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Enzymology of Electron Transport: Energy Generation With Geochemical Consequences

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INTRODUCTION

Dissimilatory metal-reducing bacteria (DMRB) are important components of the microbial community residing in redox-stratified freshwater and marine environments. DMRB occupy a central position in the biogeochemical cycles of metals, metalloids and radionuclides, and serve as catalysts for a variety of other environmentally important processes including biomineralization, biocorrosion, bioremediation and mediators of ground water quality. DMRB are presented, however, with a unique physiological challenge: they are required to respire anaerobically on terminal electron acceptors which are either highly insoluble (e.g., Fe(III)- and Mn(IV)-oxides) and reduced to soluble end-products or highly soluble (e.g., U(VI) and Tc(VII)) and reduced to insoluble end-products. To overcome physiological problems associated with metal and radionuclide solubility, DMRB are postulated to employ a variety of novel respiratory strategies not found in other gram-negative bacteria which respire on soluble electron acceptors such as O_2, NO_3^-, SO_4^{2-}, and CO_2. The novel respiratory strategies include 1) direct enzymatic reduction at the outer membrane, 2) electron shuttling pathways and 3) metal solubilization by exogenous or bacterially-produced organic ligands followed by reduction of soluble organic-metal compounds. The first section of this chapter highlights the latest findings on the enzymatic mechanisms of metal and radionuclide reduction by two of the most extensively studied DMRB (*Geobacter* and *Shewanella*), with particular emphasis on electron transport chain enzymology. These advances have drawn significantly upon genomic data for isolated microorganisms from the genera *Geobacter* and *Shewanella* (see chapter by Nelson and Methé 2005). The second section emphasizes the geochemical consequences of DMRB activity, including the direct and indirect effects on metal solubility, the reductive transformation of Fe- and Mn-containing minerals, and the biogeochemical cycling of metals at redox interfaces in chemically stratified environments.

ENZYMATIC BASIS OF IRON AND MANGANESE REDUCTION

The electron transport systems of gram-negative bacteria are generally described as inner membrane (IM)-associated electron and proton carriers that 1) mediate electron transfer from

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primary donor to terminal electron acceptor and 2) conserve energy released during electron transfer to the generation of ATP (Madigan and Martinko 2006). Figure 1 displays the electron transport chain enzymology of *Escherichia coli* respiring high concentrations of dissolved O_2 as electron acceptor. The *E. coli* electron transport system is modular in design with a membrane-soluble quinone pool (Q) linking dehydrogenase complexes at the head end with terminal reductase complexes at the terminus. Dehydrogenase complexes include electron donor-specific oxido-reductases (e.g., NADH dehydrogenase) that couple oxidation of specific electron donors to reduction of a series of membrane-associated electron carriers arranged in order of increasingly more positive electric potential (E_0'). These electron carriers include flavoproteins (Fp) and FeS proteins that translocate protons across the IM to the periplasm and direct electrons to the Q pool, respectively. Reduced Q is subsequently protonated to QH_2 at the inner aspect of the IM. QH_2 carries protons across the IM to the periplasm and transfers electrons to cytochrome b_{556} and b_{562}, two components of the terminal reductase complex that transfer electrons to cytochrome *o* (topologically located at the inner aspect of the IM) and ultimately to O_2. Cytochrome *o* catalyzes both the translocation of a proton across the IM to the periplasm and the terminal reduction of O_2 to H_2O. A proton motive force (PMF) is generated by 1) proton translocation across the IM by dehydrogenase complexes, QH_2 and cytochrome *o*, and 2) proton consumption during the terminal reduction of O_2 to H_2O by cytochrome *o*. PMF generated in this manner drives ATP synthesis as protons are translocated back into the cytoplasm through an IM-localized ATPase, catalyzing the phosporylation of ADP to ATP (Madigan and Martinko 2006).

Fe(III)- and Mn(IV)-respiring DMRB, on the other hand, are presented with a unique physiological problem: they are required to respire anaerobically on terminal electron acceptors found largely in crystalline form or as amorphous (oxy)hydroxide particles presumably unable to contact IM-localized electron transport systems. A DMRB culture actively respiring solid Mn(IV) oxides as anaerobic electron acceptor is displayed in Figure 2. To overcome the problem of respiring solid electron acceptors, Fe(III)- and Mn(IV)-respiring DMRB are postulated to employ a variety of novel respiratory strategies not found in other gram-negative bacteria that respire on soluble electron acceptors such as O_2, NO_3^-, SO_4^{2-} and CO_2 including 1) direct enzymatic reduction of solid Fe(III) and Mn(IV) oxides via outer membrane (OM)-localized metal reductases (Myers and Myers 1992, 2003a; Beliaev and Saffarini 1998; DiChristina et al. 2002) 2) a two-step, electron shuttling pathway in which exogenous electron shuttling compounds (e.g., humic acids, melanin, phenazines, antibiotics, AQDS) are first enzymatically reduced and subsequently chemically oxidized by the solid Fe(III) and Mn(IV) oxides in a second (abiotic) electron transfer reaction (Lovley et al. 1996;

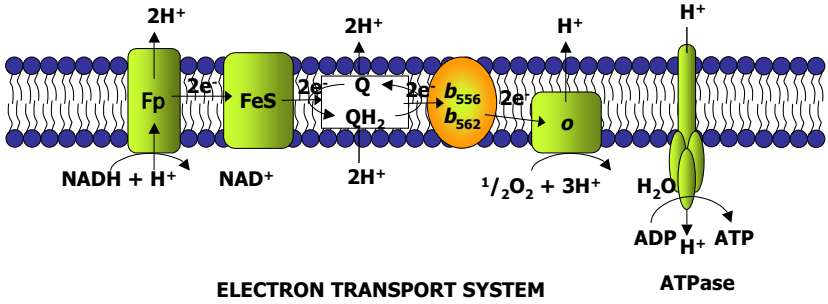

Figure 1. Electron transport and proton translocation processes of the *E. coli* aerobic respiratory chain at high O_2 concentrations with $NADH_2$ as electron donor. Fp, flavoprotein; FeS, iron-sulfur protein; Q, quinone pool; b_{556} and b_{562}, *b*-type cytochromes; *o*, cytochrome *o*.

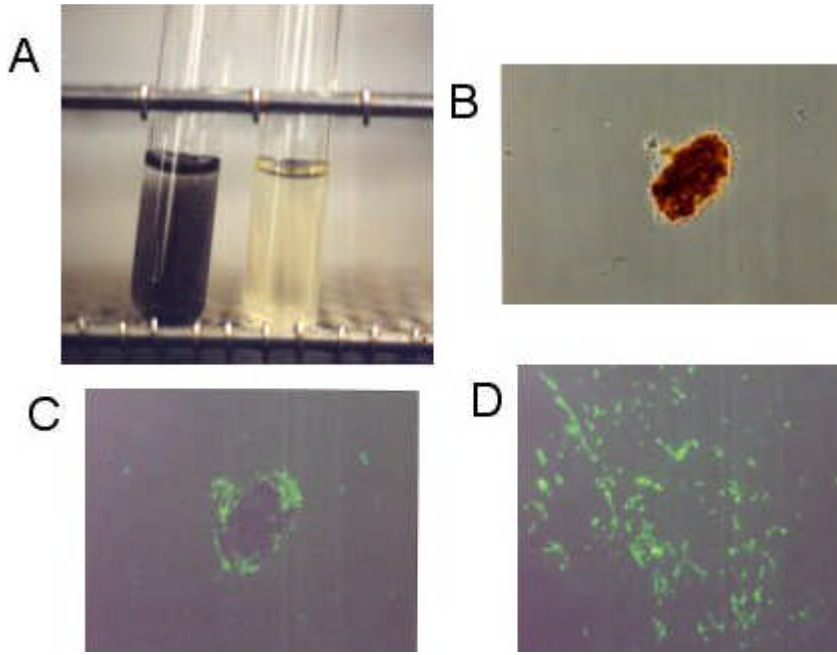

Figure 2. DMRB *Shewanella putrefaciens* strain 200 actively respiring solid Mn(IV) oxides as anaerobic electron acceptor. (A) Anaerobic cell suspensions at the beginning (tube on left side) and end (tube on right side) of a 24-hour anaerobic incubation period (note color change indicative of reductive dissolution of black Mn(IV) particles to clear, soluble reduced Mn), (B) phase contrast micrograph of cells coating the surface of a Mn(IV) oxide particle at the beginning of the anaerobic growth period, (C) epifluorescence micrograph of same field of view as in (B) with acridine orange-stained cells, (D) epifluorescence micrograph of acridine orange-stained cells at the end of the 24-hour anaerobic incubation period (note the absence of the solid Mn(IV) particles).

Coates et al. 1998, 2002; Newman and Kolter 2000; Turick et al. 2002; Hernandez et al. 2004; DiChristina et al. 2005) 3) an analogous two-step reduction pathway involving endogenous, electron shuttling compounds (Newman and Kolter 2000; Saffarini et al. 2002) and 4) a two-step, Fe(III) solubilization-reduction pathway in which solid Fe(III) oxides are first dissolved by exogenous or bacterially-produced organic complexing ligands, followed by uptake and reduction of the soluble organic Fe(III) forms by periplasmic Fe(III) reductases (Arnold et al. 1988; Lovley and Woodward 1996; Pitts et al. 2003). Although the number of DMRB species continues to increase rapidly and has now reached nearly 100 (Lovley et al. 2004), the enzymatic basis of electron transfer to metals has been most extensively studied in metal-respiring members of the genera *Geobacter* and *Shewanella*. The following section highlights the latest findings on the enzymatic basis of Fe(III) and Mn(IV) reduction by *Geobacter* and *Shewanella*, with particular emphasis on electron transport chain enzymology.

Direct enzymatic reduction at the outer membrane

Shewanella and *Geobacter* catalyze the direct enzymatic reduction of solid Fe(III) and Mn(III,IV) oxides via an electron transport chain arranged in a canonical, highly branched fashion. Hydrogenase and flavin-containing dehydrogenase complexes of both *Shewanella* and *Geobacter* (Myers and Myers 1993a; Lloyd et al. 2000) oxidize a variety of electron donors (e.g., H_2, NAD(P)H) and transfer electrons to a menaquinone pool (Myers and Nealson 1990; Myers and Myers 1993b; Nevin and Lovley 2002; Saffarini et al. 2002). In *S. oneidensis*,

menaquinol diffuses within the IM to the quinol oxidation site of CymA, a 21 kDa tetraheme cytochrome *c* that oxidizes menaquinol and is thought to transfer electrons to MtrA, a 32 kDa decaheme cytochrome *c* located in the periplasm (Myers and Myers 2000; Schwalb et al. 2003). In *G. sulfurreducens*, PpcB (a 36 kDa diheme cytochrome *c*) essentially carries out the same function as CymA (although CymA and PpcB display little or no amino acid sequence homology), oxidizing the menaquinol pool and transferring electrons to PpcA, a 10 kDa triheme cytochrome *c* located in the *G. sulfurreducens* periplasm (Lloyd 2003). As with PpcB, G. *sulfurreducens* PpcA does not display significant sequence similarity to any *S. oneidensis* *c*-type cytochromes, suggesting that they have a different evolutionary origin (Lloyd 2003). Electrons from the *S. oneidensis* menaquinol pool are transferred to one of four electron-accepting, c-type hemes within CymA, followed by inter-heme electron transfer (according to decreasing heme redox potential) until a final transfer is made to subsequent electron carriers in the periplasmic space (Harada et al. 2002). *cymA*-deficient mutants of *S. oneidensis* are unable to reduce NO_3^-, Fe(III), Mn(IV) or fumarate as electron acceptor (Myers and Myers 1997), an indication that CymA is a central branchpoint of the *S. oneidensis* electron transport system. *ppcB*-deficient mutants of G. *sulfurreducens*, on the other hand, are unable to reduce Fe(III), but retain the ability to reduce fumarate (Butler 2003). The principles of, and rationale for genetic manipulation (including generation of metal respiration-deficient mutants) is discussed in the chapter by Newman and Gralnick (2005).

Electron transport from MtrA in *S. oneidensis* and PpcA in G. *sulfurreducens* to solid Fe(III) oxides is postulated to proceed via an electron transport chain that spans the periplasmic space and terminates on the outside face of the OM (Myers and Myers 1993a; Leang et al. 2003). Electron transfer to solid Fe(III) and Mn(IV) in *G. sulfurreducens* proceeds via OmcB, an 87 kDa, 12-heme cytochrome *c* tentatively assigned to the inner aspect of the OM (Leang et al. 2003). Correspondingly, Fe(III)-grown *G. sulfurreducens* cells display higher levels of *omcB* transcripts (Chin et al. 2004; Methé et al. 2005). All *G. sulfurreducens* OM cytochromes, however, are not necessarily involved in electron transport to Fe(III) since OmcC, an OM cytochrome displaying 73% identity to OmcB, is not required for Fe(III) reduction (Leang et al. 2003; Leang and Lovley 2005) and correspondingly, Fe(III)-grown G. *sulfurreducens* cells do not display higher levels of *omcC* transcripts (Chin et al. 2004). The terminal electron transfer step to solid Fe(III) and Mn(IV) oxides in G. *sulfurreducens* is postulated to be catalyzed by either OmcD or OmcE, two *c*-type cytochromes that may be exposed on the cell surface (Methé et al. 2005). Results of DNA microarray analysis (as described in the chapter by Nelson and Methé 2005) will identify electron transport chain components with elevated transcript levels during growth on specific electron acceptors.

Similar to *Geobacter*, the *Shewanella* OM proteins involved in terminal steps of electron transfer to solid Fe(III) and Mn(IV) oxides have not been definitively identified, yet most likely include several *c*-type cytochromes (Myers and Myers 1992, 2003a). Fe(III) reduction activity is detected in wild-type *Shewanella* OM fractions (Myers and Myers 1993a), an activity that is severely impaired in *Shewanella* mutants lacking OM proteins, including several multi-heme *c*-type cytochromes. The *S. oneidensis* genome encodes 42 predicted *c*-type cytochromes (Heidelberg et al. 2002), including those in the *mtrDEF-omcA-mtrCAB* gene cluster. MtrA and MtrD are decaheme *c*-type cytochromes that display 99% similarity (Pitts et al. 2003), suggesting they may provide complementary function. MtrD is OM-associated, but may be oriented toward the periplasm (Pitts et al. 2003) and therefore not in position to contact solid Fe(III) directly. MtrB is a putative beta-barrel protein postulated to be involved in OM localization of the *c*-type cytochromes OmcA and MtrC that are involved in electron transfer to Fe(III) and Mn(IV) (Beliaev and Saffarini 1998; Myers and Myers 2002). *mtrB* mutants display a complete inability to reduce Mn(IV) and are severely, but not completely, impaired in Fe(III) reduction activity, yet retain the ability to reduce all other electron acceptors (Beliaev

and Saffarini 1998). MtrC is an OM-localized decaheme, *c*-type cytochrome required for both Fe(III) and Mn(IV) reduction activity. OmcA, on the other hand, is an OM decaheme *c*-type cytochrome involved in electron transport to Mn(IV) and not Fe(III) (Myers and Myers 2001). *omcA*-deficient mutants reduce Mn(IV) at 45% wild-type rates. Interestingly, *mtrC* overexpression in an *omcA*-deficient mutant restores Mn(IV) reduction activity to greater than wild-type rates, an indication that the functional roles of MtrC and OmcA at least partially overlap in the electron transport pathway to Mn(IV) (Myers and Myers 2003b). The functions of MtrC and OmcA in Fe(III) reduction remain unclear, yet they are postulated to be major components of the Fe(III) terminal reductase.

Some of the most convincing genetic evidence supporting the hypothesis that *Shewanella* localizes Fe(III) and Mn(IV) reductases to the OM has been derived from genetic studies with *S. putrefaciens* (DiChristina and DeLong 1994; DiChristina et al. 2002). Genetic mutant complementation analyses (as outlined in chapter by Newman and Gralnick 2005) indicated that a 23.3 kb *S. putrefaciens* wild-type DNA fragment conferred Fe(III) reduction activity to a set of 10 Fe(III) reduction-deficient mutants of *S. putrefaciens*. The smallest complementing DNA fragment contained one open reading frame (ORF) whose translated product displayed 87% sequence similarity to *Aeromonas hydrophila* ExeE, a member of the GspE family of proteins found in Type II protein secretion systems. GspE insertional mutants (constructed by targeted replacement of wild-type *gspE* with an insertionally inactivated *gspE* construct) are unable to respire anaerobically on solid Fe(III) or Mn(IV) oxides, yet retain the ability to respire all other electron acceptors including soluble complexes of Fe(III) and Mn(III) (Kostka et al. 1995; Pitts et al. 2003). Nucleotide sequence analysis of regions flanking *gspE* revealed one partial and two complete ORFs whose translated products displayed 55-70% sequence similarity to the GspD-G homologs of other Type II protein secretion systems. A heme-containing protein complex displaying Fe(III) reductase activity is present in the peripheral proteins loosely attached to the outside face of the wild-type OM, yet is missing from this location in the *gspE* mutants. Membrane fractionation studies with the wild-type strain support this finding: the heme-containing Fe(III) reductase complex is detected in the OM but not the IM or cytoplasmic fractions. These findings provide the first genetic evidence linking anaerobic Fe(III) and Mn(IV) respiration to Type II protein secretion and provide additional biochemical evidence supporting OM localization of *Shewanella* Fe(III) and Mn(IV) reductases (DiChristina and DeLong 1994; DiChristina et al. 2002).

Gram-negative bacteria secrete soluble exoproteins to the cell periphery or exterior via five known protein secretion systems (Desvaux et al. 2004). Type II protein secretion is part of the main terminal branch of the general secretory (GSP) pathway (Pugsley 1993; Pugsley et al. 1997; Filloux 2004) and is generally comprised of 12-to-16 proteins encoded by a contiguous cluster of moderately-to-highly conserved *pul* (or *gsp*) genes, usually in the same order. Pullulanase secretion by the plant cell wall-degrading microorganism *Klebsiella oxytoca* is one of the best characterized Type II protein secretion systems and a working model for pullulanase secretion has been proposed (Pugsley et al. 1997). Nascent pullulanase is first directed into and across the cytoplasmic membrane where it folds and is transiently anchored to the periplasmic aspect of the cytoplasmic membrane. After processing (signal peptide cleavage, disulfide bond formation, fatty acylation), the mature pullulanase is guided across the periplasmic space by the Type II secretion pseudopilus (GspG, H, I, J complex) and interacts with the OM-associated, multimeric GspD channel. Pullulanase is subsequently attached to the outside face of the outer membrane via a fatty acid tail. The *ferE* homolog, *gspE*, is postulated to encode a secretion ATPase that drives the secretion process, including the rapid polymerization and depolymerization reactions associated with pseudopilus extension and retraction (Filloux 2004). Peripherally attached pullulanase cleaves alpha-1,6 linkages in branched maltodextrin polymers such as glycogen or amylopectin of plant cell wall material, thereby releasing linear

dextrins for bacterial cell uptake and metabolism. Based on the *K. oxytoca* Type II pullulanase secretion model and the previously reported involvement of *S. putrefaciens* outer membrane proteins in dissimilatory Fe(III) and Mn(IV) reduction, it has been postulated that the Fe(III) and Mn(IV) respiratory deficiencies of Type II protein secretion mutants are due to their inability to secrete Fe(III) and Mn(IV) terminal reductases to the outside face of the *S. putrefaciens* outer membrane (DiChristina et al. 2002). A working model of the direct enzymatic pathway for reduction of solid Fe(III) oxides in *Shewanella* is displayed in Figure 3.

Electron shuttling pathways

A variety of Fe(III)-respiring DMRB, including *Shewanella* and *Geobacter*, can employ redox-active compounds (e.g., humic acids, melanin, phenazines, antibiotics, AQDS) as exogenous electron shuttles to reduce extracellular Fe(III) oxides (Lovley et al. 1996). The Fe(III) and Mn(IV) reduction-deficiencies of *Shewanella* Type II protein secretion mutants are rescued by addition of AQDS (DiChristina et al. 2005). *S. oneidensis gspD* insertional mutants are unable to respire anaerobically on solid Fe(III) or Mn(IV), yet retain the ability to respire all other electron acceptors, including AQDS. The ability to respire 50 mM solid Fe(III) or Mn(IV) is rescued in the *S. oneidensis gspD* insertional mutants by addition of 50 μM AQDS, an indication that the AQDS electron shuttling pathway is able to overcome the defect in the Type II protein secretion-linked pathway for respiration on solid Fe(III) and Mn(IV). AQDS is toxic to *Shewanella* cells above a critical threshold concentration and the efflux pump protein TolC protects *Shewanella* cells from AQDS toxicity by mediating AQDS efflux (Shyu et al. 2002). Electron transfer to AQDS also requires the OM protein MtrB, although its role in AQDS reduction remains unknown (Shyu et al. 2002).

Solid Fe(III) reduction by *Shewanella* is also stimulated by redox-active antibiotics and phenazines (Hernandez et al. 2004). Phenazines are similar in structure to AQDS and function as electron shuttles between *Shewanella* cells and solid Fe(III) oxides. Redox-active antibiotics (e.g., bleomycin) also function as shuttles for extracellular electron transfer to solid electron acceptors. Bacterially-produced phenazines (e.g., synthesized by *Pseudomonas chlororaphis* PCL1391) stimulate Fe(III) reduction by bacteria unable to produce them (e.g., *S. oneidensis* MR-1) (Hernandez et al. 2004). In addition, melanin (a humic acid-like compound synthesized by *S. algae* BrY in the presence of high concentrations of tyrosine) will enhance rates of Fe(III) oxide reduction (Turick et al. 2002). Melanin may have a dual function by acting as both an electron shuttle and an Fe(II)-complexing agent that prevents Fe(II) from adsorbing to and blocking Fe(III) oxide surface sites. A working model of the exogenous electron shuttling pathway for AQDS-mediated reduction of solid Fe(III) oxides by *Shewanella* is displayed in Figure 4.

Shewanella (and *Geothrix fermentans*) may also synthesize and release endogenous compounds that shuttle electrons to solid Fe(III) oxides (Newman and Kolter 2000; Nevin and Lovley 2002). Fe(III)-reducing *G. metallireducens*, on the other hand, does not appear to produce endogenous electron shuttles (Nevin and Lovley 2000). *S. algae* BrY produces melanin as a soluble electron shuttle for reduction of solid Fe(III) oxides (Turick et al. 2002). *S. algae*-produced melanin oxidizes *c*-type cytochromes at the cell surface and reduces solid Fe(III) oxides extracellularly (Turick et al. 2002). *S. oneidensis* MR-1 mutants defective in *menC* (encoding *o*-succinylbenzoic acid synthase) are deficient in menaquinone production and are unable to reduce AQDS, fumarate, thiosulfate, sulfite, DMSO or solid Fe(III) and Mn(IV) (Newman and Kolter 2000). Menaquinone is detected in the spent media of the wild-type strain, but not the *menC* mutants. Spent medium from the wild-type strain restores Fe(III) reduction activity to the *menC* mutant, while spent media from *menC* mutant does not. *S. oneidensis* MR-1 mutants defective in either *menD* or *menB* (encoding components of the menaquinone biosynthetic pathway) are also unable to reduce solid Fe(III) oxides (Saffarini et al. 2002). Vitamin K_2 (a menaquinone analog) restores the ability of the *menD* or *menB*

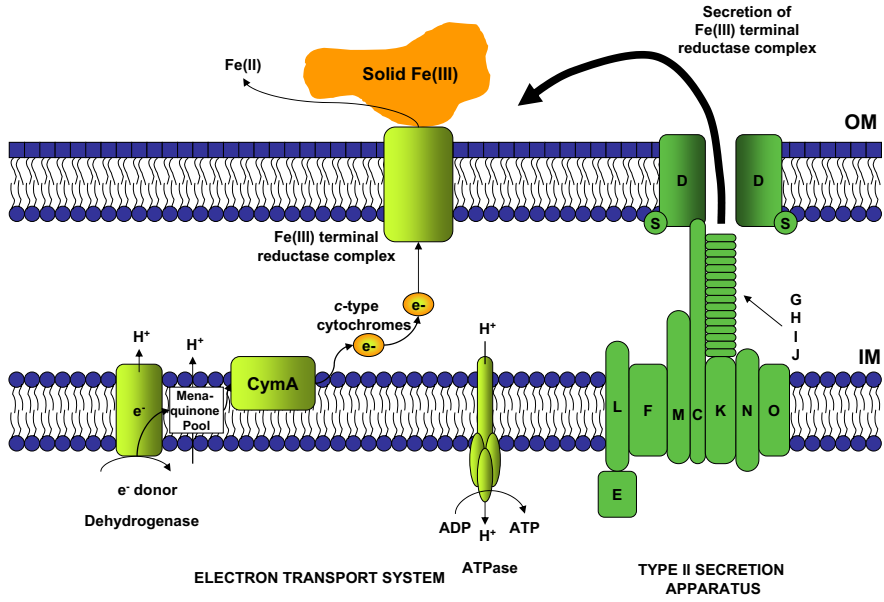

Figure 3. Working model for type II protein secretion-linked, direct enzymatic reduction of solid Fe(III)-oxides at the outer membrane.

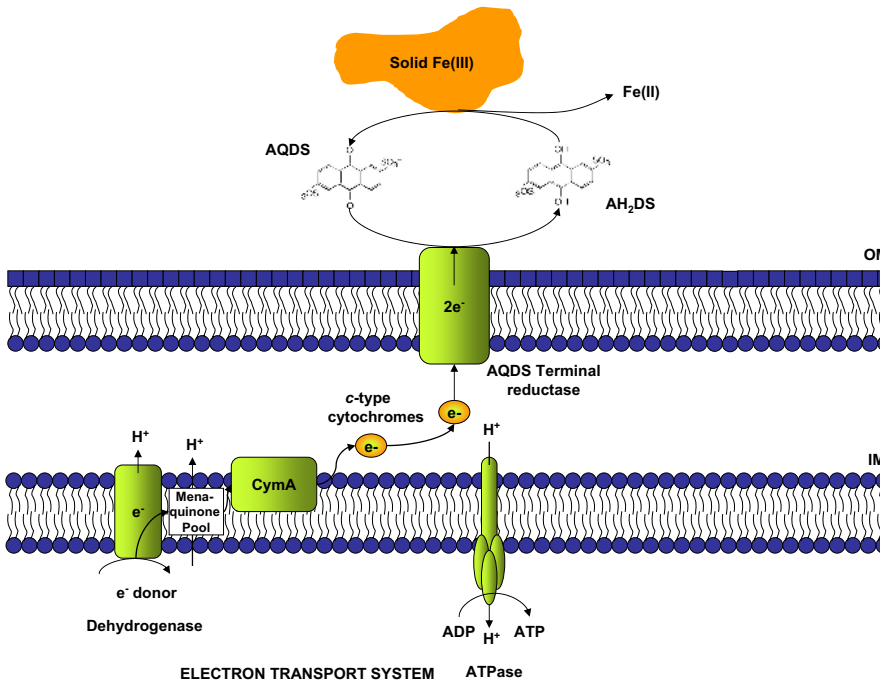

Figure 4. Working model for electron shuttling pathway with AQDS as electron shuttle.

mutants (and corresponding membrane fractions) to reduce either Fe(III) or Mn(IV) (Saffarini et al. 2002). It should be noted that the endogenous electron shuttle pathway may be the consequence of cell lysis and inadvertent spillage of menaquinol into the culture medium. Since shuttles can undergo redox cycling they can be effective at low concentrations and therefore even a small fraction of cell lysis could have a significant effect. Lipid-soluble menaquinol or vitamin K_2 then diffuses into bacterial membranes and functionally complements the *menB*, *C* or *D* mutants. Definitive evidence on the identity of the endogenous electron shuttle requires further research and will be challenging as exceedingly low concentrations may be involved.

Fe(III) solubilization by exogenous or bacterially-produced organic ligands followed by reduction of soluble organic-Fe(III)

A strong electrochemical signal indicative of soluble organic-Fe(III) is detected in a variety of marine and freshwater environments with Au/Hg voltammetric microelectrodes (Taillefert et al. 2002). Soluble organic-Fe(III) may therefore represent a dominant, yet under appreciated electron acceptor in anaerobic aquatic systems. Microbial Fe(III) reduction rates are higher with soluble organic-Fe(III) in pure cultures of *S. putrefaciens* (Arnold et al. 1988) and in freshwater sediments amended with Fe(III)-chelating compounds such as nitrilotriacetic acid (Lovley and Woodward 1996). *S. putrefaciens* reduces soluble organic-Fe(III) complexes at rates three orders of magnitude faster than amorphous or crystalline Fe(III) forms (Arnold et al. 1988). The mechanism of formation of soluble organic-Fe(III) generally involves non-reductive dissolution of amorphous Fe(III) oxides by multidentate organic ligands (forming mononuclear complexes with the Fe(III) oxides) at circumneutral pH. The strength of binding between Fe(III) and the complexing organic ligands influences soluble organic-Fe(III) reduction activity: organic ligands with strong Fe(III)-binding capability decrease (and in some cases totally inhibit) Fe(III) reduction activity by *S. putrefaciens* (Haas and DiChristina 2002).

Some Fe(III)-reducing bacteria such as *S. algae* BrY and *G. fermentans* generate relatively high concentrations of soluble organic-Fe(III) in the absence of exogenous chelating compounds, an indication that such bacteria synthesize and release organic ligands to solubilize Fe(III) prior to reduction (Nevin and Lovley 2002). Soluble organic-Fe(III) is detected electrochemically in *S. oneidensis* and *S. putrefaciens* cultures incubated anaerobically with either ferrihydrite or goethite (Taillefert and DiChristina 2005). Detection of soluble organic-Fe(III) prior to detection of Fe(II), suggests that soluble organic-Fe(III) is an intermediate in the reduction of solid Fe(III) oxides. Since lactate is the only organic ligand added to the *Shewanella* batch cultures and lactate-Fe(III) complexes do not react with Au/Hg electrodes, electrochemical detection of soluble organic-Fe(III) suggests that *Shewanella* synthesizes and releases organic ligands that complex and dissolve Fe(III) prior to reduction. The identity of the bacterially-produced, Fe(III)-solubilizing organic ligands remains unknown.

A respiration-linked, soluble organic-Fe(III) terminal reductase has yet to be definitively identified. As described above, *Shewanella* Type II protein secretion mutants are unable to reduce solid Fe(III) oxides, yet retain the ability to respire all other electron acceptors, including soluble organic-Fe(III). This finding suggests that soluble organic-Fe(III) may be reduced by terminal reductases located in subcellular compartments other than the OM. The *S. oneidensis* decaheme *c*-type cytochrome MtrA is a candidate terminal reductase for soluble organic-Fe(III): MtrA is located in the *S. oneidensis* periplasm and displays soluble organic-Fe(III) reductase activity when expressed in *E. coli* (Pitts et al. 2003). The requirement for MtrA in anaerobic respiration of soluble organic-Fe(III), however, has yet to be demonstrated in vivo. In *S. frigidimarina*, the transcriptional activator IfcR is translated in the presence of soluble organic-Fe(III) and is essential for expression of *ifcO* and *ifcA*. IfcO is a putative OM beta-barrel protein postulated to function as a soluble organic-Fe(III) transporter. IfcA is a flavin-containing *c*-type cytochrome with a small (10 kDa) tetraheme cytochrome domain that

displays soluble organic-Fe(III) reductase activity (Pitts et al. 2003). A working model of the two-step, Fe(III) solubilization-reduction pathway in *Shewanella* is displayed in Figure 5.

ENZYMATIC BASIS OF URANIUM REDUCTION

Members of the genera *Shewanella* (Lovley et al. 1991), *Desulfovibrio* (Lovley et al. 1993), *Clostridium* (Francis et al. 1994), *Geobacter* (Caccavo et al. 1992), *Thermus* (Kieft et al. 1999), *Pyrobaculum* (Kashefi and Lovley 2000), and *Desulfosporosinus* (Suzuki et al. 2002) display enzymatic U(VI) reduction activity. *Shewanella* and *Geobacter* enzymatically reduce U(VI) to U(IV) via a respiratory process that supports anaerobic growth. Although several purified *c*-type cytochromes display U(VI) reductase activity in vitro, a respiration-linked, U(VI) terminal reductase has yet to be definitively identified in vivo. Enzymatic U(VI) reduction activity is affected by U(VI) chemical speciation, electron donors, and competing electron acceptors. In the following section, the most recent findings on the enzymatic basis of U(VI) reduction by *Shewanella* and *Geobacter* are presented along with a discussion of the environmental factors affecting enzymatic U(VI) reduction activity.

Involvement of *c*-type cytochromes in enzymatic U(VI) reduction

Cytochrome c_3 of several *Desulfovibrio* species is involved in electron transfer to U(VI). Cytochrome c_3 of U(VI)-reducing (but non-respiring) *Desulfovibrio vulgaris* Hildenborough displays U(VI) reductase activity in vitro with H_2 as electron donor (Lovley et al. 1993). Cytochrome c_3 mutants of *D. desulfuricans* strain G20 are unable to reduce U(VI) with H_2 as

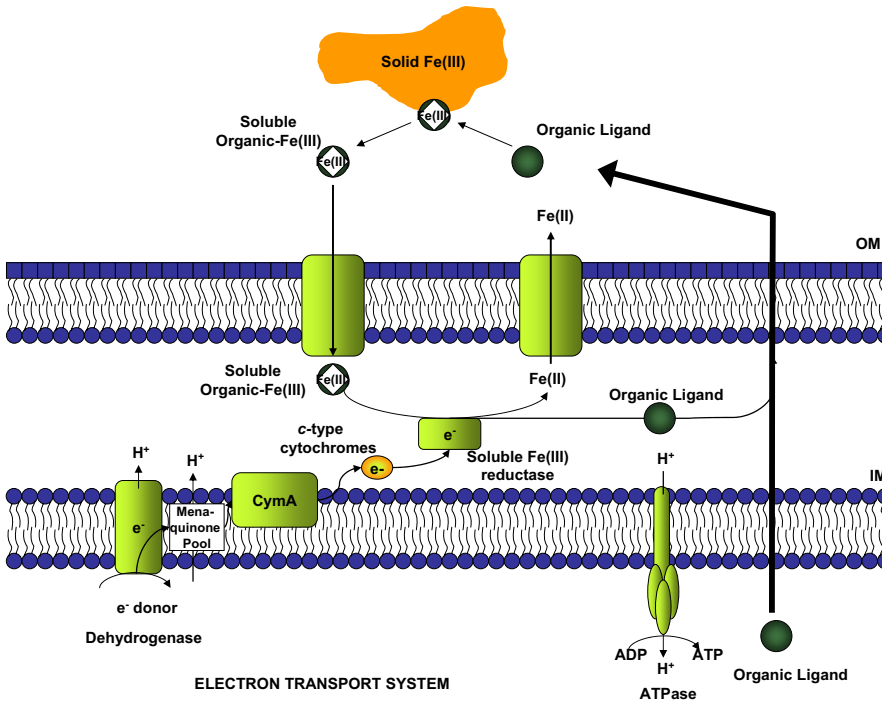

Figure 5. Working model for Fe(III) solubilization-reduction pathway with endogenous organic ligand as Fe(III)-chelating compound.

electron donor and are partially impaired in U(VI) reduction activity with lactate or pyruvate as electron donor (Payne et al. 2002). After growth of wild-type *D. desulfuricans* strain G20 in medium containing uranyl acetate, cytochrome c_3 is tightly associated with insoluble U(IV) particles (uraninite) found in the periplasm (Payne et al. 2004). Cytochrome c_7 of *G. sulfurreducens* also displays U(VI) reductase activity in vitro, however, mutants deficient in either cytochrome c_3 or c_7 retain U(VI) reduction activity in vivo (Lloyd et al. 2003). These findings suggest that either cytochrome c_3 and c_7 are not the physiological U(VI) reductases in *G. sulfurreducens* or that the electron transport pathway to U(VI) is highly branched and consists of multiple U(VI) terminal reductases. The highly branched nature of the U(VI) reduction pathway in *G. sulfurreducens* is reflected by the finding that Fe(III) reduction-deficient *ppcA* mutants (see above) are also deficient in U(VI) reduction activity (Lloyd et al. 2003).

A genetic complementation system has recently been developed to examine the enzymatic mechanism of U(VI) reduction by *S. putrefaciens* (Wade and DiChristina 2000). *S. putrefaciens* respiratory mutants unable to reduce U(VI) have been isolated and tested for the ability to respire on a suite of alternate compounds as electron acceptor, including oxygen O_2, NO_3^-, fumarate, trimethylamine-*N*-oxide (TMAO), dimethyl sulfoxide (DMSO), Mn(IV), Fe(III), chromate (Cr(VI)), arsenate (As(V)), selenite (Se(IV)), pertechnetate (Tc(VII)), thiosulfate (S(II)), and sulfite (S(IV)) (Wade and DiChristina 2000). All U(VI) reduction-deficient mutant strains also lacked the ability to respire NO_2^-. In particular, U(VI) reduction-deficient mutant strain U14 retained the ability to respire all electron acceptors except U(VI) and NO_2^-. These results suggest that the electron transport chains terminating with the reduction of NO_2^- and U(VI) share common respiratory components.

Effect of U(VI) chemical speciation on enzymatic U(VI) reduction activity

U(VI) chemical speciation is an important variable controlling enzymatic U(VI) reduction activity. In oxidizing aqueous environments at circumneutral pH (and in the absence of phosphate), U(VI) is found as soluble uranyl ion (UO_2^{2+}), often in carbonate complexed form (e.g., $UO_2(CO_3)_2^{2-}$, $UO_2(CO_3)_3^{4-}$, $CaUO_2(CO_3)_2^0$) or as crystalline solids such as metaschoepite ($UO_3{\cdot}2H_2O$), uranyl phosphates and uranyl silicates. U(IV) precipitates in reducing environments as uraninite (UO_2). The relative insolubility of U(IV) (10^{-8} M at pH > 5; Rai et al. 1990) compared with U(VI) is the basis of alternate bioremediation strategies (Lovley et al. 1991). The uranyl ion readily complexes with either inorganic (e.g., hydroxyl, carbonate, phosphate, sulfate and calcium) or organic (e.g., acetate, malonate, citrate and oxalate) ligands in aqueous solution (Grenthe 1992), and complexation markedly enhances its solubility. The type of complexing ligand changes the reduction potential of U(VI) thus affecting enzymatic reduction activity. In terms of reduction potential, hydroxo complexes are the most easily reduced forms of complexed U(VI), while complexation by carbonate decreases the reduction potential of U(VI). Complexation of U(VI)-carbonate by calcium (forming Ca-UO_2-CO_3 complexes) decreases the reduction potential to such an extent that enzymatic U(VI) reduction by *S. putrefaciens* CN32 nearly ceases (Brooks et al. 2003). Enzymatic U(VI) reduction activity by *D. desulfuricans* and G. *sulfurreducens* is also inhibited by formation of Ca-UO_2-CO_3 complexes. The effect of Ca^{2+} complexation on enzymatic U(VI) reduction activity are specific to U(VI) reduction since the enzymatic reduction of fumarate and Tc(VII) activities are not inhibited by Ca^{2+}.

In the absence of carbonate or at pH < 6 in the presence of carbonate, organic ligands bound to U(VI) also dramatically impact enzymatic U(VI) reduction activity. Citrate, for example, binds U(VI) with varying strength as a function of pH (Pasilis and Pemberton 2003). At pH > 6 and at low citrate concentrations, the highly soluble $(UO_2)_3Cit_2$ species predominates over the $(UO_2)_2Cit_2$ species. *S. alga* BrY reduces U(VI) bound to citrate and other multidentate aliphatic complexes such as malonate and oxalate more rapidly than U(VI)

bound to monodentate aliphatic complexes such as acetate, while the opposite trend is found with *D. desulfuricans* (Ganesh et al. 1997). U(VI) also adsorbs to carboxyl, phosphoryl and amine functional groups on the *S. putrefaciens* 200 cell surface, and a ligand exchange reaction may take place between the cell surface or U(VI) terminal reductases and the U(VI) complexes prior to reduction (Haas and DiChristina 2002).

Electron donors and competing electron acceptors

U(VI) reduction by *Shewanella* is coupled to oxidation of hydrogen, lactate, formate or pyruvate (Lovley et al. 1991). U(VI) reduction rates are highest with H_2 as electron donor (Liu et al. 2002b). Two explanations have been proposed to account for the increased rate of U(VI) reduction coupled to H_2 oxidation (Aubert et al. 2000; Liu et al. 2002b). First, electron flow through the electron transport chain may be more rapid when coupled to H_2 rather than lactate oxidation. Periplasmic H_2 hydrogenases may pass electrons through the electron transport chain more rapidly than those generated from cytoplasmic membrane-localized lactate dehydrogenase. Secondly, mass flux of neutrally charged H_2 to the enzymatic site of oxidation may be faster than negatively charged lactate. The negative charge of the lactate ion inhibits diffusion across the cell surface to the cytoplasmic membrane, thereby requiring an active transport system.

The presence of competing terminal electron acceptors also interferes with microbial U(VI) reduction. Thermodynamic calculations predict that electron acceptors should be utilized in order of highest free energy yield, a possible explanation for the inhibition of U(VI) reduction in the presence of nitrate (Finneran et al. 2002). Although the reduction of U(VI) coupled to the oxidation of organic compounds should yield greater free energy than Fe(III) (Cochran et al. 1986), the half-cell potentials of both U(VI) and Fe(III) can vary markedly with their coordination environment and whether they exist in the form of aqueous complexes or solid phases. For example, the half-cell potentials at pH 7 for many common, environmental Fe(III) forms vary from +0.35 V to −0.30 V (Stumm 1992), while those for U(VI) vary from +0.284 V to −0.042 V (Brooks et al. 2003). These variations in half-cell potential are compounded by the effects of reactant concentrations and other aqueous complexants, the similarity in half-cell potential of many Fe(III) and U(VI) forms and their uncertainty, the interfacial chemistry of solid phase electron acceptors, and the poorly understood redox chemistry of surface complexed Fe(II). All of these considerations complicate a rigorous thermodynamic analysis. The effects of pH are also strong because of the proton stoichiometry of reaction, and the redox stability of U(VI) over Fe(III), or vise-versa, may change if pH is not controlled, if reactant concentrations are varied appreciably, or if mineral biotransformation products exhibit different redox chemistry. In spite of these complexities and chemical interrelationships, there are some consistent thermodynamic observations. Ferrihydrite, with its higher redox potential (~−0.070 V at pH = 7 and Fe(II) = 10^{-5} mol/L) was observed to inhibit bacterial U(VI) reduction, while goethite did not (~-0.250 V at pH = 7 and Fe(II) = 10^{-5} mol/L) (Wielenga et al. 2000).

Electron transport to Mn(IV) provides a greater free energy yield than electron transport to U(VI), and is therefore predicted to be a preferred electron acceptor (Cochran et al. 1986; Langmuir 1997). Bioavailable Mn(IV)-oxides such as birnessite and bixbyite follow this prediction, however, U(VI) is reduced concurrently with less soluble forms of Mn(IV) (Fredrickson et al. 2002). To determine if this finding is due to electron acceptor competition or abiotic oxidation of U(IV) by Mn(IV), *S. putrefaciens* CN32 was incubated with U(VI) and pyrolusite (β-MnO_2) (Liu et al. 2002b). Extracellular, cell surface-associated, and periplasmic UO_2(s) aggregates were detected by Transmission Electron Microscopy (TEM) when cells were incubated only with U(VI). Upon addition of pyrolusite, extracellular UO_2(s) was depleted but periplasmic and cell surface-associated UO_2(s) remained. These results suggest

that U(IV) functions as an electron shuttle and is oxidized by the extracellular pyrolusite. U(VI) is completely reduced provided the OM of intact cells physically separates (sequesters in the periplasmic space) UO_2(s) from extracellular pyrolusite.

Humic acids have recently gained attention for their potential role as shuttles for electron transfer between anaerobically respiring *Shewanella* and solid Fe(III)-oxides (see above). Addition of AQDS to *S. putrefaciens* CN32, however, does not enhance the reduction rate of either soluble or insoluble forms of U(VI) (Fredrickson et al. 2000). AQDS actually inhibits U(VI) reduction activity, possibly by diverting electrons away from the U(VI) reduction pathway.

Subcellular location of enzymatic U(VI) reduction activity

The subcellular location of enzymatic U(VI) reduction in *Shewanella* has also been recently examined: Insoluble U(IV) particles are detected extracellularly, on the cell surface and within the periplasmic space of *S. putrefaciens* CN32 after reduction of soluble U(VI) (Liu et al. 2002a). U(IV) is not detected in the cytoplasm (Fig. 6). U(VI) reductases may therefore be localized within the OM, diffuse (or be transported) across the OM to contact U(VI) reductases located in the periplasm or IM, or both. U(VI) reduction products of *Desulfosporosinus* have been detected as nanometer-sized UO_2(s)-particles (Suzuki et al. 2002). Nanoparticles produced in the periplasm either diffuse or are exported to the cell exterior where they organize extracellularly to form larger aggregates. Aggregation of U(IV) particles prior to export from the cell may result in the periplasmic deposits detected on TEM images of U(VI)-respiring cells (Liu et al. 2002a). U(IV) particles detected in the culture supernatant also leads to the intriguing possibility that anaerobically-respiring *Shewanella* are able to actively secrete U(IV) particles as a means of avoiding build-up of toxic insoluble U(IV) end-products during U(VI) reduction. *S. putrefaciens* CN32 is also capable of reducing solid forms of U(VI) such as metaschoepite (Fredrickson et al. 2000), although solid forms of U(VI) have been found to be resistant to microbial reduction *in situ* (Ortiz-Bernad et al. 2004). The mechanism by which *Shewanella* species reduce metaschoepite is unknown, but U(VI) terminal reductase localization to the OM to contact solid U(VI) is possible.

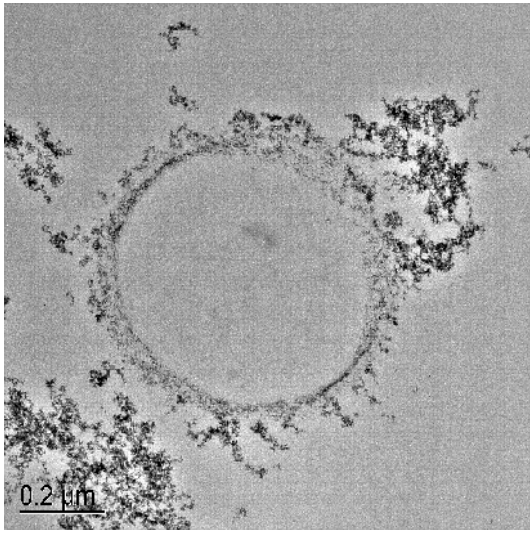

Figure 6. Transmission electron microscopy image of an unstained thin section from *Shewanella putrefaciens* strain CN32 cells incubated with H_2 and U(VI) in pH 7 bicarbonate buffer, illustrating the accumulation of nano-size U(IV)O_2 particles extracellularly and in association with the periplasmic space and cell surface.

ENZYMATIC MECHANISM OF TECHNETIUM REDUCTION

Enzymatic studies on Tc(VII) reduction have largely been focused in *E. coli*, however the ability to reduce Tc(VII) has been recently found in *S. putrefaciens* CN32, *S. oneidensis* MR-1 and *S. putrefaciens* 200 (Lyalikova and Khizhnyak 1996; Lloyd et al. 1997; Wildung et al. 2000; Payne and DiChristina 2005). Tc(VII) is also reduced under acidic conditions by *Thiobacillus thiooxidans* (Lyalikova and Khizhnyak 1996), under alkaline conditions by *Halomonas* strain Mono (Khijniak et al. 2003) and at high temperature by *Pyrobaculum islandicum* (Kashefi and Lovley 2000). Reduction of soluble Tc(VII) results in formation of Tc(IV) which precipitates as insoluble $TcO_2{\cdot}nH_2O$ (hereafter termed TcO_2) and may be immobilized *in situ*. In the absence of aqueous complexing agents, Tc(IV) may also be immobilized via formation of strong surface complexes with hydroxylated surface sites on Al and Fe oxides and clays (Rard 1983; Haines et al. 1987; Meyer et al. 1991; Eriksen et al. 1992; Wildung et al. 2000).

Involvement of hydrogenases in Tc(VII) reduction

E. coli possesses four hydrogenases, designated as hydrogenases 1-4. Hydrogenases-1 and 2 share little homology to hydrogenases-3 and 4. Hydrogenases 3 and 4 share high homology to each other and are both expressed as part of the formate-hydrogen lyase complex in *E. coli* (Bagramyan and Trchounian 2003). The Tc(VII) reductase in *E. coli* has been identified as the Ni-Fe hydrogenase-3 component of the formate-hydrogen lyase complex (Lloyd et al. 1997). Hydrogenase expression is determined by pH: hydrogenase-4 (encoded by the *hyf* operon) is expressed under alkaline conditions while hydrogenase-3 (encoded by the *hyc* operon) is expressed under acidic conditions (Bagramyan and Trchounian 2003). The formate-hydrogen lyase complex in *E. coli* is composed of formate dehydrogenase plus multiple components of the respective hydrogenase encoding operons (i.e., *hyf* and *hyp* operons). The bi-directional nature of hydrogenase-3 (HycE) enables both the production of H_2 during formate oxidation and the direct oxidation of H_2 under other conditions.

S. oneidensis MR-1 does not possess a formate-hydrogen lyase complex and possesses only two hydrogenases, neither of which share significant homology to hydrogenases-3 or 4 of *E. coli*. The first *S. oneidensis* MR-1 hydrogenase (Locus SO2098; HyaB) displays high homology to the IM-bound Ni-Fe hydrogenase HydB of *Wolinella succinogenes*, while the second *S. oneidensis* MR-1 hydrogenase (Locus SO3920; HydA) displays high homology to the putative D subunit of the NADP-reducing hydrogenase of *Thermotoga maritima* (Payne and DiChristina 2005). In terms of hydrogenase function, *S. oneidensis* MR-1 hydrogenases appear most similar to those of *Alcaligenes eutrophus* in which the cytoplasmic, soluble hydrogenase (HydA) regenerates NADH, while the membrane bound Ni-Fe hydrogenase (HyaB) generates reducing power (Lengeler et al. 1999). In organisms containing only membrane bound hydrogenases, reducing power is generated by reverse electron transport, generally carried out by membrane bound bi-directional hydrogenases (e.g., hydrogenases-3 and 4 in *E. coli*). *S. oneidensis* MR-1 therefore appears to share close similarity to the hydrogen uptake and utilization systems of *A. eutrophus* and little sequence or physiological similarity to the H_2 uptake and utilization systems of *E. coli*. Further work needs to be carried out to determine if the *S. oneidensis* MR-1 hydrogenases display Tc(VII) reductase activity.

Subcellular location of enzymatic Tc(VII) reduction activity

The enzymatic reduction of Tc(VII) is electron donor-specific. H_2 serves as electron donor in all known Tc(VII)-reducing organisms (Lloyd et al. 2000; Wildung et al. 2000; De Luca et al. 2001), while the ability to couple the oxidation of other carbon sources to the reduction of Tc(VII) occurs in only a small subset of organisms. *G. sulfurreducens* and *D.*

fructosovorans have an exclusive requirement for H_2 as electron donor for Tc(VII) reduction while *E. coli* is limited to formate and H_2 as electron donor. *S. oneidensis* MR-1 and *S. putrefaciens* CN32 couple the oxidation of formate, lactate, and H_2 to Tc(VII) reduction (Wildung et al. 2000; Payne and DiChristina 2005), but Tc(VII) reduction rates are markedly higher with H_2 as electron donor. The reduced Tc(IV) product is generally nanometer-sized $TcO_{2(s)}$ in buffers or media without high carbonate. The identity of the electron donor does not seem to influence the mineralogic nature of the reduction product. The black-colored precipitate is observed in the periplasm, and as 20-50 nm dome-like structures consisting of aggregates of many individual crystallites on the cell surface (Fig. 7). The $TcO_{2(s)}$ is nanocrystalline and exhibits insufficient long-range order to yield a discernable diffraction pattern. The precipitate maintains a Tc solubility ($\approx 10^{-8}$ mol/L) that approximates measured values for $TcO_2 \cdot xH_2O$ (as reported by Rard 1999 and associated citations). The physiologic relationship between subcellular and surface associated $TcO_{2(s)}$ is unclear. Limited evidence implies that bioreduced Tc [e.g., Tc(IV)] may exist in the form of carbonate aqueous complexes, or perhaps carbonate precipitates, in high-bicarbonate media (Wildung et al. 2000). Further research on the biogeochemistry of Tc(VII)/Tc(IV) in bicarbonate-containing media and other ligand solutions of geochemical relevance is needed.

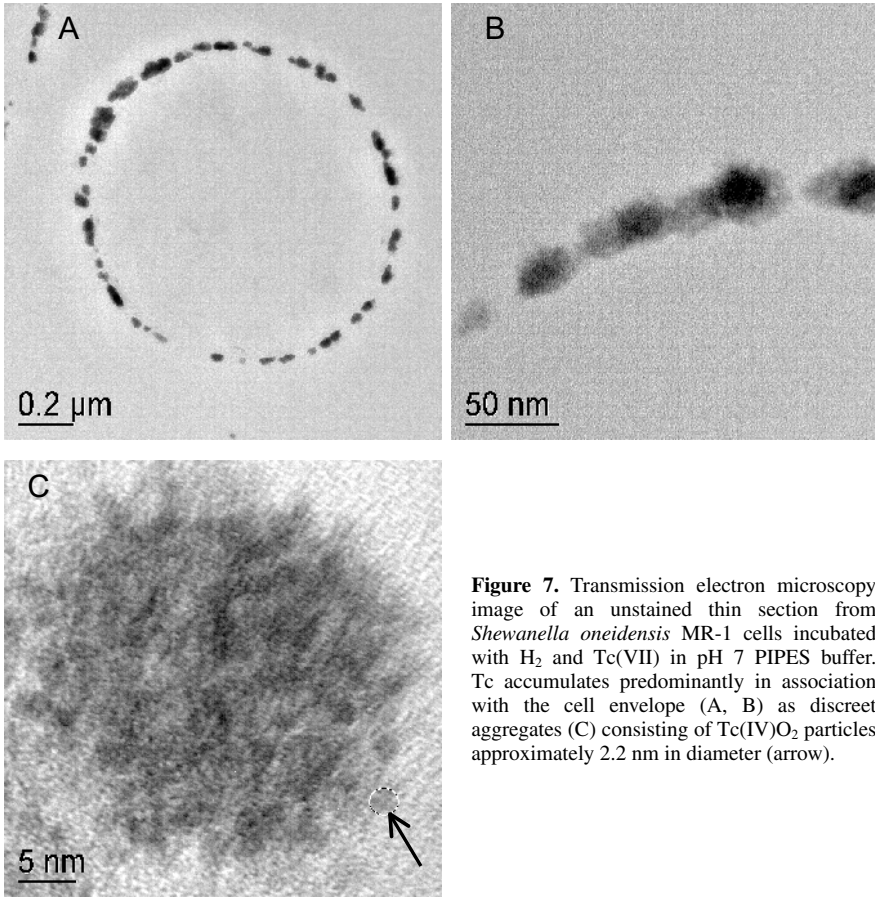

Figure 7. Transmission electron microscopy image of an unstained thin section from *Shewanella oneidensis* MR-1 cells incubated with H_2 and Tc(VII) in pH 7 PIPES buffer. Tc accumulates predominantly in association with the cell envelope (A, B) as discreet aggregates (C) consisting of $Tc(IV)O_2$ particles approximately 2.2 nm in diameter (arrow).

MICROBIAL REDUCTION-INDUCED CHANGES IN METAL BIOGEOCHEMISTRY

Direct enzymatic effects of dissimilatory metal-reducing bacteria (DMRB) on metal solubility

The majority of electron acceptors commonly used by prokaryotes (oxygen, nitrate, sulfate, carbon dioxide) exhibit relatively high levels of solubility before and after reduction. In contrast, many of the metals used as microbial electron acceptors exhibit substantially different solubility properties in the oxidized (e.g., pε = 10) versus the reduced (pε = 4 or 2) states (Table 1). Because Fe(III) and Mn(III,IV) exist predominantly as oxyhydroxide minerals in oxic environments (e.g., ferrihydrite and goethite, or birnessite and manganite), DMRB must overcome the fundamental problem of engagement of the cell electron transport system (ETS) with the mineral surface across a solid-liquid interface. DMRB have developed several novel mechanisms for overcoming this problem, as described in preceding sections, including the "shuttling" of electrons by humic acids (Lovley et al. 1996; Lovley and Woodward 1996) or cell metabolites (Newman and Kolter 2000) from terminal points of the ETS to the mineral surfaces, possibly the direct transfer of electrons to metal in the centers of mineral surfaces by multiheme cytochromes associated with the OM (Richardson 2000; Leang et al. 2003), or the solubilization of solid phase-associated Fe(III) as subsequent engagement of Fe(III) reductase(s) as a soluble Fe(III)-organic complex. Regardless of the mechanism, the microbial reduction of Fe and Mn has a profound impact on the geochemical behavior of these metals as

Table 1. Solubilities (aqueous concentrations) of select phases of Fe, Mn, U, and Tc at pH 7 in water as a function of *p*e ($pCO_2 = 10^{-3.46}$ atm, $I = 0.01$).

Solid phase	*Oxidizing*	*Reducing*	
	pε = 10 mol/L	(pε = 4) mol/L	(pε = 2) mol/L
$Fe(OH)_3$ (ferrihydrite)	2.06×10^{-8} (Fe^{3+})	2.06×10^{-8} (Fe^{3+}) 1.29×10^{-7} (Fe^{2+})	2.06×10^{-8} (Fe^{3+}) 1.29×10^{-5} (Fe^{2+})
α-FeOOH (goethite)	8.40×10^{-13} (Fe^{3+})	8.36×10^{-13} (Fe^{3+}) 5.24×10^{-12} (Fe^{2+})	8.36×10^{-13} (Fe^{3+}) 5.24×10^{-10} (Fe^{2+})
$MnO_{1.8}$ (birnessite)	6.03×10^{-5} (Mn^{2+})	Soluble as Mn^{2+} [a]	Soluble [a]
γ-MnOOH (manganite)	2.87×10^{-6} (Mn^{2+})	Soluble as Mn^{2+} [a] (2.83 mol/L maximum)	Soluble [a]
UO_2 (uraninite)	2.67×10^{-7} [$U(VI)O_2^{2+}$] [b]	2.67×10^{-7} [$U(VI)O_2^{2+}$] [b]	1.46×10^{-7} [$U(VI)O_2^{2+}$] 2.0×10^{-17} [$U(IV)(OH)_{4(aq)}$] [c]
$TcO_2{\cdot}nH_2O$	Soluble as $Tc(VII)O_4^-$	Soluble as $Tc(VII)O_4^-$	10^{-8} [$Tc(IV)O(OH)_{2(aq)}$]

a. Will precipitate as rhodochrosite [$MnCO_{3(c)}$]
b. 1×10^{-4} mol/L precipitated as schoepite [β-$UO_3{\cdot}2H_2O_{(c)}$], the primary aqueous complex under the given conditions is $UO_2(CO_3)_2^{2-}$, solubility increases with $CO_{2(g)}$ partial pressure and total aqueous carbonate concentration
c. 9.98×10^{-5} mol/L precipitated as uraninite [$UO_{2(c)}$], pε is 1.8, U(VI) concentrations decrease with decreasing pε.

well as broader impacts on the overall geochemical and mineralogic properties of solids and sediments where these processes occur.

In contrast to Fe and Mn, U, Tc, and Cr are relatively soluble in oxic environments and typically exist as anionic uranyl carbonate $UO_2(CO_3)_3^{4-}$, $UO_2(CO_3)_2^{2-}$ complexes, pertechnetate (TcO_4^-), or chromate (CrO_4^{2-}), respectively. The solubility of U(VI) at circumneutral pH is strongly dependent on dissolved carbonate concentration and other associated ligands such as silica or phosphate. Upon reduction to the +4 oxidation state and in the absence of strong complexants, U and Tc can precipitate as the hydrous oxides, UO_2 (uraninite) and TcO_2, phases that have been identified in anaerobic suspensions of DMRB cells incubated with U(VI) (Gorby and Lovley 1992) or Tc(VII) (Wildung et al. 2000) and appropriate electron donors. The direct enzymatic reduction of U(VI) results in the formation of relatively uniformly-sized nanoparticles (Fredrickson et al. 2002; Suzuki et al. 2002), a factor that can impact their subsequent reactivity and transport. For example, if U(IV) nanoparticles are less than approximately 2-to-5 nm in diameter, they may behave as large molecular clusters and be mobile in solution, while U(IV) particles with larger diameters may not be transported as readily (as described in chapter on nanoparticles by Gilbert and Banfield 2005). It is these marked changes in solubility that have prompted consideration of manipulating the activities of DMRB for the bioremediation of soils and sediments contaminated with metals and radionuclides (Lovley 1995).

Indirect effects of DMRB on metal solubility

DMRB can also indirectly influence the biogeochemical behavior of redox active and non-redox active metals. Because the mass content of Fe and Mn is typically higher than trace metals and contaminants in most soils and sediments they tend to have a dominant effect on redox reactions and function as a major sink for electrons from DMRB respiration. Due to their relatively higher mid-point potential, Mn oxides can provide a "buffer" against the net reduction of other metal ions including Fe(III) (Lovley and Phillips 1988; Myers and Nealson 1988) and U(VI) (Liu et al. 2002b). Because Mn(III,IV) oxides are relatively strong oxidants they can also oxidize reduced forms of metals such as $Cr(OH)_3$ (Fendorf and Zasoski 1992) and UO_2 (Fredrickson et al. 2002), hence impeding their net microbial reduction unless there are mechanisms that prevent their physical interaction such as the accumulation and isolation of UO_2 nanoparticles in the cell periplasm (Fredrickson et al. 2002). Although Fe(III) oxides are not as effective oxidants as Mn oxides, they can potentially impede the reduction of other metals such as U(VI) via competition mechanisms (Wielinga et al. 2000). Hence, the microbial reduction of Mn and Fe oxides can result in redox conditions that are more favorable for the reduction for trace metal contaminants. In addition, Fe(II) and Mn(II) can potentially provide buffer against re-oxidation.

Biogenic Fe(II) can also function as a facile reductant of trace metal and radionuclides including U(VI) (Liger et al. 1999), Tc(VII) (Lloyd et al. 2000; Wildung et al. 2004), and Cr(VI) (Wielinga et al. 2001). The rate of $Tc(VII)O_4^-$ reduction by sediment-associated biogenic Fe(II) was shown to be related directly to the extent of sediment Fe(III) reduction but there was extensive variation among different sediments indicating that the effectiveness of Fe(II) as a reductant was highly dependent upon molecular speciation as opposed to Fe(II) concentration alone (Fredrickson et al. 2004; Hansel et al. 2004). In general, aqueous Fe(II) is poorly reactive with Tc(VII) (Cui and Eriksen 1996) and U(VI) (Fredrickson et al. 2000), probably due to kinetic limitations. In contrast, the rates of chromate reduction by $Fe(II)_{aq}$ are relatively rapid (Wielinga et al. 2001). One area that warrants further investigation is whether biosorbed Fe(II), which can form complexes and precipitates on cell surfaces (Liu et al. 2001a), can also function as a reductant in a manner similar to Fe(II) sorbed on mineral surfaces. The fact that Fe oxides can impede i.e., via competition (Wielinga et al. 2000)

or promote, i.e., via surface complexation of Fe(II) (Fredrickson et al. 2004), reflects the similarity of the mid-point potentials of these metals and the need to pay careful attention to factors including speciation, concentration, and solubility that can greatly impact the direction and extent of such redox reactions.

Trace metals can associate with Fe or Mn oxides as adsorbed or co-precipitated species and are therefore subject to biogeochemical reactions resulting from utilization of the oxide as a terminal electron acceptor by DMRB. Ni^{2+} and Co^{2+} co-precipitated with goethite were released when oxide suspensions were subject to reduction by *S. putrefaciens* CN32, resulting in a net increase in aqueous concentrations of the metal ions (Zachara et al. 2001). Similarly, Ni^{2+} was also released from a Ni-substituted hydrous ferric oxide upon reduction by strain CN32 although under select conditions a Ni-substituted magnetite ($Fe^{III}_2Fe^{II}_{1-x}Ni_xO_4$) formed (Fredrickson et al. 2001). Ni^{2+} was found to inhibit the overall reduction reaction by an undefined chemical mechanism that could be circumvented by addition of AQDS as an electron shuttle. Aluminum release during the bioreduction of an Al-substituted goethite associated with an Atlantic coastal plain sediment was congruent with the production of Fe(II) but the released Al was associated with a sorbed phase (Kukkadapu et al. 2001). DMRB can also promote the mobilization of arsenic as arsenate via the reductive dissolution of the ferric arsenate mineral scorodite ($FeAsO_4{\cdot}2H_2O$) and from iron oxide sorption sites within sediments (Cummings et al. 1999). It is interesting to note that some DMRB are also capable of dissimilatory reduction of arsenate (AsV) to arsenite (AsIII) (Saltikov et al. 2003) but such a reduction reaction was not observed. Although there is considerable potential for mobilization of trace elements associated with metal oxides during bioreduction, the extent to which trace metals remain associated with the solid phase or are released to solution will be a function of the aqueous and solid-phase geochemical composition that ultimately controls the adsorption and precipitation reactions.

REDUCTIVE TRANSFORMATION OF Fe- AND Mn-CONTAINING MINERALS

The rate and extent of microbial reduction of Fe(III) and Mn (III,IV) oxides in soils and sediments is a function of complex and highly coupled biological, chemical, and physical factors. Mineralogy plays a critical role with factors such as surface area (Roden and Zachara 1996), extent of structural disorder (Zachara et al. 1998), surface speciation (Roden and Urrutia 2002), and thermodynamics (Liu et al. 2001b) all influencing, to some extent, the reduction process. The physiological state of the organisms, including effects resulting from growth medium composition (Glasauer et al. 2003) and electron donor-acceptor ratios (Zachara et al. 2002), are other key variables that can affect bioreduction of Fe and Mn minerals but are currently poorly understood. The role of cell physiology on metal oxide reduction is currently under-appreciated and its importance warrants further research using physiologically and compositionally defined cultures that better represent and span the range of environmental conditions.

Laboratory studies

A general observation made from laboratory studies is that poorly crystalline Fe(III) oxides such as ferrihydrite (Lovley and Phillips 1986) exhibit a greater degree of bioavailability than more crystalline phases such as lepidocrocite (γ-FeOOH), goethite (α-FeOOH), or hematite (Fe_2O_3), although as a crystalline phase, lepidocrocite is far more bioavailable than the others. The same general trend appears to hold true for Mn oxides in that more highly crystalline phases such as pyrolusite (β-MnO_2) are reduced more slowly than amorphous MnO_2 or birnessite (Burdige et al. 1992). This effect has been attributed to differences in solubility of these phases and is supported by experiments demonstrating that the maximum rate of Fe(III) reduction was found to correlate positively with the solubility of the oxide (Bonneville et al. 2004). Caution must be exercised when using rates of abiotic reduction of Fe oxides as an

indicator of their susceptibility to enzymatic reduction as the surface area-normalized rates of bacterial reduction of ferrihydrite, lepidocrocite, goethite, and hematite were found to be quite similar, in contrast to reduction of the same phases by ascorbic acid (Roden 2003). Naturally-occurring (geologic) Fe oxides have been found to be equally or more reducible than their synthetic counterparts with crystalline disorder and microheterogeneities potentially being dominant factors controlling microbial reduction (Zachara et al. 1998).

The presence of solid phase sorbents and organic complexants can also facilitate microbial reduction of Fe oxides, presumably via the removal of reduction product (Fe^{2+}) from oxide and bacterial surfaces (Urrutia et al. 1999). A similar enhancement in the extent of Fe oxide reduction can be achieved by continual replacement of the aqueous phase in semi-continuous cultures (Roden and Urrutia 1999) or in continuous flow columns where soluble Fe(II) is constantly removed (Roden et al. 2000). The products of oxide bioreduction can hence impede further reduction by passivating oxide and cell surfaces (Liu et al. 2001a,b) or can promote reduction by removing products from solution via secondary precipitation reactions.

Transformation of Fe- and Mn-bearing minerals to secondary phases is similarly a function of a complex set of biogeochemical variables. A number of laboratory studies have probed the bioreductive transformation of poorly crystalline ferrihydrites to a wide range of phases including more highly ordered Fe(III) oxides such as 6-line ferrihydrite, goethite, lepidocrocite (Zachara et al. 2002, Fredrickson et al. 2003; Hansel et al. 2003; Kukkadapu et al. 2003, 2005), mixed valence oxides such as magnetite and green rust $[Fe^{II}_{(6-x)}Fe^{III}_{(x)}(OH)_{12}]^{x+}[(A^{2-})_{x/2}{\cdot}yH_2O]^{x-}$ (Lovley et al. 1987; Fredrickson et al. 1998), or Fe(II) phases including siderite ($FeCO_3$), vivianite ($Fe_3(PO_4)_2{\cdot}8H_2O$), and ferrous hydroxy carbonate ($Fe_2(OH)_2CO_3$) (Fredrickson et al. 1998; Hansel et al. 2003; Kukkadapu et al. 2003). The extent to which each of these phases may form is influenced by a range of factors including pH and aqueous solution composition (Fredrickson et al. 1998), the relative concentrations of the oxide (electron acceptor) and electron donor (Zachara et al. 2002; Fredrickson et al. 2003), the presence of co-precipitated ions (Fredrickson et al. 2001; Kukkadapu et al. 2004), and hydrodynamic-induced distributions of reduction products (Hansel et al. 2003). The formation of secondary mineral phases is less common or extensive when more highly ordered phases such as hematite or goethite are reduced by DMRB but nonetheless Fe(II) biominerals such as siderite and vivianite have been observed to form under conditions consistent with their solubility (Zachara et al. 1998). The lower solubility of more highly ordered phases such as goethite and hematite support lower concentrations of $Fe(III)_{aq}$ and DMRB-generated $Fe(II)_{aq}$, relative to ferrihydrite, and therefore hinder precipitation of secondary minerals such as magnetite beyond reorganization on the mineral surface into nanometer-size spinel-like domains (Hansel et al. 2004).

In addition to Fe oxides and oxyhydroxides, DMRB can also reduce structural Fe in clay minerals facilitating their dissolution (Kostka et al. 1996, 1999) or changes in clay morphology, structure, and composition (Dong et al. 2003a,b). The extent of microbial reduction of structural Fe(III) in smectite can be substantial, ranging to >90% (Kostka et al. 1999). The microbial reduction of structural Fe in clay can significantly alter clay chemical and physical properties such as was reported for studies with smectite where reduction resulted in reduced swelling pressure, total surface area, and surface charge density (Kostka et al. 1999). Structural Fe(II) in bioreduced ferruginous clays can also promote the reductive dehydrochlorination of organic contaminants such as pentachloroethane and trichloroethane (Cervini-Silva et al. 2003).

Field studies

Controlled single-phase, single organism laboratory experiments can provide important mechanistic insights but it is often difficult to predict field behavior from such results due to environmental complexities and heterogeneities. One of the more well studied field sites is

a crude oil-impacted shallow groundwater aquifer located near Bemidji, Minnesota, USA. Research at this site has documented aspects of microbial community structure (Rooney-Varga et al. 1999) and associated geochemical changes (Baedecker et al. 1993) over the length of the plume as the predominant respiratory process shifted from Fe(III) and Mn(III,IV) reduction to methanogenesis (Anderson and Lovley 1999). A detailed analysis of sediments from within the plume and from the pristine aquifer revealed significant differences in the mass content and identify of Fe(III) oxides consistent with microbial-driven reduction processes (Zachara et al. 2004). Comparisons between the texturally-similar source where bioavailable Fe(III) had been exhausted and Fe(III)-reducing zone sediments where bioavailable Fe(III) remained indicated that dispersed crystalline Fe(III) oxides and a portion of the poorly crystalline Fe(III) oxide fraction had been depleted from the source zone sediment. The presence of residual ferrihydrite in the anoxic plume sediment indicated that some fraction of the Fe(III) oxides were biologically inaccessible, possibly due to their residence in microfractures in the interior of lithic fragments. Interestingly, little evidence was found for biogenic ferrous mineral phases with the exception of thin siderite or ferroan calcite surface precipitates. It is clear that additional field-based research including characterization of samples in concert with modeling and laboratory-based experiments is needed to improve our ability to predict biogeochemical behavior of redox active metals in natural and engineered systems, particularly with regard to mineral biotransformation products and biominerals.

ROLE OF MICROBIAL METAL REDUCTION IN REDOX CYCLING

As is the case for most microorganisms, DMRB rarely function alone but are members of complex communities whose collective, intertwined activities are responsible for catalyzing the cycling of elements in the biosphere. Many DMRB, particularly members of the genus *Shewanella*, are well-adapted to geochemically stratified environments where there is a gradient in electron acceptors available for oxidizing organic matter and H_2. These adaptations include a relatively robust and diverse electron transport system that can engage a wide range of electron acceptors and an extensive network of regulatory genes, both two-component and transcriptional regulators (Heidelberg et al. 2002), that allow the organisms to sense and respond to their environment. These organisms play a critical role in such environments by oxidizing fermentation products, such as low molecular weight organic acids, coupled to respiration of Fe and Mn.

Redox cycling in chemically stratified environments

The Black Sea is a prime example of a chemically (redox) stratified environment that exists over distances of tens of meters in the water column and where Fe and Mn undergo biogeochemical redox cycling (Nealson and Myers 1992). Fe and Mn are chemically unique ions in redox gradient environments because of their relatively low solubility in the oxidized state. As these metal ions are oxidized, either microbially or abiotically, they can precipitate and be subjected to gravitational settling into anoxic zones (Nealson and Saffarini 1994). As they enter the anoxic zones these precipitates can be reduced by DMRB coupled to organic matter oxidation at which point soluble species of Fe(II) and Mn(II) can diffuse upward into oxic zones. A number of excellent reviews have been published on this subject and the reader is directed to those for more information and examples (Burdige 1993; Nealson and Saffarini 1994; Nealson and Little 1997; Nealson et al. 2002). Previous investigations of redox stratified environments have justifiably focused on detailed geochemical characterization and baseline investigations into associated microbial properties using a combination of cultivation and cultivation-independent methods. The availability of whole genome sequences and the ability to sequence entire microbial communities (metagenomics) provide powerful tools to probe

microbial processes in these environments in the future (for additional discussion see chapters by Whitaker and Banfield 2005 and Nelson and Methé 2005).

Microscale redox cycling

In addition to participating in redox cycling in chemocline environments that can span many meters, more recent research indicates that DMRB also participate in redox cycling over much shorter length scales. Because Fe(II) is rapidly oxidized by O_2, neutrophilic microbial Fe oxidation is constrained to microaerobic environments where the abiotic oxidation of Fe(II) is limited by O_2 availability and microbiologic rates of Fe(II) oxidation are competitive with abiotic rates. Hence, there is a potential for tight coupling between metal reduction and oxidation steps over short distance scales in environments with sharp microaerobic and anaerobic boundaries. In fact, it has been proposed that Fe(II)-oxidizing organisms localize themselves into a narrow band of cells and associated Fe(III) oxides to facilitate interfacing with DMRB (Roden et al. 2004). Such a coupling was experimentally investigated in microcosms consisting of ferrihydrite coated sand and a co-culture consisting of a lithotrophic Fe(II)-oxidizing bacterium (strain TW2) and the DMRB *Shewanella alga* strain BrY (Sobolev and Roden 2002). The co-culture exhibited minimal Fe oxide accumulation at the sand-water interface despite measurable dissolved O_2 to a depth of 2 mm below the interface whereas a distinct layer of Fe oxide formed at this same interface in microcosms containing BrY alone. Direct microscopic observations revealed close juxtapositioning of both organisms in the upper few mm of sand. Subsequent investigations using the identical experimental system noted relatively low concentrations of Fe(II) in the co-culture relative to the microcosm containing BrY alone and suggested that Fe(III)-binding ligands impeded the formation of Fe(III) oxides and were responsible for a soluble/colloidal Fe(III) phase that facilitated the redox cycling of Fe (Roden et al. 2004). These results established the potential for a tight coupling between microbial metal reduction and oxidation processes to promote rapid microscale cycling of Fe. More research, however, is needed to better define the nature and role of Fe(III)-complexing ligands in microscale Fe cycling and whether interactions between metal-reducing and metal-oxidizing extend beyond simply Fe(II)-Fe(III) cycling.

SUMMARY

Most of the electron acceptors respired by prokaryotes (O_2, NO_3^-, SO_4^{2-}, and CO_2) are soluble both before and after reduction, while many of the metals respired by DMRB exhibit substantially different solubility properties in the oxidized versus the reduced states. Because Fe(III) and Mn(III,IV) exist predominantly as oxyhydroxide minerals in oxic environments, DMRB must overcome the fundamental problem of engagement of the electron transport system with poorly soluble minerals. Other metals, such as U(VI) and Tc(VII), are relatively soluble in oxic environments, typically as anionic uranyl carbonate complexes and as pertechnetate, respectively. Aqueous Tc(VII) and U(VI) and other soluble electron acceptors are therefore free to enter the cell periplasm through porins or channels in the OM. Upon reduction to the +4 oxidation state, however, U and Tc precipitate as uraninite and hydrous Tc(IV) oxides, phases that have been identified in anaerobic suspensions of DMRB cells incubated with U(VI) or Tc(VII) and appropriate electron donors. The dilemma of reducing soluble electron acceptors to insoluble end-products is no less serious than the one dealing with reduction of solid electron acceptors. The first section of this chapter has highlighted the latest findings on the novel respiratory strategies employed by DMRB to overcome this dilemma, including direct enzymatic reduction, electron shuttling pathways and metal solubilization by exogenous or bacterially-produced organic ligands followed by reduction of soluble organic-metal compounds. The second section has emphasized the geochemical consequences of DMRB activity, including the direct and indirect effects on metal solubility, the reductive

transformation of Fe- and Mn-containing minerals, and the biogeochemical cycling of metals at redox interfaces in chemically stratified environments.

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