Electron transfer at the cell–uranium interface in Geobacter spp.

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Abstract
The in situ stimulation of Fe(III) oxide reduction in the subsurface stimulates the growth of Geobacter spp. and the precipitation of U(VI) from groundwater. As with Fe(III) oxide reduction, the reduction of uranium by Geobacter spp. requires the expression of their conductive pili. The pili bind the soluble uranium and catalyse its extracellular reductive precipitation along the pili filaments as a mononuclear U(IV) complexed by carbon-containing ligands. Although most of the uranium is immobilized by the pili, some uranium deposits are also observed in discreet regions of the outer membrane, consistent with the participation of redox-active foci, presumably c-type cytochromes, in the extracellular reduction of uranium. It is unlikely that cytochromes released from the outer membrane could associate with the pili and contribute to the catalysis, because scanning tunnelling microscopy spectroscopy did not reveal any haem-specific electronic features in the pili, but, rather, showed topographic and electronic features intrinsic to the pilus shaft. Pili not only enhance the rate and extent of uranium reduction per cell, but also prevent the uranium from traversing the outer membrane and mineralizing the cell envelope. As a result, pili expression preserves the essential respiratory activities of the cell envelope and the cell’s viability. Hence the results support a model in which the conductive pili function as the primary mechanism for the reduction of uranium and cellular protection in Geobacter spp.

Background
Uranium is present at low levels in almost all soils and some waters and at higher concentrations in seawater and uranium-rich ores [1]. It has no known biological role and it is both radioactive and toxic [2]. As a result, its accumulation over time, even at trace amounts, in cells and tissues may lead to cancer, leukaemia, kidney failure, birth defects, neurological problems and lung disease [3–5]. Uranium accumulates to toxic levels in some environments as a result of its anthropogenic use in nuclear research, fuel production, coal combustion, phosphate fertilizers and weapons manufacturing. It is, however, the high-level radioactive waste and intense radionuclide mining since the Cold War era as well as accidental releases from nuclear plants that have resulted in vast volumes of soils, groundwater and sediments contaminated with uranium in complex mixtures with other radionuclides and toxic metals [6]. In these environments, uranium is easily oxidized into the mobile uranyl cation (UO₂²⁺), resulting in highly dispersed contaminant plumes that make standard pump-and-treat approaches logistically and economically impractical.

Interestingly, micro-organisms live in environments with high uranium content and contribute greatly to its natural cycling [7]. Of special interest is the reduction of the uranyl cation by micro-organisms, which changes the U(VI) oxidation state into U(IV) and decreases its solubility [8]. As a result, uranium is precipitated from the aqueous phase and immobilized. Some dissimilatory metal reducers can also couple the reductive precipitation of uranium to cell growth under laboratory conditions [9,10], suggestive of overlapping mechanisms for the biological reduction of uranium and natural metal acceptors. The growth of indigenous metal-reducing micro-organisms can also be stimulated in situ and results in the concomitant removal of soluble U(VI) from the contaminated groundwater and detection of its sparingly soluble less mobile form, U(IV), in sediments [11–15]. This suggests that stimulating metal reduction leads to the biological reduction of U(VI) to U(IV), thereby preventing plume migration and eliminating the potential for contaminant exposure. For this reason, the possibility of stimulating the native microbial communities to precipitate and/or stabilize uranium contaminants shows promise for the environmental restoration of contaminated sites with a minimum of environmental disruption [16,17].

Uranium reduction via conductive pili in Geobacter spp.
The in situ stimulation of metal reduction in groundwater and sediments contaminated with anthropogenic uranium is often concomitant with substantial increases in the growth and activity of dissimilarity iron-reducing micro-organisms in the family Geobacteraceae [11,13,14,18]. Members of this family are also detected in environments with naturally
high uranium content, where they co-operate with iron-oxidizers to cycle the uranium [7]. The representative strain *Geobacter metallireducens* GS15 was one of the first micro-organisms shown to gain energy for growth from the reductive precipitation of uranium [9,19]. When examined by transmission electron microscopy, the cells were surrounded by copious amounts of the uranium precipitate, which preferentially localized to one side of the cell [19] (Figure 1A). The authors noted that “the precipitate was encompassed entirely extracellular” and “no intracellular uranium was detected” [19]. This contrasts with later studies using the genetically amenable [20] strain *Geobacter sulfurreducens*, which consistently showed the precipitation of uranium inside the bacterial cell envelope [21,22]. This suggested that the uranium had permeated inside the periplasmic space, where it is reduced non-specifically by the abundant low-potential electron donors of the cell envelope of Gram-negative bacteria [23]. The mineralization of the periplasm also compromises essential cellular functions such as respiration and the cell’s viability [24]. Thus the precipitation of uranium in the cell’s periplasm is unlikely to be of environmental relevance.

The lateral immobilization of uranium observed in *G. metallireducens* [19] is strikingly similar to the preferential association of the natural electron acceptor, Fe(III) oxide, with the cell’s conductive pili, which are assembled on one side of the cell [25] (Figure 1). Interestingly, the laboratory conditions routinely used to investigate uranium reduction in *Geobacter* spp. involved culture at 30°C, a temperature that promotes pilus assembly in *G. metallireducens* [26], but not in *G. sulfurreducens* [24]. However, pilus assembly in this bacterium can be induced by lowering the culture temperature to 25°C to presumably slow down growth as during the reduction of Fe(III) oxides, and triggers pilin assembly [24,25]. The lower culture temperature produces pilated strains that are otherwise indistinguishable from the non-piliated wild-type strains in the content and profile of c-type cytochromes and other haem-containing proteins of their outer membrane [24]. Yet the pilated cells removed substantially more uranium from solution than the non-piliated cells [24]. When compared with a pilin-deficient mutant (PilA−) and a hyperpiliated strain that expressed the pilin subunit from a medium-copy plasmid (pRG5::pilA), a linear correlation between pilination and the levels of U(IV) measured was demonstrated (Figure 2A). Once immobilized, all the strains reduced a similar percentage of U(VI) to U(IV) (70–85%) [24]. However, the pilated cells removed substantially more U(VI) from solution than the non-piliated strains, in proportion to the number of pili expressed per cell (Figure 2A). The pili also promoted the extracellular precipitation of uranium on the side of the cell where the pili assembled (Figure 2B). The results thus suggest that the pilus function as electronic conduits between the cell and the radionuclide.

Despite differences in the yields of uranium reduction, the uranium minerals produced by the pilated and hyperpiliated strains produced similar uranium LIII-edge EXAFS (extended X-absorption fluorescence spectroscopy) spectra that were modelled as a mononuclear phase of uranium of mostly U(IV) co-ordinated by carbon-containing ligands in bidentate and monodentate fashion and lacking any iron- or phosphorus-containing ligands [24]. The model included one carbon ligand bound to two oxygen atoms co-ordinated with the uranium atom in a bidentate fashion and followed by a distant oxygen atom [24]. There is also a carbon ligand-bonded to one oxygen atom in a monodentate co-ordination with uranium and attached to a distant oxygen atom [24]. The bidentate C1–C3 ligand is likely to be biological in nature as reported for the carboxyl co-ordinations involving amino acids.

*Figure 1 | Extracellular reduction of uranium (A) and Fe(III) oxides (B) by *Geobacter* bacteria*

Electron Transfer at the Microbe–Mineral Interface

Figure 2 | Reductive precipitation of uranium by *Geobacter* pili

(A) Linear correlation between the levels of piliation [μg of purified pili protein per OD₆₀₀ unit ('OD₆₀₀') of cell suspension] and the amount of uranium reduced by various strains of *G. sulfurreducens* over the course of 6 h. Strains from left to right: non-piliated wild-type (WTₚ₋), pilin-deficient mutant (PilA₋), pilated wild-type (WTₚ₊), and hyperpiliated pRG5:pilA strain, which expresses the PilA subunit from a medium-copy plasmid in the PilA₋ background. (B) Transmission electron micrograph of a cell of the hyperpiliated pRG5:pilA strain showing the extracellular precipitation of uranium (in black) on one side of the cell. Scale bar, 0.5 μm. Inset, higher magnification of the uranium precipitate with interspersed pili filaments. Scale bar, 10 nm.

acids and lipopolysaccharide sugars [27–29]. Furthermore, the measured spectra did not produce a uranium signal corresponding to the uranium–uraninite distance in uraninite at 3.87 Å (1 Å = 0.1 nm) [24]. The formation of mononuclear U(IV) phases has also been reported for other bacteria of relevance to uranium bioremediation [30,31]. Furthermore, evidence for the microbial reduction of U(VI) to non-uraninite U(IV) products is also emerging from field-scale studies [31,32], whereas uraninite formation has been linked to conditions of reduced bioreducing activities [33,34]. This suggests that abiotic factors may contribute to the formation of uraninite.

Role of *c*-type cytochromes in uranium reduction

It is unlikely that *c*-type cytochromes released from the cell assist the pili in their catalysis, because the extent of uranium reduction correlated well with the levels of piliation of the strains, but not with the content of loosely bound *c*-type cytochromes [24]. Most notably, the hyperpiliated pRG5:pilA strain had a reduced *c*-type cytochrome content compared with the piliated wild-type strain, yet higher uranium reductase activities in proportion to its piliation [24]. Furthermore, STM (scanning tunnelling microscopy) spectroscopy revealed electronic states in the pilus near the Fermi level, consistent with a conducting material, but did not reveal electronic states expected for haem-containing proteins such as *c*-type cytochromes [35]. STM analyses also revealed topographic and electronic substructures with periodicities similar to those reported for the grooves of the bacterial pilus shaft [35].

Evidence to date suggests, however, that cytochromes of the outer membrane could function as secondary uranium-reduction sites. Although most of the uranium reduced by piliated cells of *G. sulfurreducens* was extracellular and associated to the pili (Figure 2B), discrete regions of uranium precipitation were also observed on the outer membrane of the piliated cells [24]. In *G. sulfurreducens*, most of the redox activity of the outer membrane is provided by abundant *c*-type cytochromes that decorate the cell surface as defined foci [36]. However, molecular analyses failed to conclusively identify *c*-type cytochromes that could function as dedicated uranium reductases [22]. Mutations in outer-membrane cytochromes were often pleiotropic [37–39] and either showed no defect or only partial defects in the cell’s ability to remove U(VI) [22]. Interpretation was also difficult due to inconsistencies in the reported mutant phenotypes, with some mutations reportedly abolishing U(VI)-removal activities, yet transmission electron micrographs showed mutant cells with extensive mineralization [22]. This, and the lack of conservation in the *c*-type cytochromes of most *Geobacter* species sequenced to date [40], suggests that cytochrome abundance, rather than specific *c*-type cytochromes, may be important for uranium reduction.

Extracellular reduction of uranium as a protective cellular mechanism

In addition to promoting the reductive precipitation of uranium outside the cell, the pilus also prevented uranium from permeating inside the periplasm [24]. Periplasmic mineralization was only observed in 8% of the piliated wild-type cells and in less than 1% of the hyperpiliated pRG5:pilA cells [24]. This contrasts with the approximately 40% of non-piliated wild-type cells with a mineralized periplasm [24]. By preventing the uranium from traversing the outer membrane, the pilus also preserved the vital functions of the cell.

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envelope and the cell’s viability [24]. The respiratory activities or ‘vitality’ remaining after uranium exposure were higher, for example, in the two piliated strains in proportion to the levels of piliation [24]. Furthermore, the survival rates after exposure to uranium followed a similar correlation with the levels of pili [24].

Outer-membrane cytochrome foci also assist the pili in their extracellular catalysis and in preventing the permeation of the uranium inside the cell envelope. The PilA− mutant cells, for example, were defective in pili and in the expression of some outer membrane c-type cytochromes [24], including the OmcS c-type cytochrome that is required for the reduction of metal oxides [41]. They also had significantly more periplasmic mineralization than the non-piliated wild-type cells in inverse proportion to the outer membrane haem content (Figure 3). Furthermore, approximately 50% of the uranium atoms in the PilA− mutant cells required an additional monodentate phosphorus ligand in the uranium LIII-edge EXAFS models [24]. The phosphorus co-ordination is consistent with the permeation of uranium into the cell envelope, where it forms carboxyl- and phosphoryl-coordinated complexes with periplasmic proteins and the peptidoglycan layer [27,28] and with membrane phospholipids [42] respectively. This also compromises the respiratory functions of the cell envelope and decreased the survival rates [24]. The results therefore support a model in which the outer membrane c-type cytochromes assist the pili in maintaining the reduction of uranium extracellularly and preventing it from permeating inside the cell envelope.

Model for uranium reduction by Geobacter spp.

The co-localization of the uranium precipitate with the monolateral pili and the direct correspondence observed between piliation, extent of U(VI) reduction, cell envelope respiratory activities and cell viability support a model in which the conductive pili function as the primary mechanism for uranium reduction and cellular protection from its toxicity [24]. The expression of numerous pili per cell, which can reach several micrometres in length, increases the redox-active surface area available for binding and reducing U(VI) outside the cell. This provides an efficient mechanism for uranium respiration, but also for immobilizing the uranium before it reaches the cell. The monolateral localization of the pili, however, only promotes the immobilization of uranium on one side of the cell. Yet redox-active foci, presumably c-type cytochromes, dispersed throughout the outer membrane assist the pili in their catalytic and protective function [24]. Some of them are loosely bound and easily detach from the membrane [36,41,43], providing a natural mechanism for releasing the uranium deposits without compromising the cell envelope.

The localization of uranium reductase activities in discreet regions of the outer membrane also leaves the surrounding areas of the outer membrane exposed to the contaminant. Uranium could traverse these membrane regions unless the cell has evolved additional mechanisms to bind the uranium to the membrane and prevent its mobility. The LPS (lipopolysaccharide) that decorates the outer leaflet of the outer membrane of Gram-negative bacteria has been proposed to function as a protective barrier to prevent U(VI) from penetrating the cell envelope [24]. Studies show that LPS effectively prevents the permeation of other soluble toxic compounds [44]. Furthermore, the LPS of G. sulfurreducens is rough as it lacks the O-antigen [45]. This exposes the most highly charged region of the LPS (the core oligosaccharide) to the cell’s exterior and promotes the binding of metallic cations [46]. Models also suggest that uranyl ions are preferentially chelated and immobilized over other ions by rough LPS [29]. The binding involves carboxyl and hydroxyl

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Figure 3 | Outer-membrane haem content and uranium mineralization in the periplasm

(A) Reverse correlation between the periplasmic mineralization reported [24] for the non-piliated strains WTP− and PilA− and their outer-membrane haem content (calculated as the total density, in pixels, of the haem-stained protein bands after PAGE [24]). (B and C) Transmission electron micrographs of unstained thin sections of WTP− (B) and PilA− (C) cells showing the subcellular localization of the black uranium precipitate. Scale bar, 0.5 μm.
co-ordinations [29] consistent with the carbon and oxygen ligands modelled from the EXAFS spectra in pilated strains of *G. sulfurreducens* [24]. Alternatively, the cell may have evolved a mechanism for detoxification. As uranium is reduced in the periplasmic space, essential functions of the cell envelope such as protein folding and secretion and the functioning of respiratory components are compromised, and the cell envelope stress response is triggered [47–49]. Unwanted periplasmic materials are then packed and disposed of in membrane vesicles [50]. As energy is devoted to uranium detoxification, less energy from uranium reduction is coupled to cell growth. This could provide a plausible explanation for the finding that uranium reduction supports growth in *Geobacter* and other metal-reducing bacteria with lower biomass than theoretically possible [10].

**Implications**

The identification of pili as the primary uranium reductase in *Geobacter* bacteria provides a much-needed fundamental mechanistic understanding of uranium reduction required to design effective in situ bioremediation strategies. An insufficient knowledge of the biological mechanisms of contaminant transformation often limits the performance of in situ subsurface bioremediation and long-term stewardship strategies. However, analyses of transcript abundance for Geobacteraceae genes involved in the assembly and functioning of the pili could be useful tools to predict the activity of *Geobacter* spp. during in situ bioremediation and assess the effectiveness of in situ bioremediation schemes. Furthermore, understanding how the pili bind and reduce uranium could enable novel bioremediation technologies based on deployable devices that integrate conductive pili genetically engineered for optimal binding, catalysis and stability.

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