reduction and mineral formation in hyperthermophilic environments, metal mineralization as a result of the dissimilatory reduction of poorly crystalline Fe(III) oxide, and U(VI) reduction at 100 °C by *Pyrobaculum islandicum* was investigated. When *P. islandicum* was grown in a medium with poorly crystalline Fe(III) oxide as an electron acceptor and hydrogen as an electron donor, the Fe(III) oxide was reduced to an extracellular, ultrafine-grained magnetite with characteristics similar to that found in some hot environments and that was previously thought to be of abiotic origin. Furthermore, cell suspensions of *P. islandicum* rapidly reduced the soluble and oxidized form of uranium, U(VI), to extracellular precipitates of the highly insoluble U(IV) mineral, uraninite (UO_2). The reduction of U(VI) was dependent on the presence of hydrogen as the electron donor. These findings suggest that microbes may play a key role in metal deposition in hyperthermophilic environments and provide a plausible explanation for such phenomena as magnetite accumulation and formation of uranium deposits at *ca*. 100 °C.

INTRODUCTION

Fe(III)-reducing microorganisms play an important role in the decomposition of natural and contaminant organic compounds in marine and freshwater sediments and in the production of geologically relevant minerals in mesophilic environments (Lovley, 1991, 1995). Fe(III) also is one of the most abundant electron acceptors in various hot environments (Lovley, 1991, 1995; Thamdrup, 2000). The significance of microbial dissimilatory Fe(III) reduction in hyperthermophilic environments has only been acknowledged during the last decade (Vargas *et al.*, 1998; Lovley & Coates, 2000). The capacity for dissimilatory Fe(III) reduction is a common characteristic among hyperthermophilic microorganisms, including *Pyrobaculum islandicum* (Vargas *et al.*, 1998). In addition to reducing Fe(III), *P. islandicum* is also capable of transferring electrons to a wide variety of toxic and heavy metals, such as U(VI), Co(III), Cr(VI), Mn(IV), Tc(VII), and Au(III), with hydrogen serving as the electron donor (Kashefi & Lovley, 2000). This is of interest because the reduced products of some of these metals such as Fe(II) and U(IV) directly impact the geochemistry of hot ecosystems and also may serve as geological signatures for the activity of hyperthermophilic Fe(III) reducers.

The microbial reduction of insoluble Fe(III) in mesophilic aquatic sediments results in the production of Fe(II)-containing minerals such as magnetite and siderite (Karlin *et al.*, 1987; Coleman *et al.*, 1993). Similarly, the microbial reduction of the oxidized form of uranium, U(VI), which is highly soluble in most waters, results in the mineralization of uranium as an insoluble extracellular U(IV) mineral, uraninite (UO_2) (Gorby & Lovley, 1992; Lovley & Phillips, 1992b; Kashefi & Lovley, 2000). This is regarded as an important process for the immobilization and removal of dissolved uranium from contaminated waters and aquatic sediments as well as for the formation of uranium ores (Lovley *et al.*, 1991; Lovley & Phillips, 1992a).

Compared to the vast amount of information regarding microbial metal mineralization in mesophilic environments, little is known about the role, if any, of microbes in mineral

deposition in hot environments. In order to evaluate the contribution of hyperthermophilic Fe(III) reducers to metal mineral deposition in hot environments, we investigated and characterized mineral formation due to Fe(III) and U(VI) reduction using the model hyperthermophilic, Fe(III)-reducing archaeon, *P. islandicum*. Our previous studies (Kashefi & Lovley, 2000) have shown that *P. islandicum* can couple the reduction of Fe(III) oxide and U(VI) to molecular hydrogen. Here we report the analyses of two extracellular mineral precipitates resulting from the reduction of poorly crystalline Fe(III) oxide and soluble U(VI): the magnetic mineral, magnetite (Fe_3O_4), and the insoluble U(IV) mineral uraninite (UO_2), respectively. The implications of the biological mineralization of these abundant metals in the geochemistry of hyperthermophilic environments are also discussed. The results presented here further demonstrate that *P. islandicum* provides a suitable model organism for studying dissimilatory reduction of iron leading to magnetite formation, as well as for the formation of uranium deposits, and highlight the significance of microbial Fe(III) and U(VI) reduction in mineral deposition in hot environments (Kashefi & Lovley, 2000).

MATERIALS AND METHODS

Organism and culture conditions

Pyrobaculum islandicum (DSM 4184) was purchased from the German Collection of Microorganisms (DSM) (Braunschweig, Germany). Strict anaerobic techniques (Miller & Wolin, 1974; Balch *et al.*, 1979) were used throughout this study, as previously described (Kashefi & Lovley, 2000; Kashefi *et al.*, 2002). Unless otherwise indicated, all incubations were carried out in the dark at 100 °C.

The reduced product of Fe(III) reduction (magnetite) was isolated from cultures of *P. islandicum* grown anaerobically in DSM medium 390, modified as previously described (Kashefi & Lovley, 2000). Briefly, thiosulfate was substituted with poorly crystalline Fe(III) oxide. Yeast extract (0.01%, w/v), vitamins (10 mL L^{-1}) and trace minerals (10 mL L^{-1}) were added from stock solutions. Sodium sulfide was replaced by L-cysteine HCl (0.25 mM), and sodium bicarbonate (1.93 g L^{-1}) was also added. Poorly crystalline Fe(III) oxide was prepared as previously described (Lovley & Phillips, 1986), and was provided at 100 mmol L^{-1} as the terminal electron acceptor, with hydrogen as the electron donor (H$_2$:CO$_2$, 80:20%, v/v, 101 kPa). The pH of the autoclaved medium was about 6.2 (Kashefi & Lovley, 2000).

Cell suspension assays

U(VI) reduction was studied in cell suspension assays as previously described (Kashefi & Lovley, 2000). Briefly, cells were first grown anaerobically, in the modified, DSM medium 390, using organic compounds as the electron donor, with the following adjustments; poorly crystalline Fe(III) oxide was replaced with Fe(III)-citrate (20 mM) as the electron acceptor, concentration of yeast extract was increased to (0.02%, w/v), followed by addition of peptone (0.05%, w/v), as the carbon source and the electron donor (Kashefi & Lovley, 2000). Cells from 800 mL cultures grown to mid-late log phase (*ca.* 2.0– 2.5×10^8 cells mL^{-1}) were grown in the above medium with Fe(III) citrate as the electron acceptor. Cells were then collected by centrifugation (4300 *g* for 10 min, at 4 °C) under an atmosphere of oxygen-free N$_2$:CO$_2$ (80:20%, v/v). The cell pellet was washed twice with 80 mL of 23 mM anaerobic bicarbonate buffer under N$_2$:CO$_2$ (80:20%, v/v) and resuspended with 8 mL of the same buffer in anaerobic pressure tubes (Bellco Glass, Inc., Vineland, NJ, USA) containing the same headspace (N$_2$:CO$_2$, 80:20%, v/v). An aliquot of the cell suspension (*c.* 0.1 mL) was added to 10 mL of anaerobic bicarbonate buffer (23 mM, pH 6), amended with uranyl acetate (250 μM, pH 6) as U(VI), to provide *c.* 0.025 mg of cell protein per millilitre, under H$_2$:CO$_2$ (80:20%, v/v, 101 kPa) atmosphere. Cells in killed controls were autoclaved for 2 h and examined under the microscope to confirm total cell lysis. Incubations were done in the dark at 100 °C, unless otherwise mentioned. Concentrations of U(VI) in the cell suspensions were measured on filtrates (0.2-μm pore diameter) using a Dionex DX-500 Ion Chromatograph (Dionex Corporation, Sunnyvale, CA, USA) with an HPIC-AS5 column and 0.1 M MgSO$_4$–0.05 M H$_2$SO$_4$ as the eluent. U(VI) was monitored at *A650* following an arsenazo-III postcolumn derivitization as previously described (Kashefi & Lovley, 2000).

Microscopy

Cells from cultures grown with poorly crystalline Fe(III) oxide, and Fe(III)-citrate as the electron acceptors as well as cell suspensions of U(VI)-reducing *P. islandicum* were routinely examined either with a Zeiss standard phase-contrast microscope (Carl Zeiss Microimaging Inc., Thornwood, NY, USA) equipped with an oil immersion objective (100/1.25), or stained with acridine orange (Hobbie *et al.*, 1977), and inspected by epifluorescence microscopy (Kashefi *et al.*, 2002). When indicated, thin sections of resin-embedded samples were prepared and examined with a JEOL 100S transmission electron microscope operated at 80 V, as previously described (Kashefi *et al.*, 2002).
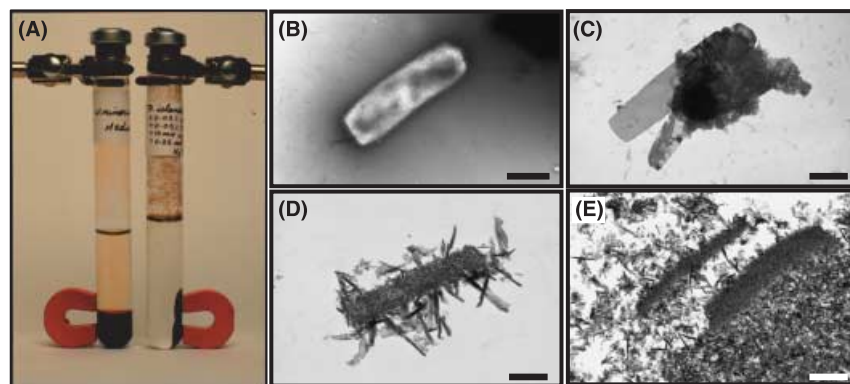
Protein assay

Protein concentrations were determined using the bicinchoninic acid protein assay (Smith *et al.*, 1985). Bovine serum albumin was used as the reference protein.

Magnetic analyses of minerals

The black magnetic mineral precipitate resulting from the dissimilatory reduction of poorly crystalline Fe(III) oxide by

P. islandicum was first collected anaerobically by slow centrifugation (119 *g* for 30 min at room temperature, while still in the pressure tubes). The supernatant was removed anaerobically using a 10-mL syringe and a needle. The black precipitate was then dried by flushing the tube with a stream of oxygen-free $N_2:CO_2$ (80:20%, v/v), using a 23-gauge needle and a 0.2-μm filter, while still stoppered. The dry mineral precipitate was washed three times with acetone and then dried once more under vacuum. Samples were immediately prepared for magnetic measurements by dispersing a known amount of the mineral precipitate in CaF_2. Magnetic hysteresis loops (magnetization vs. magnetic field) at room temperature and in fields up to 1.5 Tesla (T) were measured using a vibrating sample magnetometer. Low-temperature remanence measurements were made from 20 to 300 K with a Quantum Design's Magnetic Property Measurement System (model No. MPMS2), (Quantum Design, San Diego, CA, USA) SQUID susceptometer. The sample was given a saturation remanent magnetization in a field of 2.5 T at 20 K and then measured in approximately zero magnetic field at 5 K intervals up to 300 K. The residual field in the superconducting solenoid for remanence measurements was less than 1 μT.

X-ray diffraction analyses of magnetite and uraninite

The black magnetic mineral precipitate resulting from the dissimilatory reduction of poorly crystalline Fe(III) oxide by *P. islandicum* was first collected anaerobically as described under the section for magnetic analyses of minerals. The dried pellet mineral precipitate was scraped off with a spatula and ground to a fine powder using a mortar and a pestle. The resulting fine powder was then sprinkled, randomly oriented and mounted on a glass slide with amyl acetate and scanned from 10 to 70°2θ at room temperature on a Philips X'Pert system (PW 3040, PANalytical, Almelo, the Netherlands).

A similar procedure was followed for the characterization of the black mineral precipitate produced as a result of the reduction of U(VI) by *P. islandicum* cell suspensions. The dried pellet was scraped off and was ground to a fine powder

(as above), which was then randomly orientated on a glass slide, before it was mounted with amyl acetate for X-ray diffraction analyses, as previously described by Gorby and Lovley (1992), and scanned from 10 to 70°2θ on a Siemens X-ray diffractometer.

RESULTS AND DISCUSSION

Magnetite formation as a result of iron reduction

During growth on Fe(III) oxide, *P. islandicum* converted the nonmagnetic, brown, poorly crystalline Fe(III) oxide into ultrafine-grained magnetite, which was black in colour and strongly attracted to a magnet (Fig. 1A). The black precipitate was not present and no Fe(III) reduction was detected in any of the controls (Kashefi & Lovley, 2000). Controls included uninoculated tubes with H_2 ($H_2:CO_2$, 80:20%, v/v) or N_2 ($N_2:CO_2$, 80:20%, v/v) incubated at 100 °C; a killed control with H_2 ($H_2:CO_2$, 80:20%, v/v) incubated at 100 °C; and an inoculated tube with H_2 ($H_2:CO_2$, 80:20%, v/v) incubated at 30 °C. Thus, formation of the black precipitate was not abiotic but a direct consequence of the metabolic activity of *P. islandicum* during growth on Fe(III) oxide.

Transmission electron microscopic analysis of the culture before the poorly crystalline Fe(III) oxide is added (Fig. 1B), and during growth (after being incubated for different lengths of time at 100 °C) on Fe(III) oxide (Fig. 1C–E), revealed that it was composed of cells covered with mineral deposits and surrounded by poorly crystalline Fe(III) oxide (Fig. 1C). The Fe(III) oxide particles transitioned into needle-like mineral structures (Fig. 1D) and then into small crystalline mineral particles [less than 30 nm in diameter (d)] (Fig. 1E) in the course of its reduction. These particles were clearly extracellular, and were not arranged inline. No intracellular magnetite was observed when thin sections of embedded samples from various replicates and independent experiments were prepared and examined by transmission electron microscopy (data not shown).
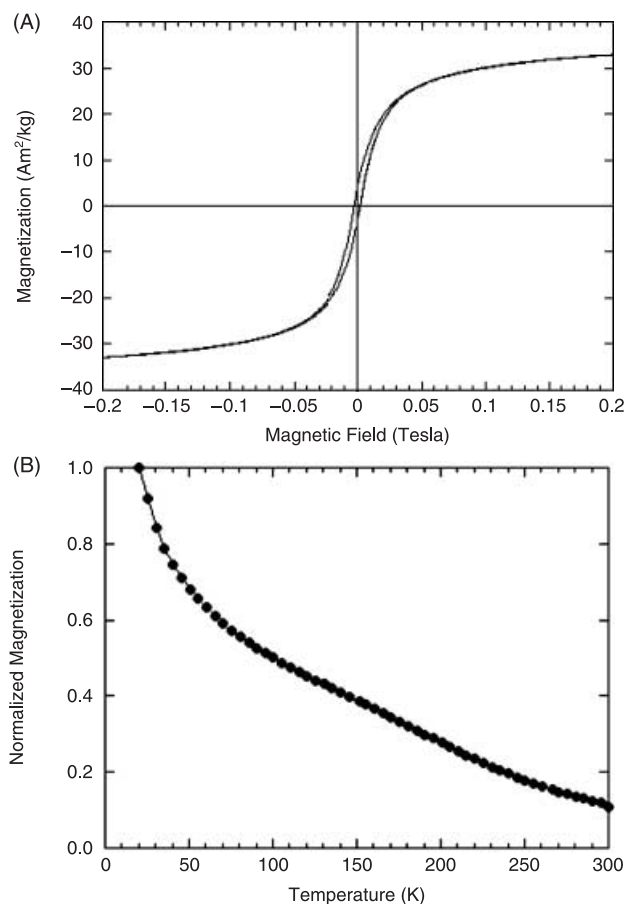
X-ray diffraction analyses of the ultrafine-grained, black magnetic precipitate indicated that the lattice spacing values
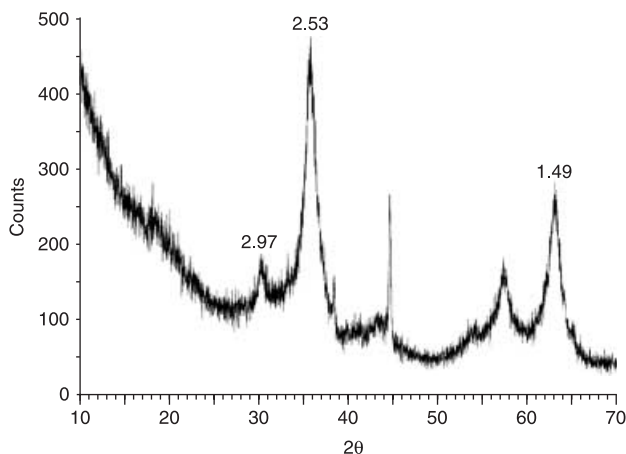
Fig. 2 X-ray diffraction pattern of the black magnetic precipitate formed as a result of the reduction of poorly crystalline Fe(III) oxide by *Pyrobaculum islandicum* 100 °C. Lattice spacing values matched those for magnetite from Joint Committee on Powder Diffraction File 2000 (data card 190629).


Fig. 3 Magnetic analyses of *Pyrobaculum islandicum* magnetite. (A) Hysteresis loop at 295 K for the magnetite precipitate produced by *P. islandicum*. The coercivity was 2.3 mT, remanence ratio was 0.09, and bulk saturation magnetization was 36.8 Am^2 kg^{-1}. (B) Thermal demagnetization of saturation remanence for the magnetite mineral produced by *P. islandicum*. The saturation remanence was given in a 2.5 T field at 20 K.

matched those of magnetite described in the Joint Committee on Powder Diffraction File 2000, data card 190629 (Fig. 2). Furthermore, electron diffraction analyses of one of the crystal aggregates revealed a diffuse ring pattern, consistent with the electron diffraction pattern characteristic of magnetite (Fe$_3$O$_4$) [National Bureau of Standards, Monograph 25, Sec. 5, 31 (1967)] (data not shown).

The magnetite particles produced by *P. islandicum* exhibited magnetic hysteresis at room temperatures (Fig. 3A). From the hysteresis loop at high magnetic fields, the mineral precipitate yielded a maximum bulk saturation magnetization (J$_s$) of 36.8 Am^2 kg^{-1}. Using the value of 92 Am^2 kg^{-1} for pure magnetite at 295 K, the weight fraction of magnetite in the precipitate was approximately 40%. The small but finite values of remanence (J$_r$/J$_{rs}$ = 0.096) and coercivity (H$_c$ = 2.3 mT) were consistent with a broad magnetite particle size distribution that peaks below 30 nm and predominantly contains superparamagnetic (SPM) particles with d < 30 nm, and a smaller fraction with sizes above 30 nm that extend into the stable single-magnetic-domain (SD, d ≈ 30–100 nm) or multidomain (MD, d > 100 nm) size ranges (Moskowitz, 1993; Bazylinski & Moskowitz, 1997). Both SD- and MD-sized particles contribute to remanence and coercivity at room temperature, whereas SPM particles have J$_r$ = 0 and H$_c$ = 0. A mixture of SD (J$_r$ > 0) and SPM (J$_r$ = 0) particles will reduce the J$_r$/J$_s$ ratio from its theoretical SD value of 0.5 for randomly orientated SD particles (Dunlop & Özdemir, 1997). For the mineral precipitate produced by *P. islandicum*, the fraction of such remanence carrying particles was small based on the observed low remanence ratio value J$_r$/J$_s$ = 0.09. In contrast, J$_r$/J$_s$ values near the theoretical SD value of 0.5 are observed for magnetite magnetosomes produced by magnetotactic bacteria, which contain narrowly sized SD particles (Bazylinski & Moskowitz, 1997). The observed value of coercivity (H$_c$ = 2.3 mT) was at

least an order of magnitude less than coercivity values for SD particles of magnetite produced by magnetotactic bacteria (Moskowitz *et al.*, 1988, 1989, 1993). The reduction in H$_c$ from typically high values associated with stable SD particles was characteristic of thermal relaxation behaviour in nanophase superparamagnetic particles. While hysteresis behaviour of magnetite particles produced by *P. islandicum* was similar to the hysteresis results from particles produced by *Geobacter metallireducens* and a marine strain of *Shewanella putrefaciens* (Moskowitz *et al.*, 1989; Bazylinski & Moskowitz, 1997), both remanence and coercivity values at 300 K were higher for the magnetite produced by *P. islandicum*. This suggests that *P. islandicum* is capable of producing a larger fraction of SD grains (> 30 nm) than either *G. metallireducens* or *S. putrefaciens*.

The thermal decay of a saturation remanence given at 20 K in a magnetic field of 2.5 T is shown in Fig. 3B. The characteristic concave downward shape is a hallmark of nanophase particles exhibiting superparamagnetism where the gradual
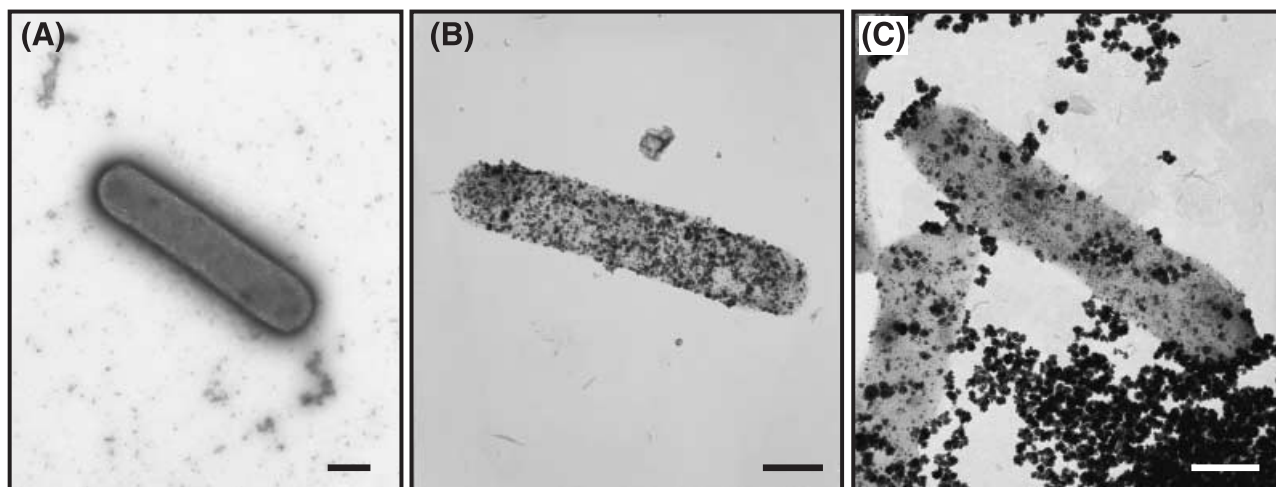
Fig. 4 Mineralization of U(VI) at 100 °C by cell suspensions of *Pyrobaculum islandicum*. Transmission electron micrograph of negatively stained cells of *P. islandicum* at time 0 (A), 60 min (B), and 140 min (C), showing the extracellular mineral produced as a result of U(VI) reduction. Bar, 0.5 µm.

decrease in remanence with increasing temperature is produced by the progressive unblocking of magnetization as stable SD particles become SPM with increasing temperature. The shape of the remanence curve reflects a distribution of unblocking temperatures, due to a lognormal distribution in particle sizes (Bazylinski & Moskowitz, 1997). Also, there is no indication in the thermal demagnetization curve for the Verwey transition in magnetite. This crystallographic phase transition, which is expressed in magnetization curves as a sharp drop in magnetization near 110–120 K, is a fingerprint for stoichiometric magnetite, and occurs for all magnetite particle sizes (SD and MD) above the superparamagnetic threshold size of approximately 30 nm (Moskowitz *et al.*, 1989; Özdemir *et al.*, 1993; Bazylinski & Moskowitz, 1997). The lack of a Verwey transition indicated that this organism produced a small fraction of particles that are SD or MD and that the remanence behaviour of the particles at low temperatures was swamped by their superparamagnetic unblocking response. In addition, partial or complete oxidation of magnetite to maghemite also may lead to suppression of the Verwey transition (Dunlop & Özdemir, 1997), which may have occurred under ambient conditions due to the ultrafine-grained nature of the precipitate. The low-temperature remanence curve (Fig. 3B) also is analogous to results obtained for particles produced by *G. metallireducens* and a marine strain of *S. putrefaciens* (Moskowitz *et al.*, 1989; Bazylinski & Moskowitz, 1997).

Uranium reduction and uraninite formation

Washed cell suspensions of Fe(III)-grown *P. islandicum* reduced U(VI) (provided as 10 µM, 250 µM or 1 mM uranyl acetate) at 100 °C with hydrogen as the electron donor. The yellowish colour of the bicarbonate buffer (23 mM) containing the U(VI)/cell suspension rapidly dissipated (10 and 250 µM

uranyl acetate were reduced in less than 30 min and in about 2.5 h, respectively). A dark (almost black) precipitate was formed, as previously observed (Kashefi & Lovley, 2000). The blackish precipitate was not produced in uninoculated control tubes or in killed controlled tubes that had been incubated at 100 °C, nor was it produced in inoculated control tubes that were incubated at 30 °C. In all control tubes the yellow colour of the U(VI)-containing bicarbonate buffer did not dissipate throughout the incubation. Furthermore, consistent with previous reports (Kashefi & Lovley, 2000), no loss of U(VI) was detected in any of the control tubes, suggesting that the reduction of soluble U(VI) to insoluble U(IV) was not abiotic but was the result of the enzymatic activity of *P. islandicum*.

The electron-dense precipitate formed as a result of enzymatic U(VI) reduction in cell suspensions of *P. islandicum* was deposited outside the cells (Fig. 4), suggesting that, similar to Fe(III) reduction, the mineralization of uranium was extracellular. X-ray diffraction analyses of the precipitate produced by cell suspensions of *P. islandicum* was consistent with the U(IV) mineral, uraninite (UO$_2$) (Fig. 5).

Geological implications

The finding that a hyperthermophilic microorganism is capable of producing extracellular ultrafine-grained magnetite and uraninite may help explain some geological phenomena. For example, it was previously suggested that the accumulation of ultrafine-grained magnetite in the deep, hot subsurface might be the result of microbial activity (Gold, 1992; McKay *et al.*, 1996). The results presented provide an example of the type of microorganism that could produce magnetite in such an environment. The magnetite mineral produced by *P. islandicum* had marked differences with the magnetite produced by magnetotactic bacteria (Blakemore, 1982; Bazylinski
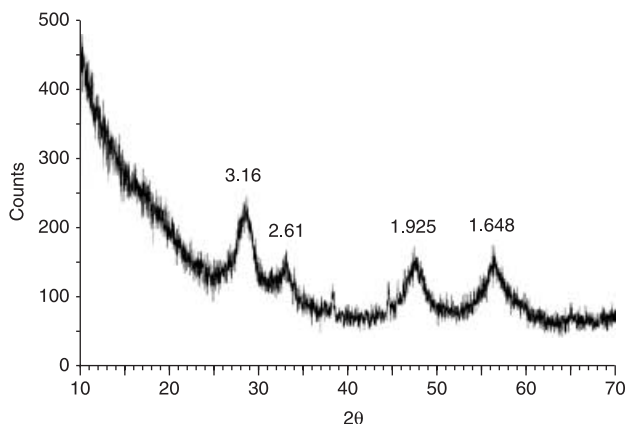
et al., 1988), but was similar to that produced by mesophilic dissimilatory Fe(III)-reducers such as *G. metallireducens* and *S. putrefaciens* (Lovley *et al.*, 1987; Moskowitz *et al.*, 1989). These mesophilic bacteria also produce extracellular magnetite (Lovley *et al.*, 1987; Glasauer *et al.*, 2002), although it has been reported that, similar to magnetotactic bacteria, magnetite formation in *S. putrefaciens* also may take place in membrane-confined vesicles in the cytoplasm (Glasauer *et al.*, 2002).

It has long been suspected that Fe(III)-reducing microorganisms might have the potential to isotopically fractionate iron during Fe(III) reduction, which could provide a clear method for identifying the differences between these two different mechanisms, biotic and abiotic, for magnetite formation (Friedman & O'Neil, 1977; Caldeira *et al.*, 1990; Beard *et al.*, 1999). However, the experimental data and theoretical understanding of the mechanisms of Fe isotope fractionation required to assign a biotic vs. abiotic origin to magnetites present in the rock record are only just emerging (Crosby *et al.*, 2007). Recent studies (Crosby *et al.*, 2005) with crystalline Fe(III) oxide phases such as haematite and goethite have shown that at least some of the observed fractionation of Fe isotopes during microbial Fe(III) oxide reduction is driven by coupled electron/Fe atom exchange at the oxide surface, which results in the production of isotopically 'light' aqueous Fe(II) and a yet unidentified form of isotopically 'heavy' Fe(III) pool (Crosby *et al.*, 2005; Johnson *et al.*, 2005). These studies along with the development of novel isotopic fractionation methods (Anbar *et al.*, 2005; Johnson & Beard, 2005) raise hopes that, in a near future, it may be possible to isotopically profile magnetite minerals such as those found in ancient rocks. This may then be indirectly used as a geological signature of the microbial activity of Fe(III)-reducing hyperthermophiles on a hot, early Earth (Anbar *et al.*, 2000). Recent studies suggest that an abundance of -δ^{56}Fe in magnetite-bearing banded iron-formations (BIFs) may indicate a source

of isotopically light Fe(II) linked to microbial Fe(III) oxide reduction (Johnson *et al.*, 2008a,b). Measurements of the Fe isotope composition of magnetite (and aqueous Fe^{2+}) produced by *P. islandicum* could provide further insight into the potential for high-temperature microbial Fe(III) oxide reduction to produce Fe isotopic 'biosignatures'.

Our finding that cells of *P. islandicum* can mineralize uranium as uraninite at 100 °C sets a new upper temperature limit at which microorganisms may have a substantial impact on uranium geochemistry and provides supporting evidence for a role of microbial reductive precipitation of U(VI) in the precipitation of U(IV) in marine sediments, uranium ore accumulations, and the formation of uranium reduction spots (Lovley *et al.*, 1991; Lovley & Phillips, 1992b; Lovley, 1993; Kashefi & Lovley, 2000). In this study, the immobilization of uranium via U(VI) reduction was of interest for its potential role in the formation of uranium deposits, which is both geochemically and economically significant (Lovley & Phillips, 1992a). The finding that *P. islandicum* can reductively precipitate uranium as uraninite at *ca.* 100 °C provides a possible explanation for the formation of sandstone-type uranium deposits, which are thought to have been formed at such high temperatures (Hostetler & Garrels, 1962). The abiotic reduction of U(VI) by lignite organic matter, which is often associated with uranium deposits, is thought to occur at temperatures above, but not below, 120 °C (Nakashima *et al.*, 1984). Our results suggest that the accumulation of reduced U(VI) minerals at hyperthermophilic temperatures below 120 °C may be explained by the biological activity of hyperthermophilic microorganisms similar to *P. islandicum*. Thus, uranium deposits formed at such temperatures (Hostetler & Garrels, 1962) could provide a geological signature for the activity of hyperthermophilic microorganisms. A well-known example of uranium deposition is the Oklo nuclear reactor, a naturally generated nuclear reactor from the Precambrian period that originated when the U(VI) that was dissolved in groundwaters ranging from 100 to 150 °C was reduced to U(IV), resulting in the accumulation of large uranium precipitates (Brookins, 1990). The microbial contribution to the initial precipitation of U(IV) at this site remains to be elucidated. The environmental parameters, however, appear adequate for a contributing role from the microbial reductive precipitation of soluble uranium at these temperatures.

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