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Summary

Anaeromyxobacter dehalogenans* strain 2CP-C reduces U(VI) and Tc(VII) to U(IV)O$_{2(s)}$ (uraninite) and Tc(IV)O$_{2(S)}$ respectively. Kinetic studies with resting cells revealed that U(VI) or Tc(VII) reduction rates using H$_2$ as electron donor exceeded those observed in acetate-amended incubations. The reduction of U(VI) by *A. dehalogenans* 2CP-C resulted in extracellular accumulation of ~5 nm uraninite nanoparticles in association with a lectin-binding extracellular polymeric substance (EPS). The electron donor did not affect UO$_{2(S)}$ nanoparticle size or association with EPS, but the utilization of acetate as the source of reducing equivalents resulted in distinct UO$_{2(S)}$ nanoparticle aggregates that were ~50 nm in diameter. In contrast, reduction of Tc(VII) by *A. dehalogenans* 2CP-C cell suspensions produced dense clusters of TcO$_2$ particles, which were localized within the cell periplasm and on the outside of the outer membrane. In addition to direct reduction, *A. dehalogenans* 2CP-C cell suspensions reduced Tc(VII) indirectly via an Fe(II)-mediated mechanism. Fe(II) produced by strain 2CP-C from either ferrihydrite or Hanford Site sediment rapidly removed ^{99}Tc(VII)O$_4^-$ from solution.

These findings expand our knowledge of the radionuclide reduction processes catalysed by *Anaeromyxobacter* spp. that may influence the fate and transport of radionuclide contaminants in the subsurface.

Introduction

During World War II and the Cold War era, significant quantities of uranium (U) and technetium (Tc) were released into the environment at the Oak Ridge and Hanford US Department of Energy (DOE) sites. In oxidized, circumneutral pH environments, U is typically found as soluble U(VI) carbonate complexes [UO$_2$(CO$_3$)$_3^{4-}$$_{(aq)}$ and UO$_2$(CO$_3$)$_2^{2-}$$_{(aq)}$] (Fredrickson *et al.*, 2000; Wang *et al.*, 2004), while ^{99}Tc is present as the pertechnetate [Tc(VII)O$_4^-$] anion (Wildung *et al.*, 2000). Under anoxic conditions, the bioreduction of U(VI) or Tc(VII) to insoluble oxides, U(IV)O$_{2(s)}$ (uraninite) or Tc(IV)O$_{2(s)}$·nH$_2$O, by dissimilatory metal-reducing bacteria (DMRB) can significantly alter the mobility of these radionuclides in contaminated groundwaters (Lovley *et al.*, 1991; Gorby and Lovley, 1992; Lloyd *et al.*, 2000; Wildung *et al.*, 2000; Anderson *et al.*, 2003). Members of the recently described genus *Anaeromyxobacter* are DMRBs indigenous to U and Tc contaminated sites such as the Oak Ridge Field Research Center (FRC) site (Petrie *et al.*, 2003; Istok *et al.*, 2004; North *et al.*, 2004). Community analysis of an *in situ* biostimulation experiment within contaminated FRC sediments showed an increase in DMRB within the δ-*Proteobacteria* group from 5% to nearly 40% of the total community population (Petrie *et al.*, 2003; North *et al.*, 2004), which to a large extent represented an increase of both *Geobacter* spp.- and *Anaeromyxobacter* spp.-related 16S rRNA gene sequences.

Anaeromyxobacter dehalogenans strain 2CP-C is the best studied member of the genus and is capable of coupling the oxidation of H$_2$, formate, acetate and other organic compounds to the reduction of metal and non-metal electron acceptors (He and Sanford, 2002; Sanford *et al.*, 2002; He and Sanford, 2003; 2004; Wu *et al.*, 2006). Analysis of the *A. dehalogenans* 2CP-C genome sequence (Thomas *et al.*, 2008) confirmed the complexity of the electron transport pathways in this organism and identified a wide array of genes encoding putative redox

proteins, including multihaem *c*-type cytochromes and hydrogenases, which previously were shown to play roles in the reduction of U(VI) and Tc(VII) in other organisms (De Luca *et al.*, 2001; Lloyd *et al.*, 2003; Bencheikh-Latmani *et al.*, 2005; Marshall *et al.*, 2006; Shelobolina *et al.*, 2007; Marshall *et al.*, 2008). Recent studies demonstrated that *A. dehalogenans* 2CP-C respires U(VI) using H_2 as the electron donor (Wu *et al.*, 2006; Sanford *et al.*, 2007); however, there is currently no information available on the nature and location of the reduced end-product uraninite. Knowledge of the structure (i.e. crystallinity, particle size and degree of particle aggregation) of the solid phase U(IV) is critical for predicting subsequent environmental behaviour including mobility. Furthermore, although the ability to reduce Tc(VII) is distributed among DMRB, the reduction of $Tc(VII)O_4^-$ by members of the *Anaeromyxobacter* genus has not been documented.

The purpose of this study was to explore the effects of electron donor type on the reduction of $Tc(VII)O_4^-$ and U(VI)-carbonate complexes by *A. dehalogenans* 2CP-C. Using high-resolution electron microscopy, we determined the localization, size and degree of aggregation of the nascent nanoparticles. These findings provide a better understanding of the importance of electron donor type on radionuclide reduction by *Anaeromyxobacter* and the nature of the insoluble oxide precipitates.

Results and discussion

Electron donor-specific U(VI) reduction

In our initial experiments, we used uranyl acetate in the sodium bicarbonate-buffered system based on the report that *A. dehalogenans* 2CP-C could not couple acetate oxidation to U(VI) reduction in growth studies (Wu *et al.*, 2006). Using the uranyl acetate/sodium bicarbonate system, *A. dehalogenans* 2CP-C rapidly reduced 250 μM U(VI) in Balch tubes supplied with H_2 as the electron donor (Fig. 1A). In the control series, which were not supplemented with H_2, the *A. dehalogenans* 2CP-C cells also displayed substantial U(VI)-reducing activity amounting to over 50% of the initial amount of U(VI) in 72 h. We hypothesized that in our experiments U(VI) reduction was coupled to acetate oxidation, which was supplied with the 250 μM U(VI) acetate. This was an unexpected result because *A. dehalogenans* 2CP-C was previously shown to require H_2, but not acetate, as electron donor for growth with U(VI) as electron acceptor (Wu *et al.*, 2006). Our hypothesis was tested by amending uranyl acetate to *A. dehalogenans* 2CP-C resting cell suspensions with exogenous acetate, which resulted in complete reduction of 250 μM U(VI) in 72 h. These results suggest that the electron donor demand of U(VI)-reducing *A. dehalogen-*
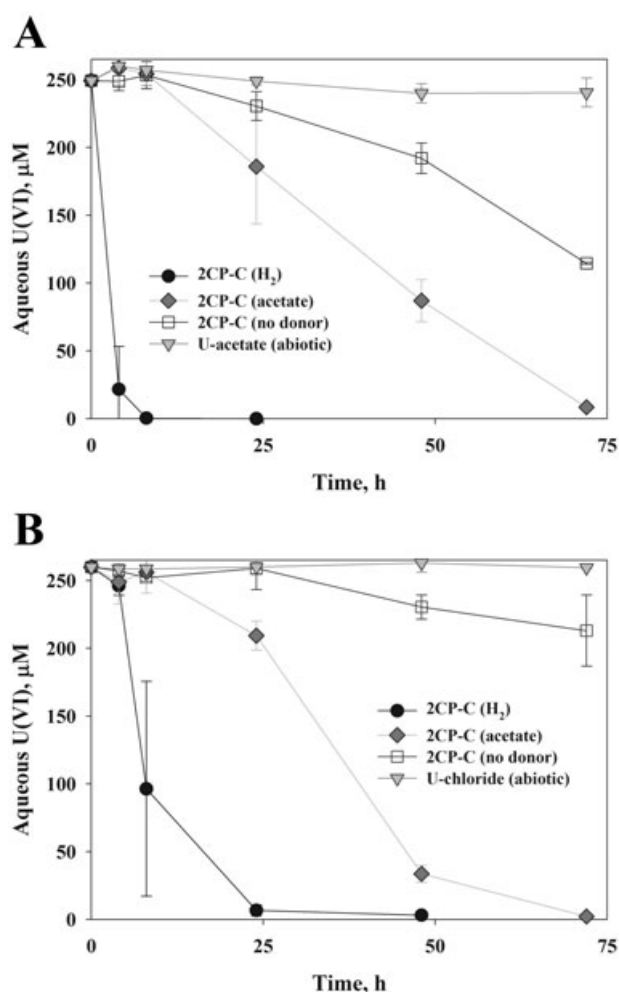

Fig. 1. Donor-specific reduction of U(VI) by *A. dehalogenans* 2CP-C cell suspensions. The assay tubes were amended with 250 μM U(VI) added as either uranyl acetate (A) or uranyl chloride (B) and received sodium acetate (10 mM) or H_2 (10 ml) as electron donors. Controls included tubes that received either no electron donor or no 2CP-C cells (abiotic). Results represent the mean of triplicates from a representative experiment with error bars indicating the standard deviation.

ans 2CP-C cells may vary between growing and non-growing conditions.

Additional experiments using U(VI) chloride corroborated the initial evidence that *A. dehalogenans* 2CP-C resting cells used either H_2 or acetate as the sole electron donor for U(VI) reduction (Fig. 1B). Cells supplied with no exogenous electron donor displayed only minimal U(VI) reduction at the conclusion of the assay. Faster U(VI) chloride reduction rates were sustained in resting cell suspensions amended with H_2 as compared with those provided acetate as the electron donor. The differences in rates for H_2- and acetate-driven U(VI) reduction are not unprecedented, and similar trends in electron donor-specific kinetics have been previously observed in *Shewanella* spp. cultures (Liu *et al.*, 2002). This difference
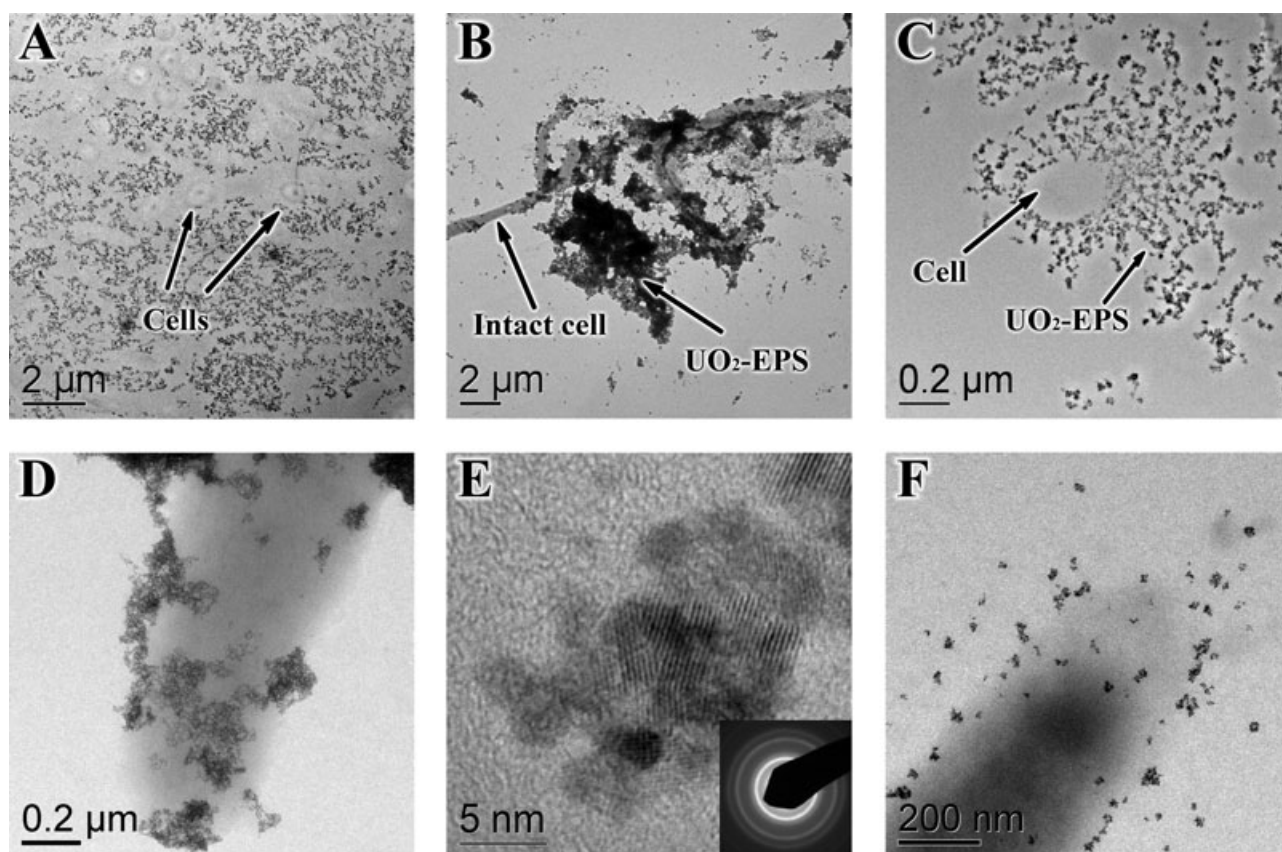
Fig. 2. Subcellular localization of uranium nanoparticles. Unstained ultra thin sections (A and C) or whole-mount (B and D) TEM micrographs of *A. dehalogenans* 2CP-C cells incubated with uranyl acetate with 10 ml H_2 for 24 h at 30°C. The localization of the $UO_{2(S)}$-EPS is shown in association with intact cells. High-resolution TEM of biogenic U(IV) and coinciding selected area electron diffraction (SAED) of $UO_{2(S)}$ nanoparticles produced by 2CP-C cells (E). Whole-mount TEM micrograph of 2CP-C cells incubated with uranyl acetate but without electron donor (F).

may be explained by the subcellular localization of H_2-oxidizing (i.e. periplasmic hydrogenase) versus acetate-oxidizing (i.e. cytoplasmic acetyltransferase or dehydrogenase) enzyme systems, or the difference in free energy ($\Delta G^{\circ\prime}$) changes associated with electron transfer from H_2 or acetate to U(VI), as was previously suggested (Wu *et al.*, 2006). Regardless of the electron donor supplied, either H_2 or acetate, U(VI) reduction was approaching completion by 72 h in resting cell experiments.

The subcellular localization and size of the U(IV) precipitates generated during the resting cell assays was studied in cells after 24-h incubation using transmission electron microscopy (TEM). Thin section TEM analysis of *A. dehalogenans* 2CP-C cells incubated in bicarbonate buffer amended with U(VI) acetate and H_2 revealed formation as loosely packed, unorganized aggregates of U nanoparticles (Fig. 2A and C). High-resolution whole-mount TEM and selected area electron diffraction (SAED) analysis demonstrated that the precipitates were uraninite ($UO_{2(S)}$) with crystallite size of ~5 nm (Fig. 2E), which formed irregular aggregates and entangled multiple *A. de-*

halogenans 2CP-C cells (Fig. 2B and D). A similar formation of extracellular $UO_{2(S)}$ precipitates has been observed in experiments with other U(VI) reducing organisms (Gorby and Lovley, 1992; Lovley and Phillips, 1992; Liu *et al.*, 2002; Marshall *et al.*, 2006). In contrast to previous studies with *Shewanella* spp. and *Geobacter* spp. (Liu *et al.*, 2002; Lloyd *et al.*, 2002; Marshall *et al.*, 2006; Shelobolina *et al.*, 2007), $UO_{2(S)}$ accumulation was not observed in the periplasm of *A. dehalogenans* 2CP-C cells (Fig. 2A and C). Although the biomolecular mechanism(s) of U(VI) reduction by *A. dehalogenans* 2CP-C cells are not fully understood, the lack of periplasmic $UO_{2(S)}$ particles may be linked to a number of complex factors including the rate of U(VI) reduction or the transport of uranyl into the periplasm. Alternatively, the absence of periplasmic $UO_{2(S)}$ particles may be linked to the distribution and reactivity of outer membrane (OM) bound *c*-type cytochromes, which have been shown to function as uranyl reductases in *S. oneidensis* MR-1 (Marshall *et al.*, 2006). *Anaeromyxobacter dehalogenans* 2CP-C cells incubated with uranyl acetate without exogenous electron donor also formed
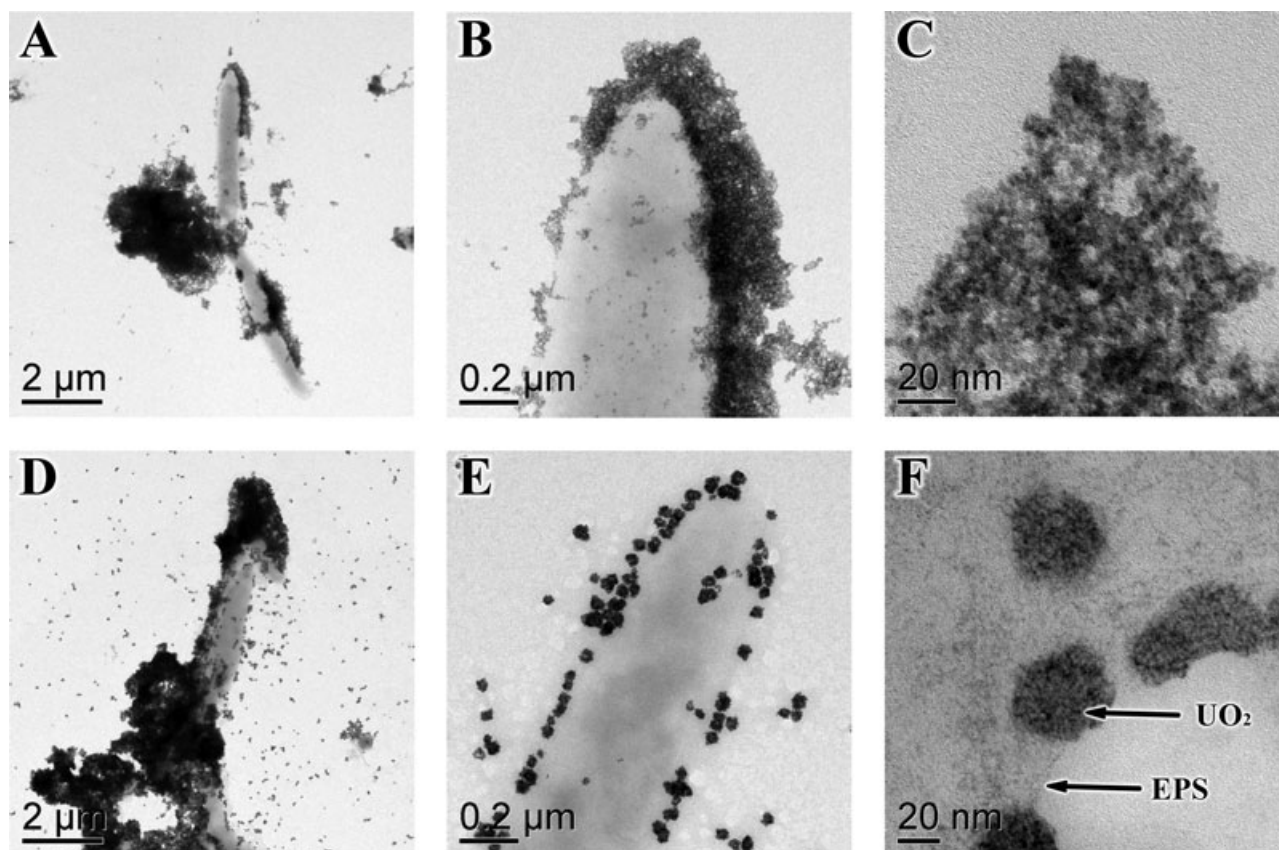
Fig. 3. The electron donor-specific localization of $UO_{2(S)}$ nanoparticles. Unstained whole-mount TEM micrographs of *A. dehalogenans* 2CP-C resting cells incubated for 24 h at 30°C with uranyl chloride with either 10 ml of H_2 (A–C) or 10 mM sodium acetate (D–F) as the exogenous electron donor. The association of EPS with large aggregates of $UO_{2(S)}$ nanoparticles is shown in (F).

some extracellular $UO_{2(S)}$ with the relative abundance of nanoparticles (Fig. 2F) being consistent with the amount of U(VI) reduced, when the acetate of the uranyl salt likely functioned as a fortuitous exogenous electron donor in the 'no donor' control series (Fig. 1A).

Extracellular uraninite was also formed in *A. dehalogenans* 2CP-C cell suspensions incubated in bicarbonate buffer amended with U(VI) chloride (Fig. 3). *Anaeromyxobacter dehalogenans* 2CP-C cells surrounded by $UO_{2(S)}$ aggregates, morphologically similar to those previously demonstrated in association with *S. oneidensis* MR-1 cells and extracellular polymeric substance (EPS) (Marshall *et al.*, 2006), were observed in suspensions amended with either H_2 (Fig. 3A) or acetate (Fig. 3D) as electron donor. However, this analysis also revealed donor-specific differences in the resulting distribution of nanoparticulate $UO_{2(S)}$. The H_2-driven U(VI) chloride reduction produced extracellular nanoparticles of $UO_{2(S)}$ (Fig. 3A–C) that were indistinguishable from those produced by cells reducing U(VI) acetate in the presence of H_2 (Fig. 2A–E). In contrast, the acetate-driven U(VI) chloride reduction revealed that extracellular nanoparticles of $UO_{2(S)}$ were distributed in a very different pattern, with

individual $UO_{2(S)}$ nanoparticles (~5 nm diameter) arranged in larger, spherical aggregates up to 50 nm in diameter (Fig. 3D–F) that were absent in the H_2 incubations. These aggregates localized predominantly in association with the cell OM but were also observed in regions distal to the cell. These observations provide further evidence that acetate serves as the source of reducing equivalents for U(VI) reduction and suggest that the $UO_{2(S)}$ nanoparticles occur in association with EPS. Although the origin of the donor-specific differences in the distribution of nanoparticulate $UO_{2(S)}$ by *A. dehalogenans* 2CP-C is currently unknown, a plausible explanation is that *A. dehalogenans* 2CP-C utilizes distinct, donor-specific terminal reductases for U(VI) reduction. We previously demonstrated that *S. oneidensis* MR-1 uses more than one *c*-type cytochrome for U(VI) reduction, although no donor-dependent differences in $UO_{2(S)}$ nanoparticle aggregate sizes were observed when reduction was coupled to the oxidation of an inorganic substrate (i.e. H_2) or in the presence of an organic substrate (i.e. lactate) (Marshall *et al.*, 2006; 2008). Although the biomolecular mechanisms of U(VI) reduction in *A. dehalogenans* have not been determined, it is plausible that electron donor-specific electron transfer
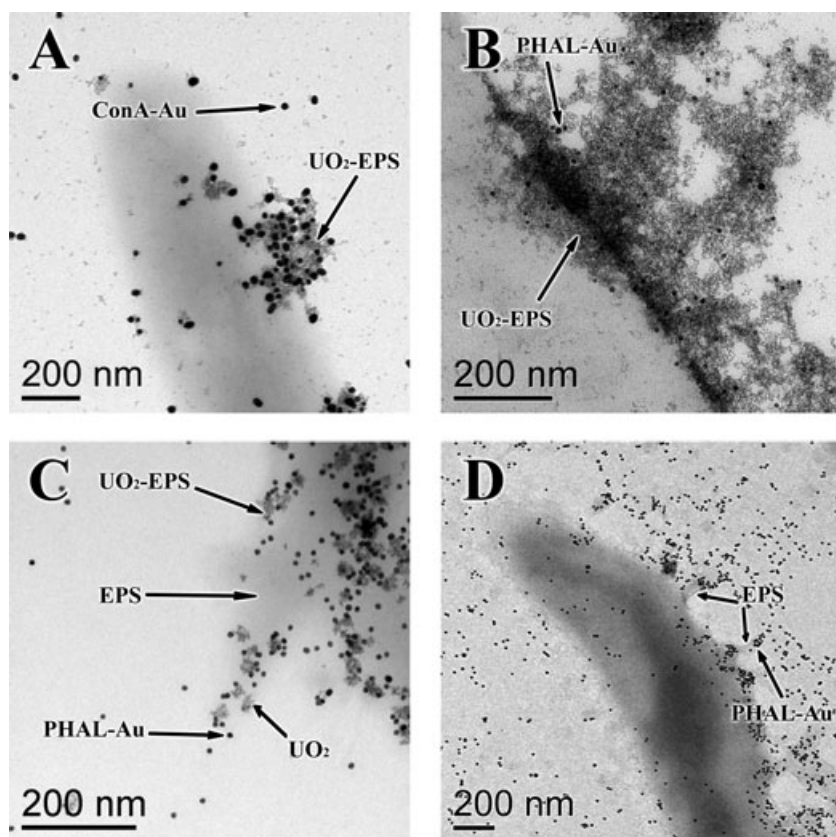
Fig. 4. Visualization of EPS and $UO_{2(S)}$-EPS with lectin stains. Unstained whole-mount TEM images of *A. dehalogenans* 2CP-C resting cells incubated with either 250 µM uranyl chloride (A–C) or 1 mM sodium fumarate (D) added as the electron acceptor and reacted with the glycoconjugate-specific lectins complexed with particles of colloidal gold (Au) for visualization. The exogenous electron donor provided was 10 ml H_2 (A,B) or 10 mM sodium acetate (C,D). The lectin Conconavalin A (ConA) was complexed with 40 nm particles of colloidal Au (A) while the *Phaseolus vulgaris* leucoagglutinin (PHAL) was complexed with 10 nm Au particles (B–D).

pathways could employ different terminal reductases, which, in turn, may influence the distribution and properties of $UO_{2(S)}$. While both *A. dehalogenans* 2CP-C and *S. oneidensis* MR-1 produce a cadre of multihaem *c*-type cytochromes with multiple (2–10) haem binding sites, the 2CP-C genome encodes several unique, high molecular weight *c*-type cytochromes predicted to contain more than 20 putative haem binding sites and associated with the OM. Studies are ongoing in our laboratory to determine the relationship between the *A. dehalogenans* 2CP-C *c*-type cytochromes and the formation of biogenic $UO_{2(S)}$.

Regardless of the source of reducing equivalents for U(VI) reduction, $UO_{2(S)}$ nanoparticles produced by *A. dehalogenans* 2CP-C were predominantly in an aggregated state. As U(VI) reduction progressed, a visible dark precipitate formed in the Balch tubes, presumably the aggregates visualized by EM (Fig. 2A or C), suggesting that these aggregates were not an artifact of the EM fixation/dehydration process. EPS-specific staining with two different gold-labelled lectins confirmed the association of the $UO_{2(S)}$ nanoparticles with EPS (Fig. 4A–C). When *A. dehalogenans* 2CP-C cells were incubated with fumarate as the exogenous electron acceptor, lectin-specific binding of EPS could also be observed (Fig. 4D). These observations were consistent with the previous observation of the EPS produced by *S. oneidensis* MR-1 possess-

ing a strong affinity towards lectin stains (Marshall *et al.*, 2006) and also suggest that the EPS produced by *A. dehalogenans* 2CP-C contains a carbohydrate component.

As previously suggested, association of $UO_{2(S)}$ with EPS could impact the environmental mobility of these particles as the nanometer-sized biogenic uraninite particles may be subject to colloidal transport in the subsurface (Suzuki *et al.*, 2002; Marshall *et al.*, 2006). Although colloidal transport of $UO_{2(S)}$ nanoparticles through porous medium has yet to be demonstrated, the interaction of biogenic $UO_{2(S)}$ produced by *A. dehalogenans* 2CP-C with EPS and/or the formation of larger, spherical aggregates could ultimately influence the environmental mobility and stability of $UO_{2(S)}$ nanoparticles and prevent their dispersion. While the concept of $UO_{2(S)}$ nanoparticle association with EPS is quite new, an early U(VI) bioreduction study demonstrated that fine-grained U(IV) precipitates produced by *Geobacter metallireducens* GS-15 ultimately resulted in the formation of large uraninite precipitates (i.e. > 0.2 µm diameter) (Gorby and Lovley, 1992). It is unknown whether the uraninite nanoparticles were associated with EPS or whether the aggregates were single- or polydomain. In the current study, the $UO_{2(S)}$ nanoparticles produced by *A. dehalogenans* 2CP-C (Figs 2 and 3) did not pass through 0.2 µm membrane filters. Retention of the ~5 nm nanoparticles by the 0.2 µm membrane filters
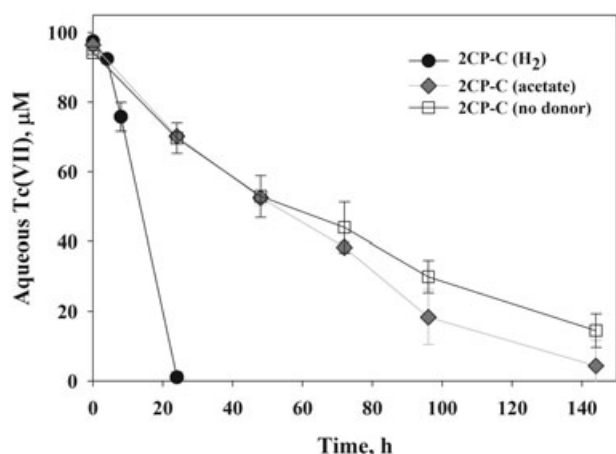
Fig. 5. Reduction of 100 μM Tc(VII) by *A. dehalogenans* 2CP-C cell suspensions. Assay tubes were supplemented with either 5 mM sodium acetate or 10 ml of H₂ as electron donor. One additional set of tubes received 2CP-C cells but no exogenous electron donor (no donor). Results represent the mean of triplicate assay tubes from a representative experiment with error bars indicating the standard deviation.

implies that the UO₂(S) particles are aggregated or bound to extended EPS. Currently, the nature and strength of the interactions between the nanoparticles and EPS are unknown and it is not possible to fully assess the effects on U environmental fate and transport. Hence, the ability of *A. dehalogenans* to produce EPS-associated UO₂(S) nanoparticles, the nature of such interactions and the biomolecular mechanisms contributing to the formation of large UO₂(S) aggregates warrant further exploration.

Electron donor-specific Tc(VII) reduction

In addition to U(VI), *A. dehalogenans* 2CP-C resting cell suspensions reduced Tc(VII)O_4^- to Tc(IV) (Figs 5 and 6). When H₂ was supplied as the sole electron donor, 2CP-C cells rapidly reduced 100 μM Tc(VII)O_4^- ($< 1.1 \times 10^{-6}$ mol 1^{-1}) within 24 h. Cell suspensions amended with acetate as the electron donor reduced Tc(VII) considerably slower than suspensions amended with H₂ requiring 144 h for nearly complete reduction. However, we cannot conclude that the observed Tc(VII) reduction was coupled with acetate oxidation as *A. dehalogenans* 2CP-C cells not supplied with an exogenous source of reducing equiva-

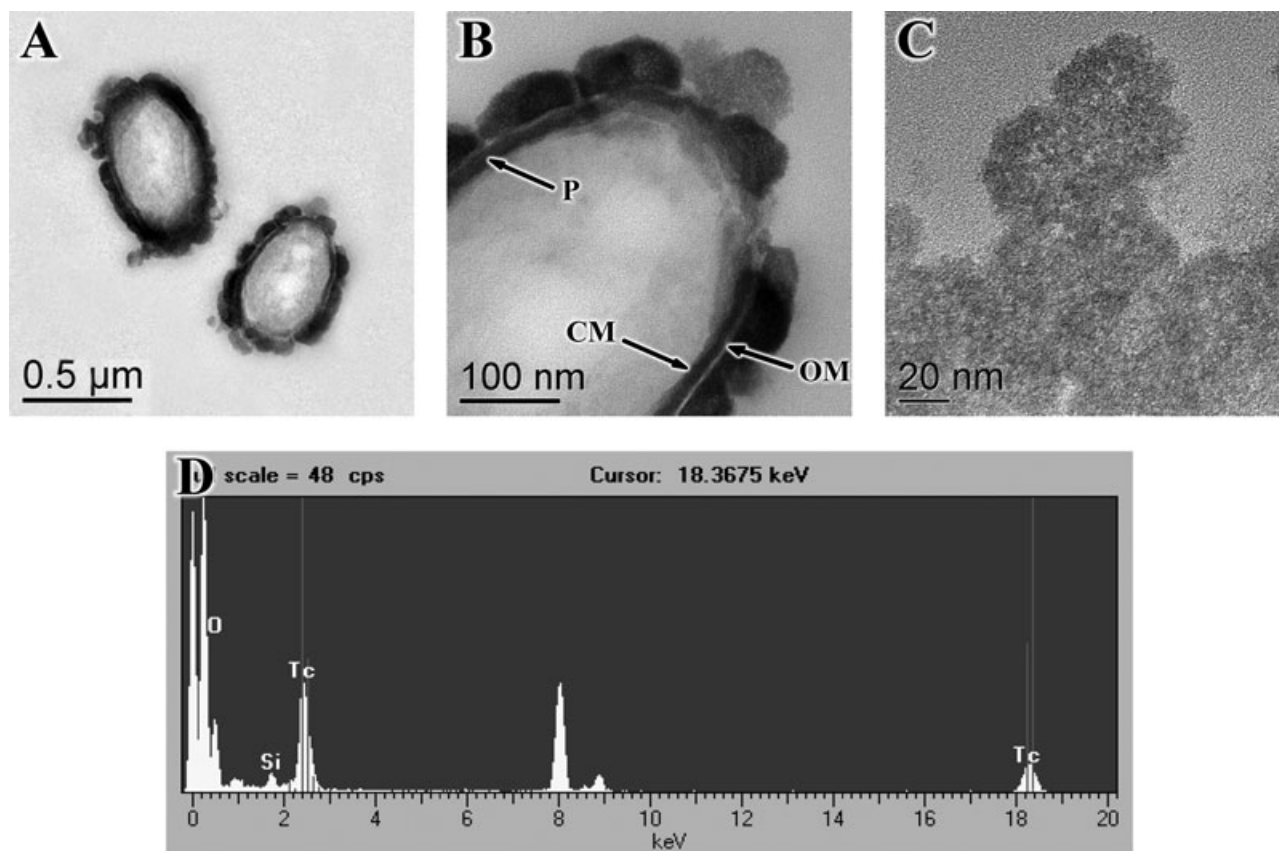**Fig. 6.** Subcellular localization of Tc(IV)O₂ nanoparticles. Unstained thin section TEM micrographs of *A. dehalogenans* 2CP-C cells incubated with Tc(VII)O_4^- and H₂ (A,B) for 24 h at 30°C. High-resolution TEM (C) of biogenic Tc(IV) and coinciding electron dispersive spectrometry (D) of TcO₂ nanoparticles produced by 2CP-C cells. Arrows indicate the cell membrane (CM), periplasm (P) and outer membrane (OM).

lents reduced similar amounts of Tc(VII) during the 144 h assay. The finding that *A. dehalogenans* 2CP-C cells couple Tc(VII) reduction with H_2, but not acetate oxidation agrees with previous reports suggesting that organisms such as *Geobacter sulfurreducens* and *Desulfovibrio fructosovorans* only couple Tc(VII) reduction with H_2 oxidation (Lloyd *et al.*, 2000; De Luca *et al.*, 2001). We cannot unequivocally elucidate the nature of the H_2-independent Tc(VII) reduction observed in the resting cell assays without H_2 amendments because residual endogenous electron carriers may serve as a pool of reducing equivalents supporting Tc(VII) reduction. We recently demonstrated that *S. oneidensis* MR-1 cells lacking functional hydrogenases reduced Tc(VII) by a cytochrome-mediated pathway (Marshall *et al.*, 2008). A similar process may be responsible for the observed H_2-independent reduction of Tc(VII) in *A. dehalogenans* 2CP-C. While our findings suggest that the primary mechanism for direct Tc(VII) reduction in *A. dehalogenans* 2CP-C involves H_2 and a hydrogenase mediated pathway, mutagenesis studies will be needed to evaluate the biomolecular mechanisms of Tc(VII) reduction.

Microscopic analysis of *A. dehalogenans* 2CP-C resting cells suspensions collected after Tc(VII) reduction with H_2 as electron donor revealed electron dense Tc(IV) particles consistent with $TcO_2 \cdot nH_2O$ (TcO_2) formation (Fig. 6). Dense clusters of TcO_2 particles were localized within the cell periplasm and on the outside of the OM (Fig. 6A and B). These findings are consistent with previous reports of TcO_2 nanoparticles localized within the periplasm and at the cell periphery of other organisms (Lloyd *et al.*, 1999; 2000; Wildung *et al.*, 2000; Liu *et al.*, 2002; Marshall *et al.*, 2008). The Tc(IV) precipitate clusters at the surface of the *A. dehalogenans* 2CP-C cells were greater than 50 nm in diameter and contained individual TcO_2 nanoparticles ~2 nm in diameter (Fig. 6C). Like biogenic $UO_{2(S)}$, the TcO_2 nanoparticles were retained by 0.2 μm membrane filters, suggesting strong aggregation and/or association with polymeric biomolecules such as EPS.

Biogenic Fe(II)-facilitated Tc(VII) reduction

To explore if *A. dehalogenans* 2CP-C could reduce Tc(VII) via an indirect biogenic, Fe(II)-mediated mechanism, a previously characterized Fe(III)- and Mn(III/IV)-oxide-containing sediment from the upper Ringold Formation, where many of the Hanford Site ^{99}Tc(VII) plumes reside, was used. After 28 days of incubation with the Ringold sediment amended with H_2 as electron donor, *A. dehalogenans* 2CP-C resting cells had produced ~145 μM HCl extractable Fe(II). This sediment contains significant quantities (235 μmol g^{-1} by XRF) of Mn(III/IV) oxides that must be reduced prior to the onset of Fe(III) reduction (Fredrickson *et al.*, 2004). After pasteurization of the
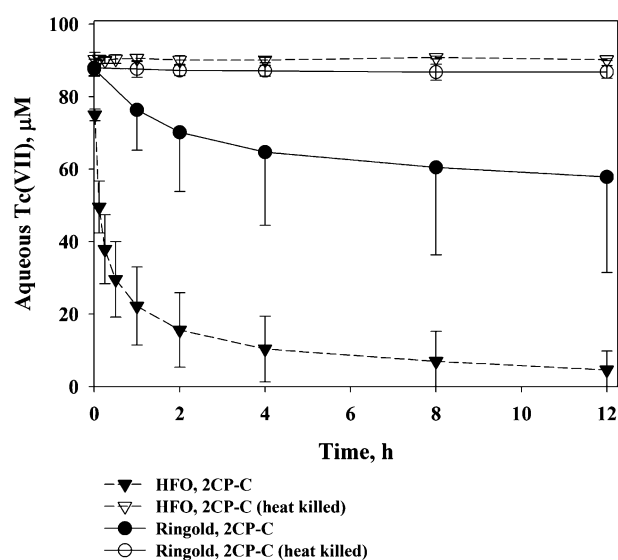

Fig. 7. The effect of biogenic Fe(II) on the solubility of Tc(VII)O_4^-. Resting cells of *A. dehalogenans* 2CP-C cells were used to reduce Fe(III) associated with Ringold sediment (1 g/10 ml) or HFO (15 mM, nominal concentration) using 10 ml of H_2 as electron donor. After pasteurization, a Tc(VII)O_4^- stock was added to the assay tubes. Additional sets of assay tubes received heat killed (autoclaved) 2CP-C cells. Results represent the mean of quadruplicate assay tubes from a representative experiment with error bars indicating the standard deviation. Only the minus error bars were included for the bioreduced Ringold sediment to increase readability.

assay tubes and the addition of Tc(VII)O_4^-, ~30% of the Tc(VII) was removed from solution in the assay tubes within 12 h (Fig. 7), consistent with the reaction of approximately 62% of the biogenic Fe(II) with Tc(VII). In contrast, the addition of heat-killed *A. dehalogenans* 2CP-C cells to the Ringold sediment failed to produce detectable levels of HCl extractable Fe(II) or exhibit Fe(II)-mediated Tc(VII) reduction (Fig. 7).

Another experiment was performed to evaluate the reactivity of biogenic Fe(II) generated by *A. dehalogenans* 2CP-C. To avoid ancillary effects from co-associated mineral components in the Ringold sediment, synthetic silica-hydrous ferric oxide (HFO; ferrihydrite) (Kukkadapu *et al.*, 2004) was used to evaluate the reactivity of biogenic Fe(II) generated by *A. dehalogenans* 2CP-C. In *A. dehalogenans* 2CP-C resting cell suspensions amended with HFO and H_2, the formation of magnetite was implied by the ferrimagnetic behaviour and Fe(II)/(III) ratio of the transformed solids [~2.1 mM HCl extractable Fe(II)]. Consistent with previous findings (Lloyd *et al.*, 2000), the biogenic magnetite was very effective at reducing Tc(VII)O_4^- as > 80% of the initial Tc(VII) added was reduced within 4 h (Fig. 7).

The bioreduced ferrihydrite reacted much more rapidly with the Tc(VII)O_4^- than the reduced Ringold sediment. It has been previously shown that the rate of Tc(VII) reduc-

tion by either sediment or synthetic Fe(II) and the subsequent formation of Fe/Tc(IV) precipitates is related to the concentration of HCl-extractable Fe(II) and its molecular speciation (Fredrickson *et al.*, 2004; Zachara *et al.*, 2007; Peretyazhko *et al.*, 2008a,b); such factors were clearly at play in these experiments. Given that *A. dehalogenans* 2CP-C efficiently reduces Tc(VII) indirectly through an Fe(II)-mediated mechanism, it is possible that Tc(VII) reduction through an indirect, Fe(II)-mediated mechanism will predominate in sedimentary environments containing bioreducible Fe(III) and where H_2 concentrations are low relative to organic carbon. In contrast, in environments where H_2 concentrations are elevated as a result of organic matter fermentation and/or mineral weathering, the dominant Tc(VII) reduction pathway may be enzymatic (i.e. direct) or possibly a more complex, mixed pathway.

Environmental implications

Recent findings suggest that members of the *Anaeromyxobacter* genus contribute to metal reduction in contaminated groundwater and sediments at the Oak Ridge FRC site (Petrie *et al.*, 2003; North *et al.*, 2004). Our studies indicate that members of the *Anaeromyxobacter* genus have physiological properties that allow them to affect the mobility of metal contaminants. Understanding the biomolecular basis for radionuclide reduction by *A. dehalogenans* is relevant for predicting and managing *in situ* reduction reactions leading to the immobilization of soluble radionuclide contaminants as poorly soluble metal oxide phases. In subsurface sediments where electron donor concentrations fluctuate and are often limiting biologically driven redox reactions, the ability of *A. dehalogenans* 2CP-C to couple oxidation of either organic electron donors or H_2 with radionuclide reduction provides a distinct advantage. Similarly, the association of $UO_{2(S)}$ or $TcO_2 \cdot nH_2O$ nanoparticles with the bacterial cells and EPS may limit particle dispersion in subsurface waters, depending upon the nature (i.e. stability) of such interactions. Future investigations will have to consider the biomolecular mechanisms contributing to metal reduction by *Anaeromyxobacter* spp. and the physiochemical properties of the nanoparticulate end-products and their long-term behaviour in porous media. While the precise biomolecular mechanisms used by *A. dehalogenans* 2CP-C to reduce U and Tc remain poorly understood, our data suggest that, similar to other metal-reducing bacteria such as *Geobacter* and *Shewanella*, *Anaeromyxobacter* utilizes OM *c*-type cytochromes and/or hydrogenases to facilitate radionuclide reduction. Because of their widespread distribution and versatility, members of the *Anaeromyxobacter* genus may play relevant roles in the biogeochemical transformation of contaminants in the subsurface.

Experimental procedures

Chemicals, materials and medium

All chemicals used in this study were purchased from Sigma Chemical (St Louis, MO) unless specified otherwise. Materials used for electron microscopy studies were purchased from Electron Microscopy Sciences (Fort Washington, PA). R2A medium was purchased from BD Diagnostics (Sparks, MD).

U(VI) reduction assay conditions

The resting-cell U(VI) reduction assays were performed using modifications of a previously described protocol (Marshall *et al.*, 2006). Late-log phase *A. dehalogenans* 2CP-C (ATTC BAA-259) cells grown at 37°C in R2A medium amended with 10 mM sodium fumarate were harvested by centrifugation, washed in 30 mM sodium bicarbonate buffer (pH 7.0, 4°C), and suspended in fresh buffer to a titre of 2×10^9 cells ml^{-1}. Sodium acetate (10 mM) or H_2 (10 ml, ~410 µmol) was used as the electron donor for all experiments. Anoxic 25 ml assay tubes, purged with $N_2:CO_2$ gas mixture (80:20, v/v), contained 250 µM U(VI) added as either uranyl acetate or uranyl chloride in 30 mM sodium bicarbonate buffer (pH 7.0). Assays were initiated by adding 1 ml of cell suspension to 9 ml of assay mix, resulting in a final titre of 2×10^8 cells ml^{-1} and were incubated in a horizontal position at 30°C with slow gyratory shaking (25 r.p.m.). At multiple time points, samples were collected inside an anoxic chamber (COY Laboratory Products, Grass Lake, MI) and analysed for soluble U(VI) remaining in filtrates (< 0.2 µm pore size) using a kinetic phosphorescence analyser (KPA-10; Chemchek Instruments, Richland, WA), as previously described (Brina and Miller, 1992).

Direct and Fe(II)-facilitated Tc(VII) reduction

All Tc(VII) reduction assays were performed using resting cell suspensions prepared similarly to U(VI) experiments except that cells were washed and suspended in 30 mM piperazine-N,N′-bis(ethanesulfonic acid) (PIPES) buffer (pH 7.0, 4°C). Anoxic assay tubes, purged with N_2, contained 100 µM ammonium pertechnetate ($NH_4{}^{99}Tc(VII)O_4$) (Perkin-Elmer, Boston, MA), 30 mM PIPES buffer, either 5 mM sodium acetate or 10 ml of H_2 as electron donor and were incubated at 30°C. Kinetic studies were initiated by adding 1 ml of washed cell suspensions to each assay tube followed by horizontal incubation at 30°C with gyratory shaking (50 r.p.m.). At multiple time points, samples were collected in an anoxic atmosphere and the concentrations of aqueous $^{99}Tc(VII)$ remaining in filtrates (< 0.2-µm pore size) were directly measured by liquid scintillation counting of ^{99}Tc (0.292 MeV beta).

The reduction of $Tc(VII)O_4{}^-$ by biogenic Fe(II) was determined using two sources of Fe(III) oxide: a synthetic silica-ferrihydrite (Si-HFO, 2% Si) (Kukkadapu *et al.*, 2004) and a previously characterized, Fe(III) oxide-containing sediment from the Hanford site, referred to as Ringold 6-6/6-7 (Fredrickson *et al.*, 2004). Assay tubes contained 0.1 g ml^{-1} Ringold 6-6/6-7 sediment or 15 mM (nominal concentration)

hydrous ferric oxide (both prepared in 9 ml of 30 mM PIPES buffer) and slightly over-pressurized with 10 ml of H_2 as electron donor under a N_2 headspace. The initial Fe(III) reduction was performed by adding 1 ml of N_2 purged-*A. dehalogenans* 2CP-C resting cells in PIPES buffer to assay tubes followed by horizontal incubation at 30°C with gyratory shaking (25 r.p.m.). Additional Ringold 6-6/6-7 or HFO tubes received heat killed (autoclaved) 2CP-C resting cells. After 28 days of incubation, representative tubes were extracted overnight with 0.5 M HCl followed by determining the acid-extractable Fe(II) concentration at 562 nm using the ferrozine assay (Stookey, 1970). The remaining tubes were then pasteurized for 1 h at 80°C and frozen at −20°C until use. The reduction of Tc(VII) by biogenic Fe(II) was accomplished by the addition of Tc(VII)O_4^- from a concentrated stock to all assay tubes. Tc(VII) was quantified at experiment initiation and the tubes were incubated at ambient temperature (~25°C) with gentile agitation. Aqueous Tc(VII) concentration remaining in filtrates was determined periodically as described above.

Transmission electron microscopy

Three millilitres of resting cell suspensions incubated for 24 h with U(VI) or Tc(VII) were centrifuged [2300 *g*, 5 min, ambient temperature (~25°C)] and the cell pellets were fixed, embedded and sectioned using strictly anoxic techniques prior to visualization of unstained sections at 200 kV using a JEOL 2010 high-resolution transmission electron microscope as previously described (Marshall *et al.*, 2006). Unstained whole mounts were prepared from U(VI) reduction assays by directly adding 20 μl of reduction assays to 200 mesh copper grids with carbon-coated formvar support film prior to air drying. To evaluate the extracellular carbohydrate composition during resting cell assays, two gold bead-conjugated lectin stains (Conconavalin A or *Phaseolus vulgaris* leucoagglutinin, EY Laboratories, San Mateo, CA) were added to whole mounts after 24 h incubations with 250 μM U(VI) chloride or 1 mM sodium fumarate as previously described (Marshall *et al.*, 2006). The elemental compositions of precipitates were determined using electron dispersive spectroscopy (Oxford Instruments, Fremont, CA) equipped with a SiLi detector and analysed with ISIS software (Oxford Instruments). Images and SAED patterns were digitally collected and analysed using DigitalMicrograph software (Gatan, Pleasanton, CA).

Acknowledgements

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