Johannes Gescher
Andreas Kappler *Editors*

Microbial Metal Respiration

From Geochemistry to Potential Applications

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Editors
Johannes Gescher
Institute for Applied Biosciences
Karlsruhe Institute of Technology
Karlsruhe
Germany

Andreas Kappler
Center for Applied Geoscience (ZAG)
Eberhard-Karls-University Tuebingen
Tuebingen
Germany

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Preface

Metal respiration is an exciting research field. Researchers with various scientific backgrounds and interests have developed hypotheses and proven concepts that allowed the identification of mechanisms and the evaluation of implications of microbial metal respiration. This includes the biochemistry of this respiratory process, the environmental impact on soils and sediments, mineral transformation and the potential application in remediation and even microbial fuel cell development. The interdisciplinary character of this research field should be motivation for students to get involved in this field, since they have the opportunity to experience different ways of thinking and to learn methods from molecular biology to synchrotron radiation-based analyses.

In this field we generally build on the initial work of microbiologists who were open enough to believe that rock respiration is possible, which led to the first isolation of metal reducing bacteria. In this book the editors brought together "second or third generation" experts studying metal respiration, building on the initial work done in the 1980s. In different chapters, we cover a substantial amount of what we know about the mechanisms and applications of microbial metal respiration. We are aware that certain aspects such as metal reduction by Archaea and Gram-positive microorganisms are only insufficiently covered, which is mostly due to the limited amount of knowledge we have so far in these research directions. Nevertheless, future editions will most probably include chapters about the biochemistry of Archaeal and Gram-positive metal reduction, and the environmental influence of these organisms, as well as their influence on the formation of (secondary) minerals.

Gratitude is expressed to the participating authors, all of them experts in the field that took some of their valuable time to work together with us on this project. We would also like to thank Anette Lindqvist from Springer publishing for patience and help throughout the production process.

A. Kappler
J. Gescher

Contents

Minerals and Aqueous Species of Iron and Manganese as Reactants and Products of Microbial Metal Respiration

Juraj Majzlan

Abstract Minerals and aqueous species of redox-active elements are common participants in the processes of microbial metal respiration. Redox-active elements may be major or minor constituents of minerals and mineraloids. They are often adsorbed onto the surfaces on minerals that may or may not be involved in microbial metal respiration. They may be adsorbed onto or incorporated in solid-like organic matter; harvested by and contained in living cells; associated with aqueous colloidal matter, organic or inorganic; dissolved and complexed with humic substances; dissolved in the aqueous phase, possibly complexed with inorganic anions. Given their sheer abundance, iron and manganese are the most important elements from this point of view. Mineralogy of Fe is controlled by the two common oxidation states, +2 and +3. The three commonly available oxidation states of Mn (+2, +3, +4) make the mineralogy of this transition metal even more variable. Besides the chemical and crystallographic aspects of minerals of Fe and Mn, this chapter also briefly refers to the mounting evidence that essentially all near-surface minerals of Fe and Mn are involved in microbial metal respiration. In addition to the minerals of Fe and Mn, minerals with layered structure are discussed. These embrace clay minerals and layered double hydroxides; the latter group includes the ephemeral but important green rusts. Redox potentials for many of the minerals of Fe and Mn are calculated and the dependence of redox potentials on the particle size of iron oxides is quantitatively evaluated.

J. Majzlan (✉)
Institute of Geosciences, Friedrich Schiller University, Burgweg 11,
07749 Jena, Germany
e-mail: Juraj.Majzlan@uni-jena.de

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1 Introduction

Near-surface geomaterials, be they soils, sediments, or perhaps anthropogenic wastes, are complex mixtures which consist of crystalline inorganic phases, materials of inorganic or organic compositions with a poorly defined structure, liquid or gas phases, and living organisms. The interaction among these constituents determines the evolution of these geomaterials. The importance of the individual constituents for the biogeochemical cycling of elements may vary, not only in time and space, but also from different perspectives and perception points from which this importance is evaluated.

Here, we focus on the inorganic constituents, not necessarily inorganic by their origin (e.g., iron oxide biominerals) but inorganic in their composition. The phases with redox-active elements stand in the spotlight of this contribution, primarily because they can be exploited by the microbial life as deposition sites of electrons. Yet, other phases present in the near-surface geomaterials are also mentioned and briefly described to complete the mineralogical, chemical, and thermodynamic portrait of these settings.

2 Inorganic Phases

From the point of their origin, the solid inorganic phases in the near-surface geomaterials can be divided into two large groups, the *detrital phases* and the *authigenic phases*. The former comprise minerals derived directly from the bedrock by mechanical disintegration, for example, quartz grains weathered out from a granite. Some of these detrital minerals are relatively chemically inert, as the already mentioned quartz, and will not contribute significantly to the element cycling within the geomaterials under "normal" conditions (that is, conditions usual—not extreme—in terms of temperature, pressure, pH, Eh, and elemental concentrations for unpolluted natural environments). Other detrital minerals may give way to newly formed, that is, authigenic minerals. These may form as a result of a solid-state reaction from the detrital minerals. More commonly, the authigenic phases precipitate within the near-surface geomaterials either inorganically, upon the action of the liquid and gaseous phases (usually aqueous solutions and air) or as a result of biological activity.

All elements present in the geomaterials participate in the cycling of matter within the near-surface geomaterials. Some of them may be more important than the others simply because of their sheer abundance. Others may be more central if one considers their solubility in the aqueous phase. Yet, others may derive their significance from their ability to switch between oxidation states, as mentioned above. We should not be, however, fooled by a false impression that there are elements, which are completely inert, apart from the gaseous noble elements. We know that gold is mobile in near-surface weathering zones of ore deposits and the

release of silver nanoparticles appears to modify the inorganic and biotic element cycling in polluted sediments (Kaegi et al. 2011). In "normal" near-surface geomaterials, elements such as Au or Ag are, of course, not of prime importance. The key elements in the normal settings are *iron* and *manganese* because of their abundance and redox activity, and *silicon* and *aluminum* because of the omnipresence. Common minerals of these elements are dealt with in the following text.

According to their bulk chemical composition, the minerals which store and release iron and manganese can be broadly divided into two large groups. The first group comprises *those minerals whose major components are Fe and Mn*; many members of this group are described below. The second group contains *minerals in which Fe and Mn are minor components*. For natural environments, these are especially clay minerals, also described below. The importance of these two groups is reservoirs of Fe and Mn vary in time and space. Canfield (1997) studied suspended particulates in 23 North American rivers and found that in most of them, Fe is carried in the silicate fraction, either as primary rock-forming silicates or secondary clay minerals. The non-silicate fraction can be split about equally to the crystalline and poorly crystalline Fe oxides. Later studies have indicated that much of the reactive iron oxides dispersed in near-surface waters form nanoparticles, which adhere to silicates (Poulton and Raiswell 2005). Canfield (1997) also determined that most, sometimes all Mn, were carried in the suspended river particulates in the form of Mn oxides. The silicate fraction was only subordinate for this element. Under reducing conditions, both Mn and Fe form simple carbonates or can be incorporated into sheet silicates. Iron associates strongly with sulfide, whereas manganese does not.

3 The Mineralogical Perspective

A *mineral* is defined as "a naturally occurring solid with a highly ordered atomic arrangement and a definite (but not fixed) chemical composition. It is usually formed by inorganic processes" (Klein 2002). The clause "usually formed by inorganic processes" was added relatively recently to allow the biologically formed phases to be called minerals. As the definition clearly states, a mineral must have a defined chemical composition or a range thereof and a periodic structure. Phases such as quartz and hematite comply with these criteria and are minerals. Materials such as ferrihydrite, a compound central to iron cycling in oxidized near-surface geomaterials, do not possess a long-range three-dimensional periodic structure and therefore are not minerals *sensu stricto*. For such compounds, the term *mineraloid* has been introduced (Klein 2002). In the literature, they are still often referred to as minerals *sensu lato*.

Metal cations, such as Fe^{3+} or Mn^{4+}, are surrounded in a structure by anions, for example O^{2-} or S^{-}. The anionic nearest neighbors constitute a coordination sphere and their number is the coordination number. The overall geometry of the coordination sphere is simply called the coordination, for example, tetrahedral or

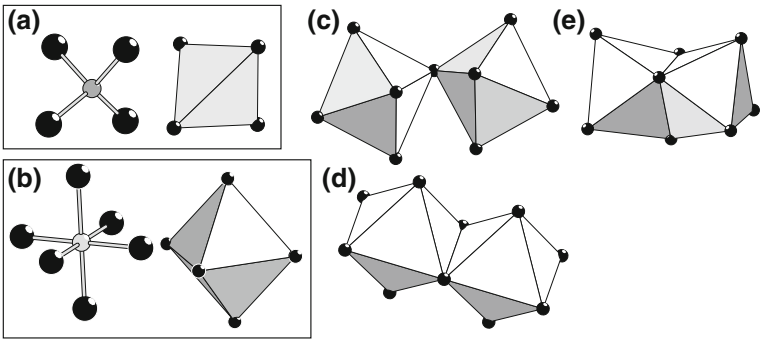

Fig. 1 Ball-and-stick (*left*) and polyhedral (*right*) representation of **a** a tetrahedral and **b** an octahedral coordination. Polyhedral representation of **c** corner-, **d** edge-, and **e** face-sharing octahedra. *Black spheres* are the O atoms

octahedral coordination (see Fig. 1a, b). These two coordination types are common in minerals and aqueous species of all elements considered in this review. The polyhedra (for example, octahedra) then attach by sharing a corner, edge, or a face—that is, one, two, or three anions, respectively (Fig. 1c–e). A final word of caution fits very well into this section. A lattice is a mathematical construct which represents the translational symmetry of a structure. A motif is a unit which periodically repeats in a structure, as dictated by its translational and non-translational symmetry. Hence, a structure can be broken down to a lattice and a motif and the terms structure and lattice are not synonymous, as very often presented in the literature. A lattice does not comprise any atoms; therefore, a lattice can accept no substitution; there is no "structure lattice" or "lattice structure", as reported in many papers.

3.1 Iron Minerals

Mineralogy of iron is controlled by the two usual oxidation states of this element, +2 and +3. Both of them are found abundantly in near-surface minerals and mineraloids. In addition, microbial life is the most important agent for the turnover of Fe(II) and Fe(III) and the formation and dissolution of Fe(II) and Fe(III) minerals in nature (Konhauser et al. 2011). Hence, the microbial metal respiration plays a dominant, not a subordinate role in cycling of Fe in the near-surface environments.

A general portrait of the non-silicate mineralogy of iron emerges rapidly from a pH–pe phase diagram of the system Fe–C–S–O–H (Fig. 2a). Under reducing conditions, pyrite, FeS_2, is occupying a large portion of the diagram. As will be discussed below, there is an entire group of iron sulfides reported from reduced sediments, pyrite being the stable one. Under conditions chosen for the construction of Fig. 2a, siderite, $FeCO_3$, has also a small stability field. This phase is indeed seen to form in natural settings. Iron oxides span large areas of the diagram. Under

reducing conditions and absence of sulfur, magnetite, Fe_3O_4, may form. Other mixed-valence iron phases, metastable and ephemeral in their existence, are also known and described below. Under oxidizing conditions, hematite, Fe_2O_3, is the most stable phase. The Fe^{3+} oxides, however, are not restricted to hematite; a number of metastable phases which do not show up in Fig. 2a is known from natural systems (Konhauser 1998) and described below.

Common minerals of divalent *ferrous iron* are listed in Table 1. Minerals of ferrous iron include oxides, carbonates, sulfides, and silicates. Note that the discussion of layered compounds, including phyllosilicates, whose chemical variability extends significantly beyond ferrous and ferric iron, is deferred to the section *Layered compounds* below.

The oxide mineralogy of ferrous iron is dominated by *magnetite*, $Fe^{2+}Fe^{3+}{}_2O_4$, a mixed ferrous–ferric oxide (Fig. 3). Magnetite belongs to the spinel group, a group of minerals whose structures are based on cubic close packing of oxygen anions (Fleet 1981). The cations fill tetrahedral and octahedral interstices in the array of the oxygen anions. Magnetite in sediments has been extensively studied, especially with respect to the magnetic properties of these sediments (Dunlop and Özdemir 1997). The biological origin of some sedimentary magnetite has been recognized early and the biologically induced and controlled mechanisms has been distinguished (Lowenstam 1981). The biologically controlled synthesis of magnetite in the magnetotactic bacteria attracted massive attention (Schüler 2010); interestingly, the biologically induced magnetite production by dissimilatory Fe(III)-reducing bacteria exceeds that of the magnetotactic bacteria by a factor of several thousand (Konhauser 2007). As an alternative to Fe(III) reduction, Chaudhuri et al. (2001) proposed that anaerobic microbial oxidation of Fe(II) may also account for the magnetite formation. Interestingly, they argue that this Fe(II) does not have to be present as a dissolved ion in an aqueous solution but the microorganisms can also respire Fe(II) in the crystal structure of relatively insoluble minerals. The microbial formation of magnetite depends on many factors, such as concentrations of iron, humic acids, ferrihydrite, oxyanions, bacterial cells, and redox state of iron in the system (O'Loughlin et al. 2010; Piepenbrock et al. 2011).

Ferrous carbonate, $FeCO_3$, is the mineral *siderite*. Siderite is isostructural with a series of simple carbonates (Effenberger et al. 1981), the most common one being calcite, $CaCO_3$. The structures of these minerals comprise octahedrally coordinated divalent cation (Fe^{2+}, Ca^{2+}, Mn^{2+}) and relatively rigid $(CO_3)^{2-}$ groups. Siderite is an important inorganic phase which couples the biogeochemical cycles of Fe and C (e.g., Roh et al. 2003). The formation of siderite is thought to be often associated with the bacterial respiration of organic matter coupled with dissimilatory Fe reduction (Pye et al. 1990). Siderite forms under reducing conditions and oxidizes readily in contact with today's atmosphere. Under metamorphic conditions, siderite may react to form Fe-bearing silicates. Because of its instability, the preservation of siderite in the geological record may be fragmental (Kholodov and

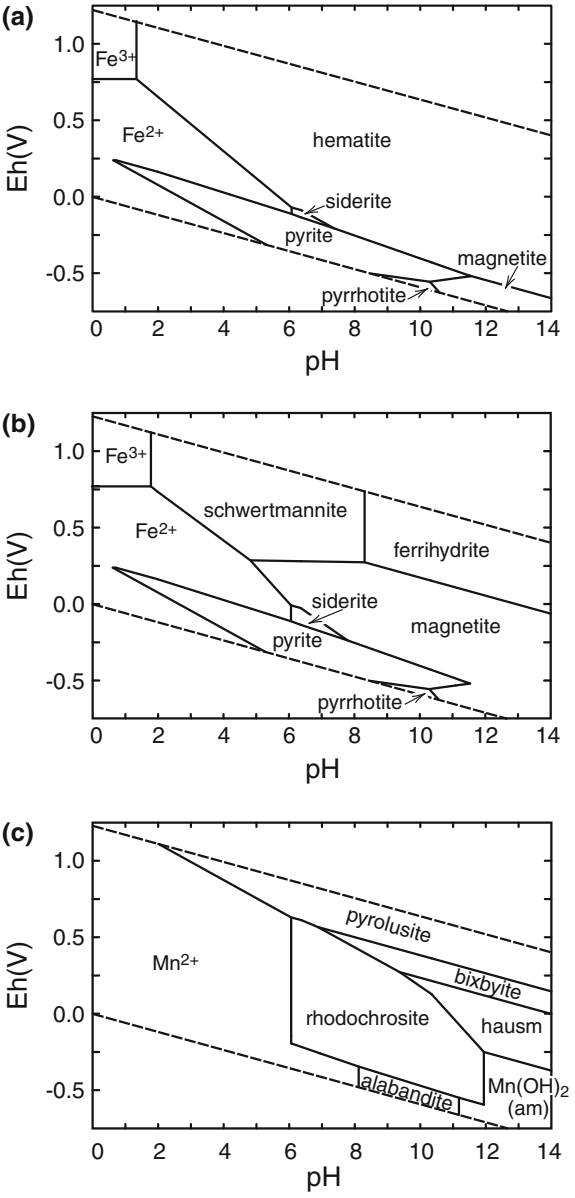

Fig. 2 Eh–pH diagrams for the systems **a** Fe–S–C–O–H, **b** Fe–S–C–O–H, with crystalline Fe(III) oxides suppressed from the calculations, **c** Mn–S–C–O–H. In all diagrams, the fugacities or activities were fixed at these values: fugacity of CO_2 at 10^{-2}, activity of dissolved sulfur at $10^{-4.5}$, activity of the dissolved metals (either Fe or Mn) at 10^{-4}. All minerals are discussed in the text except: pyrrhotite, $Fe_{1-x}S$; bixbyite, Mn_2O_3; hausm = hausmannite, Mn_3O_4; alabandite, MnS; $Mn(OH)_2$ (am), amorphous $Mn(OH)_2$. Calculated with Geochemists' Workbench

Butuzova 2008) and this mineral may have formed in greater quantities than those assumed from the present day occurrences.

The mineralogy of iron sulfides is much more complex than that of ferrous oxides or carbonates. The ultimate product of the many transformations among the iron sulfides is the mineral *pyrite*, FeS_2, the most common sulfide of all (Schoonen 2004). The structure of pyrite is built by octahedrally coordinated Fe^{2+} cations and

Table 1 Minerals in which iron is the major cation

Minerals of ferrous or ferrous–ferric iron		
Oxides	Carbonates	Sulfides
Magnetite	Siderite	Mackinawite
	Green rust	Pyrite
Minerals of ferric iron		
Oxides	Oxyhydroxides	
Hematite	Goethite	
Maghemite	Lepidocrocite	
	Akaganeite	
	Feroxyhyte	
	Schwertmannite	
	Ferrihydrite	

covalently bonded $(S_2)^{2-}$ pairs. Hence, strictly speaking, pyrite is a disulfide and not a sulfide. It is generally believed that pyrite does not crystallize directly from reduced solutions in which Fe^{2+} and reduced sulfur come into contact (Schoonen and Barnes 1991). The initial precipitates are *X-ray amorphous Fe sulfides* of variable stoichiometry. Detailed studies on these precipitates show that their local structure can be likened to the bulk structure of the mineral *mackinawite*, FeS (Michel et al. 2005). This structure contains clusters of tetrahedrally coordinated Fe^{2+}. Another Fe sulfide often reported from sediments is the mineral *greigite*, Fe_3S_4, isostructural with magnetite. The generally accepted transformation sequence is amorphous Fe sulfides—mackinawite—(greigite)—pyrite. The nature of iron sulfides in soils and sediments is usually determined by sequential acid digestion with the associated limitations (Billon et al. 2001); detailed mineralogical information is usually missing. Szczepanik and Sawlowicz (2010) reported a direct observation of mackinawite, greigite, and pyrite in Jurassic sediments.

The mineralogy of *ferric iron* (Table 1) is dominated by oxides and oxyhydroxides; carbonates or sulfides of Fe^{3+} are unknown. The oxides and oxyhydroxides are collectively called for simplicity iron oxides. All conceivable properties of iron oxides have been reviewed in more than 600 pages by Cornell and Schwertmann (2000). Hydroxides of Fe^{3+} are also known but are extremely rare (the mineral bernalite, Birch et al. 1993). The structures of the iron oxides are based on cubic or hexagonal close packing of oxygen anions. The interstices occupied by Fe^{3+} have mostly octahedral, rarely tetrahedral coordination.

The most common crystalline ferric oxides are *hematite*, α-Fe_2O_3, and *goethite*, α-FeOOH (Fig. 3). Both structures are based on hexagonal close packing array of oxygens. The structure of hematite can be described as a densely packed assembly of octahedral sheets where pairs of octahedra from adjacent sheets share a face. Face sharing is rare among minerals and phases in general and usually results in instability of such phase; not so in hematite and related compounds. The structure of goethite is built by octahedral double chains. Within the chains, the octahedra share edges. The chains attach to each other by corners and create the 2×1 tunnels.

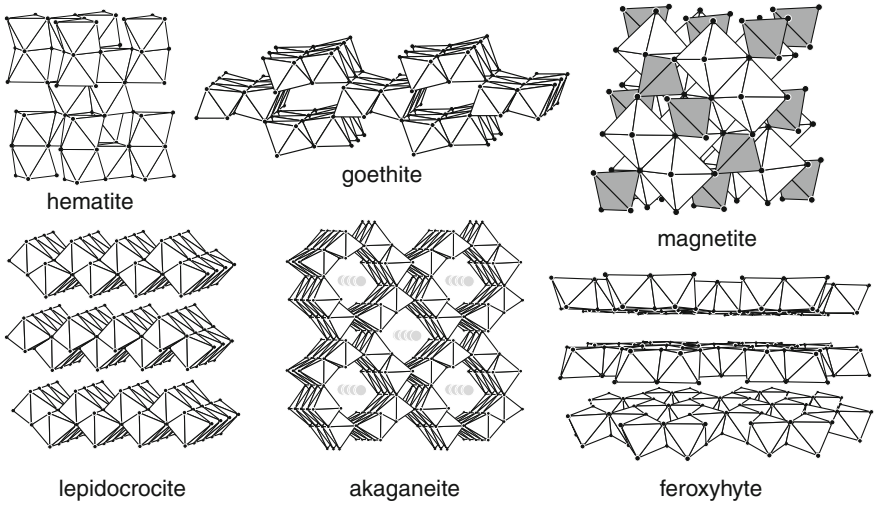

Fig. 3 Polyhedral representations of the crystal structures of Fe(III) oxides. *White polyhedra* are octahedra, *medium gray polyhedra* are tetrahedra. The *light gray spheres* in akaganeite represent the Cl^- atoms in tunnels. For clarity, hydrogen atoms are not shown

Hematite and goethite are the thermodynamically stable iron oxides and the most common crystalline iron oxides in soils and sediments. As the most stable phases, they are the ones to be attacked least by various extraction protocols, for example Na dithionite. The fraction of the Fe extractable by Na dithionite is used as an indicator of soil maturity (Cornell and Schwertmann 2003). Hence, broadly speaking, the fraction of hematite and goethite is a measure of advancement of ferric oxide transformation to the stable assemblage. Goethite is the most common soil Fe oxide in all regions, hematite is also common but missing in temperate and cool regions (Schwertmann 1985). Goethites, less commonly hematites, are the products of slow transformation of the poorly crystalline Fe oxides such as ferrihydrite; the transformation mechanism may involve intermediate metastable phases (e.g., Nesterova et al. 2003). Goethite is a common, although probably not the most common Fe oxide formed via biologically induced Fe(III) precipitation. The most common biologically induced Fe(III) oxide is most likely ferrihydrite. Precipitation of goethite via microbial metal respiration has been documented, for example by Miot et al. (2009) or Larese-Casanova et al. (2010).

Maghemite, γ-Fe_2O_3, is the other anhydrous ferric oxide beside hematite, of some importance in near-surface environments. Maghemite is also a spinel mineral, just like magnetite, based on a cubic close packed array of oxygen anions. Fe^{3+} cations occupy octahedral and tetrahedral positions (Fig. 3) but the stoichiometry and the structure requires some of the positions to remain vacant. It is a matter of ongoing debate on which positions are vacant and which ones are filled by the Fe^{3+} ions (e.g., Grau-Crespo et al. 2010). The easiest and by far the most common route for maghemite formation is the oxidation of magnetite, both detrital and authigenic (the latter can be of inorganic or biological origin). In either case,

maghemite carries the chemical features of the original magnetite, for example, elevated Ti content from the primary titanomagnetite (Allan et al. 1989).

The minerals *lepidocrocite*, γ-FeOOH, *akaganeite*, β-FeO(OH,Cl), and *feroxyhyte*, δ'-FeOOH (Fig. 3) are not as common as hematite or goethite. The structure of lepidocrocite is constructed by corrugated layers of edge-sharing octahedra. Akaganeite has a tunnel structure, with 2×2 tunnels. The tunnels are large enough to host an additional ion, most commonly the Cl^- anion. The chloride ion may or may not be an essential component of the structure. Natural feroxyhyte is usually quite disordered and its structure is not known well (Manceau and Drits 1993). Better crystallinity can be achieved in the laboratory (Koch et al. 1995) and these materials are made of flat octahedral sheets, similar to those found in the structure of gibbsite (see below). Of these three FeOOH polymorphs, lepidocrocite is the most common one, although far less common than goethite. Inorganically, lepidocrocite forms in environments with a sufficient aqueous Fe(II) concentration and relatively rapid oxidation of Fe(II); the presence of CO_2 or Al supports the crystallization of goethite at the expense of lepidocrocite (Carlson and Schwertmann 1990). Experiments with microorganisms have shown that lepidocrocite and goethite may be formed simultaneously by nitrate-reducing, Fe(II)-oxidizing bacteria in natural environments (Larese-Casanova et al. 2010).

An intermediate, ephemeral group of phases which are produced by microbial respiration and rapidly transformed to other minerals are the *green rusts*. These minerals are Fe(II)–Fe(III) double hydroxides with additional anions in their structure. The entire group of these phases will be described below, in the section *Layered structures*, with emphasis on green rusts, their structure, and role in metal respiration and biomineralization.

Schwertmannite, nominally $Fe(OH)_{3/4}(SO_4)_{1/8}$, belongs to the group of iron oxides despite that sulfate appears to be an essential component of this material. Schwertmannite is poorly crystalline and in the original description, Bigham et al. (1990) proposed that schwertmannite is structurally related to akaganeite. The tunnels in schwertmannite should be then occupied by the $(SO_4)^{2-}$ anions. The only caveat of this assumption is that the sulfate anions are too large for these tunnels, a fact that is willingly ignored in the literature since then (Fernandez-Martinez et al. 2010). The tunnel-like units may, in fact, exist, but sulfate may attach to the surfaces of the very small particles of schwertmannite as hydrogen-bonded complexes and not reside within the tunnels (Majzlan and Myneni 2005). Schwertmannite is restricted to environments rich in Fe(III) and sulfate ions under moderately acidic conditions (Fig. 2b) where it forms via inorganic or biologically induced precipitation (e.g., Hedrich et al. 2011). Under near-neutral conditions, ferrihydrite (see below) will precipitate.

The most poorly crystalline compound among iron oxides is *ferrihydrite*, a hydrated ferric oxide-oxyhydroxide. There is no agreement in the literature as to what should be the nominal composition of ferrihydrite. Because of its great importance in the near-surface environments, properties and structure of ferrihydrite is a subject of intensive research. The latter, the structure, is also a subject of a heated debate. The question of the structure of ferrihydrite was addressed by

Drits et al. (1993), Manceau and Drits (1993), Janney et al. (2000a, b, 2001), Jansen et al. (2002), Michel et al. (2007, 2010), and Manceau (2011). Ferrihydrite is considered to consist of a range of nanocrystallites with variable structure or a single structure without long-range order.

Ferrihydrite is likely the most common initial precipitate from aqueous solutions which reach saturation with respect to Fe(III). This substance is produced in copious amounts by the action of Fe(II)-oxidizing bacteria or during inorganic oxidation of Fe(II). Konhauser (1998) mentioned springs, sediments, soil pores, aquifers, hydrothermal systems, mine wastes, and water distribution systems as examples of environments where ferrihydrite may be formed.

Ferrous and ferric iron have a rich mineralogy in environments with abundant *sulfate, phosphate, or arsenate* anions. Such conditions are limited to specific settings, for example acid mine drainage (Majzlan 2010), and will not be considered here in detail. For the mineralogy and crystallography of Fe sulfates and phosphates, the reader is referred to Hawthorne et al. (2000) and Huminicki and Hawthorne (2002), respectively.

4 Manganese Minerals

Manganese occurs in natural systems in the oxidation states +2, +3, and +4. An additional possible oxidation state, as compared to iron, adds to the richness of the mineralogy of manganese. As in the case of iron, mineralogy and geochemistry of manganese can be elucidated in a pH–pe diagram (Fig. 2c). The Mn carbonate, rhodochrosite, has a prominent stability field at broad circumneutral and moderately reducing conditions. The only other phase relevant to common natural systems in Fig. 2c is pyrolusite, MnO_2. This phase is stable under oxidizing conditions over a range of pH values attainable in normal natural settings. It is necessary to note that there are more than 30 Mn^{4+} oxides known (Post 1999), all metastable with respect to pyrolusite. This means that the formation conditions of all these Mn^{4+} oxides overlap with those of pyrolusite. In other words, the stability field of pyrolusite in Fig. 2c represents the formation conditions of a large group of Mn^{4+} oxides. The other minerals depicted in Fig. 2c are of little interest for the processes in near-surface geomaterials.

Divalent manganese may be immobilized in *rhodochrosite*, $MnCO_3$, a mineral isostructural with calcite and siderite (Effenberger et al. 1981). Rhodochrosite precipitates if the aqueous concentrations of Mn^{2+} and CO_2 build up sufficiently to exceed the solubility product of this mineral. In fact, rhodochrosite is the most abundant manganese mineral in sedimentary ores (Maynard 2010), meaning that such conditions are met in oceanic sediments. Roy (2006) argued that these conditions can be established only in O_2-stratified oceanic basins where the insoluble Mn^{4+} oxides (see below) precipitate in the upper, oxidized strata and "rain" to the ocean floor. There, they will dissolve under reducing conditions and combine with biologically derived bicarbonate ions to form rhodochrosite (see

references in Roy 2006). Yet, in contact with the atmosphere, Mn^{2+} will be oxidized to Mn^{4+} and give way to a different group of minerals.

The oxides, oxyhydroxides, and hydroxides of Mn^{4+} are collectively called Mn oxides. They contain predominantly Mn^{4+} but Mn^{3+} seems to be present almost always in a small concentration. The replacement of Mn^{4+} by Mn^{3+} must be compensated by an additional positive charge, usually supplied in a form of mono- or divalent cations which reside in the structure.

There is no shortage of review publications on oxides of manganese. The mineralogical aspects were examined by Post (1999) and Vodyanitskii (2009). Technical aspects of manganese oxides, very important as energy storage media in batteries, were summarized by Brock et al. (1998). A treatise on biogenic manganese oxides was published by Tebo et al. (2004). For mineralogy and chemistry of manganese ores, see Maynard (2010).

Mn oxides are divided in terms of their crystal structures into tunnel and layer structures (Post 1999). The stable Mn oxide, *pyrolusite*, MnO_2, represents the simple tunnel structure, with 1×1 tunnels between the octahedral chains (Fig. 4). Another anhydrous Mn oxide, *ramsdellite*, MnO_2, possesses 2×1 tunnels (Fig. 4), hence is isostructural with goethite. Further expansion of the framework leads to 2×2 tunnels in the structure of *hollandite* (Fig. 4), $Ba_x(Mn^{4+},Mn^{3+})_8O_{16}$, a mineral isostructural with akaganeite. Larger tunnels, namely 2×3 and 3×3, are found in the structures of *romanechite*, $Ba_{0.66}(Mn^{4+},Mn^{3+})_5O_{10}{\cdot}1.5H_2O$, and *todorokite*, $(Ca,Na,K)_x(Mn^{4+},Mn^{3+})_6O_{12}{\cdot}3.5H_2O$ (Fig. 4). As the mineral formulae suggest, the structures with larger tunnels can tolerate the coexistence of Mn^{4+} and Mn^{3+} within their framework and therefore must accept cations (Ba, Ca, Na, K) to compensate the charge deficit. The mono- and divalent cations reside in the tunnels. Some of these phases, especially todorokite, may encapsulate cations such as Ni or Co in economically interesting amounts, for example in the deep-sea manganese nodules (Burns and Burns 1978).

The layer structures of manganese oxides include especially *birnessite*, $(Na,Ca)Mn_7O_{14}{\cdot}2.8H_2O$ (Fig. 4). Other minerals have been described and characterized (see Post 1999) but they require elements normally not present in high amounts in the natural settings (e.g., Zn in chalcophanite or Li in lithiophorite). The structure of all these minerals, however, is based on octahedral sheets of Mn^{4+}- or Mn^{3+}-centered octahedra. These sheets show an amazing flexibility in terms of Mn^{4+}/Mn^{3+} ratio, possible substitutions at the octahedral sites, and attachment of additional polyhedra (octahedra, but also tetrahedra) to the sheets. The other important variable is the composition of the space between the sheets which can be occupied by cations and/or H_2O molecules.

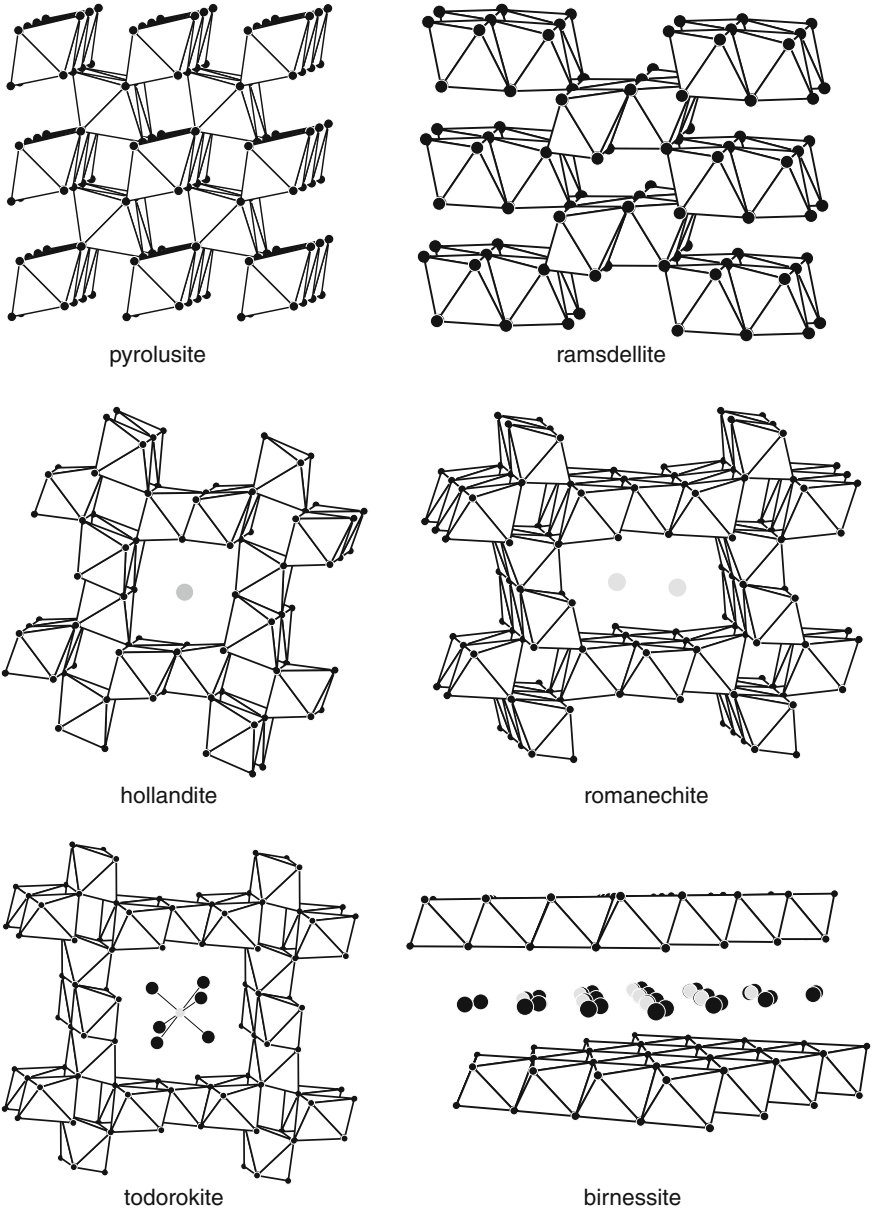

Fig. 4 Polyhedral representation of the crystal structures of Mn(IV) oxides. The *light gray spheres* in the tunnels or interlayers of the structures represent cations other than $Mn^{3+}/^{4+}$. In todorokite, a coordination sphere of such cation is also shown. The *black sphere* in the interlayer of birnessite is made of O atoms (possibly water molecules) which coordinate the interlayer cations. For clarity, hydrogen atoms are not shown

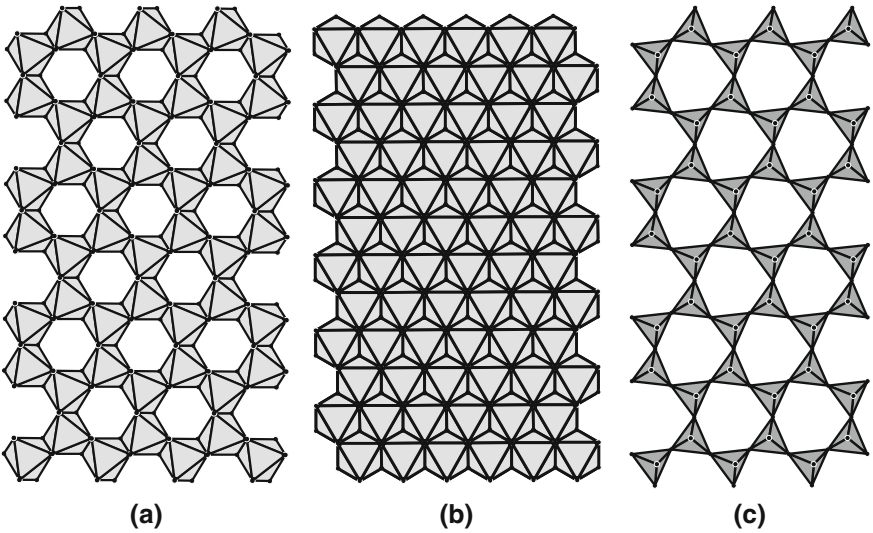

Fig. 5 Sheets found in the structures of common clay minerals and hydrotalcites. **a** Dioctahedral, **b** trioctahedral, **c** tetrahedral sheet. To highlight the vacancies in the dioctahedral sheets, the octahedra are *light gray*, unlike in the other figures. Tetrahedra are *medium gray*

5 Layered Structures: Layered Double Hydroxides and Clay Minerals

Although we encountered some layered structures already among the iron and manganese oxides, there are still two groups of minerals with an important role in the near-surface geomaterials. They are chemically variable and it is better to describe them separately, although some of them could be broadly assigned to the iron oxide phases.

Structurally, all these phases are built by octahedral and tetrahedral sheets. There are two types of octahedral sheets, the dioctahedral and the trioctahedral sheets, found in the structures of gibbsite, $Al(OH)_3$, and brucite, $Mg(OH)_2$, respectively (Fig. 5a, b). The prefixes di- and tri- refer to the coordination of the $(OH)^-$ anions within the sheets. The tetrahedral sheet (Fig. 5c) has an overall composition of $[Si_2O_5(OH)]^{3-}$; because of its charge, it cannot occur separately but must be charge-compensated, for example, by combining with an octahedral sheet.

The first group of layered compounds of interest are the *layered double hydroxides* (LDH) with a general formula $\left[M^{2+}{}_{1-x}M^{3+}{}_{x}(OH)_2\right]^{x+}\left(A^{n-}{}_{x/n}\right)\cdot mH_2O$ The mineral hydrotalcite, $Mg_6Al_2(OH)_{16}(CO_3)\cdot 4H_2O$, is an example of the architecture of these phases (Fig. 6). There is a partial substitution of Mg^{2+} by Al^{3+}(or, in general, M^{2+} by M^{3+}) in the brucite-like sheets in hydrotalcite. This heterovalent substitution (i.e., substitution of ions with unlike oxidation states) causes the sheets to have a permanent positive charge. This positive charge

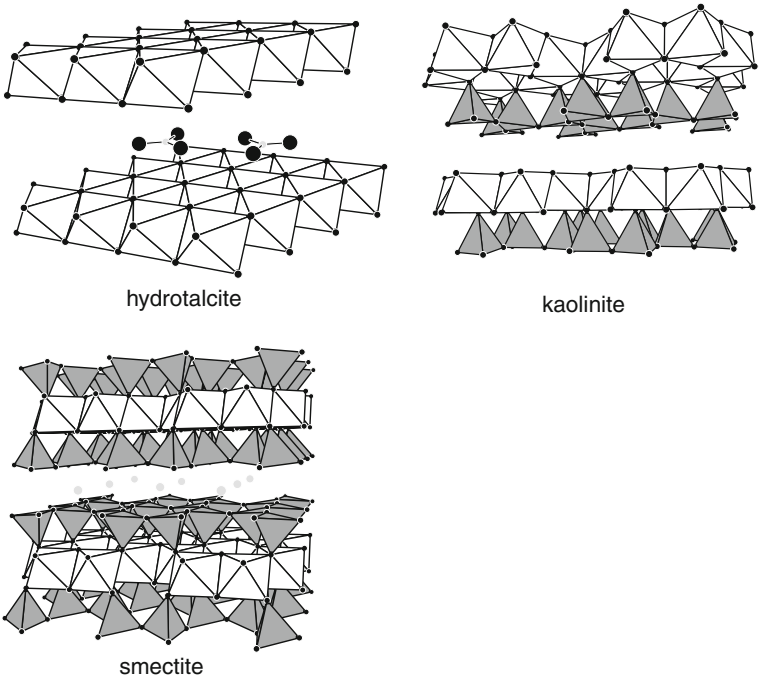

Fig. 6 Polyhedral representation of the crystal structure of layered compounds mentioned in the text. *White polyhedra* are octahedra, *medium gray polyhedra* are tetrahedra. For hydrotalcite, two carbonate (CO_3^{2-}) groups are shown in the interlayer. For kaolinite, two T–O layers are displayed. For smectite, two T–O–T layers are shown, with *gray spheres* representing the interlayer cations, most commonly Na^+ or Ca^{2+}

must be compensated by the anions A^{n-} which reside in the space between the sheets. In hydrotalcite, these are the carbonate anions, but other anions, such as chloride, sulfate, hydroxide, nitrate, perchlorate (Brindley and Kikkawa 1980), carboxylate, or aromatic anions (Meyer et al. 2010) have been reported. Because of their ability to store and exchange anions in the space between the octahedral sheets, the LDH phases are sometimes also called anionic clays.

The LDH phases relevant within context of redox processes are the so-called *green rusts* (GR), $Fe^{2+} - Fe^{3+}$ layered double hydroxides (Chaves 2005). Being LDH phases, their structure is the same as described above. In the literature, the green rusts with monoatomic anions (Cl^-, Br^-, I^-, F^-) are referred as GRI. The green rust with the planar carbonate anion is called the GR-CO_3 and the green rusts with non-planar oxyanions (SO_4, SeO_4) as GRII. Green rusts were initially reported as a product of oxidation of steel (Stampfl 1969) but were since then found in soils (Trolard et al. 1997) or ochreous sediments (Bender Koch and Mørup 1991). Owing to their high reactivity, green rusts are believed to play a very important role in the redox element cycling in soils (Myneni et al. 1997).

Green rust is formed by biological reduction of Fe(III) substrates where goethite, lepidocrocite, or ferrihydrite (Ona-Nguema et al. 2009; Salas et al. 2010; Ekstrom et al. 2010) serve as the terminal electron acceptors. A phase which accompanies the precipitation of GR is magnetite. The rate of GR precipitation and the abundance of magnetite can be controlled by the degree of metal (e.g., Al) substitution in the Fe(III) oxides, adsorption of species (e.g., As) on the surfaces of the Fe(III) oxides, or the concentration of extracellular polymeric substances. In addition, GR itself can serve as a terminal electron acceptor (Zegeye et al. 2007); an example is the reduction of sulfate in GRII-SO_4 to sulfide and the conversion of the green rust to GR-CO_3.

The other important group of layered structures are the phyllosilicates and, as a subgroup of the phyllosilicates, the *clay minerals*. The structures of phyllosilicates in general are constructed by combinations of the tetrahedral and octahedral sheets. In this case (as opposed to LDH), the sheets actually attach to each other to define layers. These layers may contain one tetrahedral and one octahedral sheet (Fig. 6) and then are labeled as the T–O layers. An example in this group is the mineral kaolinite. Similarly, there are T–O–T layers (Fig. 6), made of an octahedral sheet sandwiched between two tetrahedral sheets. An example with interlayer cations Na^+ or Ca^{2+} is the minerals of the smectite group. The last combination is the T–O–T layers with an intervening O sheet. The octahedral sheets are most commonly populated by Al^{3+} or Mg^{2+} and are then either di- or trioctahedral, as already described above. A very common substitution of Al^{3+} for Si^{4+} is encountered in the tetrahedral sheets. This substitution causes a permanent negative charge of the layers and the need to compensate the negative charge with cations placed in the interlayer space. These cations may be fixed or exchangeable, further adding to the complexity of the phyllosilicate minerals. For a detailed geometric and crystal chemical analysis of the structures of clays, see the recent book of Meunier (2010).

The definition of a clay mineral is somewhat nebulous. In mineralogy, a clay mineral is a mineral from the phyllosilicate group with particle size of less than 2 μm (Murray 2007). Other definitions exist but are all based on particle size; a particularly vague definition states that the clay minerals should occur in a form of "extremely small crystalline particles" (see Murray 2007). Some of the phyllosilicates hardly ever attain the small size of 2 μm; examples are the rock-forming mica minerals. Others, for example the smectites, essentially never exceed this size. There are minerals, as chlorites, which may or may not satisfy the operational definition of being a clay mineral depending on their size.

So far, the clay minerals have been described here as dominated by Al, Mg, Si, O, and H. They may, however, contain other elements, in particular iron. Fe^{3+} can substitute for Al^{3+} in the octahedral sheets or rarely for Si^{4+} in the tetrahedral sheets. Fe^{2+} substitutes for Mg^{2+} in the octahedral positions. Although Fe-rich clay minerals are not as common as their Al- and Mg-dominated counterparts, the Fe-rich clay minerals are interesting from two points of view. First and obvious, Fe is a redox active element and these minerals may play an active role in the redox

cycling of elements in the environment. Kostka et al. (2002) have shown that iron(III)-reducing bacteria may utilize Fe(III)-rich clay minerals as the only electron acceptors. Second and less obvious, it seems that particularly those clay minerals which are a by-product of biological activity are rich in Fe (e.g., Konhauser and Urrutia 1999).

Other cations may be found, occasionally also in higher concentrations, in the clay minerals and in phyllosilicates in general. These are Mn^{2+}, Mn^{3+}, Cr^{3+}, Ni^{2+}, Ti^{4+}, and many others.

6 The Aqueous Perspective

The aqueous speciation of Fe and Mn can be described from the Eh–pH diagrams (Fig. 2). It is necessary to understand that these diagrams were constructed with an assumption that the concentration of ions or molecules with a strong tendency for Fe or Mn complexation is negligible. Such ions and molecules and their influence on metal solubility will be briefly discussed later in this section. The following discussion is based on diagrams (Fig. 2) which were calculated from the available thermodynamic data literature. It is crucial to be aware that thermodynamics deals with models of reality, not reality itself (Anderson 2005). This fundamental fact about thermodynamics is not to be confused with the observation that kinetic constraints may hinder formation of species or minerals in nature. In natural solutions and near the solubility limits of minerals, mineraloids, and organometallic substances, there is probably a whole zoo of species in the aqueous solutions whose characterization escapes us now with the techniques available to us.

Ferric iron is coordinated octahedrally in strongly acidic to mildly basic aqueous solutions, up to pH of $\sim$8.5. The only aqueous species with a substantial solubility is the one dominant at the lowest pH, that is, the hexa-aquo $\left[Fe(H_2O)_6\right]^{3+}$ complex. This complex deprotonates in several steps with an increasing pH and the deprotonated species have a great tendency to condense into a solid, that is, one of the iron oxide minerals. The solubility of ferric iron reaches its minimum at circumneutral values. Hence, under the usual natural conditions, very little ferric iron can be expected to be truly dissolved in an aqueous system.

Ferrous iron is also coordinated octahedrally but the hexa-aquo complex $\left[Fe(H_2O)_6\right]^{2+}$ deprotonates first at pH $>$ 10. The preference of this species to remain fully coordinated by H_2O molecules over a wide pH range and its limited tendency to associate with other ions (sulfate, carbonate, nitrate) are the reasons for the much higher solubility of Fe(II) in aqueous solutions.

The *aqueous speciation of manganese* is essentially the speciation of **Mn(II)**. The oxides of tetravalent manganese are so sparingly soluble that aqueous **Mn(IV)** can be principally considered as non-existent. Aqueous **Mn(III)** is unstable and dissociates spontaneously in Mn(II) and Mn(IV); the reasons for this reaction can be traced to the electronic configurations of these oxidation states of Mn

(Burns 2005). The speciation of Mn(II) is similar to that of Fe(II). The hexa-aquo complex $\left[Mn(H_2O)_6\right]^{2+}$ deprotonates first at pH > 10 and remains in the solution under a wide range of pH values.

The aqueous concentration of metal cations can be significantly enhanced via a process of *complex formation*. In this process, the cation associates with another molecule and remains dissolved. The association of metals with small carboxylic molecules (acetate, citrate, oxalate) has been recognized and utilized early, for example for extraction of Fe(III) by Schwertmann (1964) or in the process of $Al(OH)_3$ precipitation by Wang et al. (1983). Perhaps, more important is the association of metals with the carboxylic groups in the natural humic and fulvic acids; these complexes were shown to be of importance for Fe(II) (e.g., Luther et al. 1996), Fe(III) (e.g., Kuma et al. 1999; Tipping et al. 2002; Gustafsson et al. 2007), or Al(III) (Tipping et al. 2002).

An association of metals with larger organic molecules with an extremely high affinity for metals has been a subject of research in many studies. These molecules, *siderophores*, are known to associate strongly with iron (see Kraemer et al. 2005 for a review). Iron is, however, not the only metal captured and complexed by siderophores. Manganese (Duckworth and Sposito 2007), uranium (Mullen et al. 2007), or chromium, aluminum, galium, copper, and zinc (Zou and Boyer 2005) were shown to be complexed with various siderophore molecules.

7 The Surface Perspective

Most of the environmental reactions which involve minerals take place at the surfaces of the minerals. The surface structure of minerals certainly differs from simple cuts of the bulk structure. Once a solid is broken and a surface is created, some of the cations located directly at the new surface may have an incomplete coordination sphere. The missing–dangling bonds are compensated by additional bonding from the surrounding aqueous or gaseous phase, most commonly by attachment of H_2O molecules, sometimes their dissociation (Brown et al. 1999). Surfaces may reconstruct, create, re-arrange or annihilate vacancies, steps, terraces, and kinks. Surfaces are essentially defects in otherwise periodic crystal structures and as defects, they may be highly reactive.

Our understanding of crystal surfaces is not as thorough as the understanding of crystal structures. Bulk properties of surfaces have been carefully measured but the atomistic view is often incomplete or missing altogether. Because of methodological limitations, only surfaces of a few substances for which large single crystals can be grown, are known with some certainty. This restriction means that essentially nothing is known about the surfaces of many environmentally crucial substances, ferrihydrite for example.

Given that the interactions between the surfaces and their surrounding are controlled by hydrogen bonds, ionic interactions, and charge-transfer reactions

(Hansen 2011), an important bulk property of surfaces is their charge. In the simplest approximation, the charging behavior of the surfaces can be described by their point-of-zero-charge (pzc); below this point, the surfaces are positively charged and vice versa. The pzc values for iron oxides vary between 7 and 9 (Cornell and Schwertmann 2003), meaning that under neutral and acidic conditions, the surfaces of iron oxides are positively charged. Hence, iron oxides are potent adsorbents for inorganic and organic anions, such as phosphate (Dzombak and Morel 1990) or carboxylate anions (Hanna 2007) and surface binding of these ions and organic molecules (e.g. humics) changes the surface charge of Fe oxides significantly. The inorganic and organic moieties may compete for the adsorption sites (Kreller et al. 2003). In contrast, the pzc values for manganese oxides is much lower, between 1 and 4 (e.g., Tan et al. 2008) and under the usual environmental conditions, the surfaces of manganese oxides are negatively charged. Inorganic or biologically produced manganese oxides are, therefore, well known as adsorbents of cations such as Ni^{2+}, Zn^{2+}, Cu^{2+}(e.g., Manceau et al. 2003; Peña et al. 2010, and others). Surface chemistry of clay minerals is largely controlled by the (OH) groups which are an integral part of their structure (see Johnston 2010). Hydrogen bonding to the immediate surrounding and structuring of water molecules near the clay surfaces determines the nature of interactions of clays with inorganic and organic molecules. Naturally, the consideration of the overall surface charge conceals the protonation–deprotonation reactions at the specific binding sites. Yet, the simple models have worked very well for the description of surface reactivity of inorganic solids.

It is interesting to notice that similar bulk models may be applied to the surfaces of microorganisms, with success in describing the interactions between their surfaces and aqueous ions. Ginn and Fein (2008) studied proton and metal adsorption on bacterial walls of phylogenetically diverse microorganisms. They concluded that most of these organisms behave very similarly in terms of ions adsorption and their surface properties can be understood with a simple model. This model, proposed by Fein et al. (1997), assumes that the surface reactivity of bacterial walls is to be assigned to the carboxyl, phosphate, and hydroxyl groups, with pK_a values of $\sim$4.8, 6.9, and 9.4, respectively. Hence, the pzc values of such materials should lie within this range, thus dictating the electrostatic interactions with oxides of iron and manganese and clay minerals. The pzc values for 137 virus species scatter in the broad range of 1.9–8.4, with most common values being located between 3.5 and 7.0 (Michen and Graule 2010). These authors assigned the variation of surface charge on viruses to the protonation state of the carboxyl and amine groups.

The last decade has seen a rise of the number of the studies whose research question is the atomistic structure of mineral surfaces (see Fenter and Sturchio 2004). For a few model compounds, respectable amount of data is available, for example for the phyllosilicate mineral muscovite, a model compound for clay minerals (Lee et al. 2010). In some cases, the differences between individual faces on a crystal of a single phase have been investigated and documented

(Yanina and Rosso 2008). Redox reactions occur upon adsorption of Fe(II) onto the surfaces of Fe(III) oxides as the electron from Fe(II) is transferred to the bulk structure of the oxide substrate (Williams and Scherer 2004).

Experimental studies are complemented by computational ones (e.g., Kirkpatrick et al. 2005). These studies provide an unprecedented detailed view of the surfaces; surfaces of isostructural minerals corundum (α-Al_2O_3) and hematite (α-Fe_2O_3) have been shown to be similar but not identical (Catalano 2011). Furthermore, the electron transfer chains in redox reactions in iron oxides can be tracked to the minute detail in computational work (Wang and Rustad 2006).

8 Pools of Metals in Near-Surface Geomaterials

The overview of the possible metal species shows a great diversity of the reservoirs of metals in the near-surface geomaterials. An exhaustive characterization and fully quantitative assignment of the different hosts is very demanding. We know, however, that Fe and Mn in the near-surface geomaterials can be found

- in minerals (i.e., well-crystalline solid phases) in which these metals represent the dominant cations. Most of the minerals of iron and manganese mentioned above belong to this category. In natural settings, pure phases are an exception, substitution on structural sites the rule. The substitutions are a function of the major ion size and charge.
- In minerals in which these metals represent a minor cation. For Fe and Mn, clay minerals are the best examples; only rarely, Fe and Mn are the dominant octahedral cations in clay minerals.
- In mineraloids (poorly crystalline solids) in which these metals are also dominant phases. Examples are ferrihydrite, schwertmannite, and some of the other iron oxides, probably also green rust. Poorly crystalline and difficult-to-identify Mn oxides have been collectively called wad. Al–Si-rich substances such as imogolite and allophone also belong to this group.
- Adsorbed on the surfaces of minerals. For example, a positively charged Fe(III) species can adsorb onto the surface of an Mn oxide.
- Adsorbed onto or incorporated in solid-like organic matter, including, but not limited to biofilms, extracellular polysaccharide, and decaying remnants.
- Harvested by and contained in living cells, for example, iron in magnetotactic bacteria.
- Associated with aqueous colloidal matter, organic or inorganic.
- Dissolved and complexed with humic substances.
- Dissolved in the aqueous phase, possibly complexed with inorganic anions, for example phosphate or arsenate.

Note that this list was compiled from the perspective of metal ions. As the list indicates, organic matter, either solid-like or colloidal (nanoparticulate), adsorbs metal ions. On the other hand, surfaces of metal oxides, for example Mn oxides,

have been shown to adsorb organic matter (e.g., Tipping and Heaton 1983). By analogy, metal ions can be complexed in the aqueous solutions by small inorganic cations, such as phosphate. The surface of metal oxides, for example iron oxides, is known to adsorb phosphate very strongly (Cornell and Schwertmann 2003).

9 Redox Potentials of Fe and Mn Minerals

Redox potentials express the amount of energy that can be extracted, under given conditions, by redox transformation of a given species. Of course, this energy depends on the nature of the phase or species. The largest redox potential in the systems considered here is assigned to the aqueous Fe^{3+}/Fe^{2+} couple. As discussed briefly in the section on aqueous speciation, the only Fe(III) species soluble in significant concentrations is the hexa-aquo $[Fe(H_2O)_6]^{3+}$ complex and this species predominates at very acidic conditions (see Fig. 2a, b). Hence, the high redox potential of the Fe^{3+}/Fe^{2+} couple can be exploited only by acidophilic bacteria (Thamdrup 2000). The redox potentials of MnO_2 oxides/Mn^{2+} is about +550 to +610 mV (Fig. 7). The potentials of the Mn^{3+} phases scatter around +270 mV.

A fascinating and rich landscape comes to sight if one inspects the redox potentials for iron oxides where more thermodynamic data exist. The highest potentials are those for the reduction of poorly crystalline compounds such as ferrihydrite or schwertmannite (Fig. 7). As expected, the lowest potentials are calculated for the well-crystalline stable phases hematite and goethite. It has been known for a long time that the energy of finely divided aggregates depends on their surface area (Langmuir 1971); if the particle size reaches some limit, the energy stored in surfaces will no longer be negligible (Navrotsky 2001). The particle size or surface effect on the thermodynamics of iron oxides has been explored and summarized in Navrotsky et al. (2008). Pictorially, this effect is shown in Fig. 8. The redox potentials of the iron oxides are a function of their surface area and vary over a considerable range. At particle sizes comparable to those usually seen in ferrihydrite (2–20 nm), the energies and redox potentials of all iron oxides are very similar. In other words, in the nanoscale regime, all iron oxides are thermodynamically equal. Adsorption of anions onto the surfaces of iron oxides drives their stability in an opposite direction, that is, stabilizes them (Majzlan 2011). Phosphate adsorption onto ferrihydrite has an astonishing stabilizing effect on this substance (Fig. 7). Arsenate stabilizes ferrihydrite too, but not to the same extent. Adsorption of cations, such as Al, also inhibits the transformation of ferrihydrite to crystalline iron oxides (Hansel et al. 2011) and has probably very similar thermodynamic effect like phosphate or arsenate.

A further complication is the aqueous complexation of the Fe(III), Fe(II), or Mn(II) species. Holmén et al. (1999) have shown that the potential of reduction of Fe(III) complexed to hydroxamate ligands is negative at pH = 7. It is certain that complexation of the metal cations with smaller or larger organic molecules has a

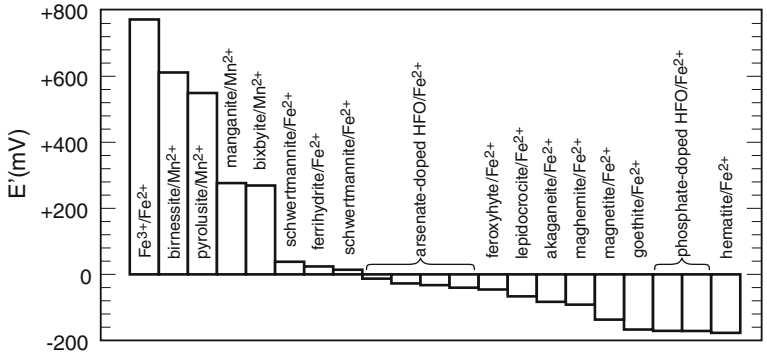

Fig. 7 Redox potential for the aqueous Fe^{3+}/Fe^{2+} pair and minerals of Mn and Fe. For completeness, two Mn^{3+} minerals are included here although they were not discussed in the text: bixbyite, Mn_2O_3, and manganite, MnOOH. HFO = hydrous ferric oxide, a generic term for X-ray amorphous ferric oxyhydroxides, including, but not limited to, ferrihydrite. The redox potentials were calculated for pH = 7 and assuming activities of 10^{-5} for the dissolved metals (Mn^{2+} or Fe^{2+}), 10^{-4} for dissolved sulfate, and 10^{-5} for dissolved phosphate or arsenate. Data from Robie and Hemingway (1995), Navrotsky et al. (2008), Majzlan (2011), Lemire et al. (2011)

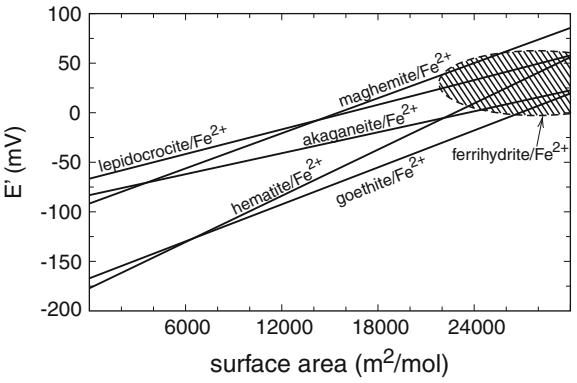

Fig. 8 Variation of the redox potential for iron oxide minerals as a function of their surface area (that is, particle size). The redox potentials were calculated for pH = 7 and assuming activity of 10^{-5} for the dissolved Fe^{2+}. Data from Navrotsky et al. (2008)

profound influence on the redox potentials encountered in near-surface geomaterials.

There appears to be a significant confusion in the literature about the redox potentials of minerals relevant to redox element cycling in soils and sediments. Liu et al. (2011) stated that "The redox potential of the goethite Fe(III)/Fe(II) couple at neutral pH is 799 mV (Fredrickson et al., 2000)." Fredrickson et al. (2000), however, report 799 mV for this reaction at standard state conditions that is activity of H^+ is 1.0, far away from pH of 7. Fredrickson et al. (2000) also reported a value of −88 mV at $[Fe^{2+}] = 10^{-6}$, presumably also at pH = 7, but the latter condition was not specified. Thamdrup (2000) calculated the redox potentials for

Table 2 Redox potentials for minerals of Mn and Fe. For comparison, potentials for several simple redox pairs relevant to bacterial metabolism are also listed.The listed potentials for minerals of Mn and Fe are pictorially shown in Fig. 7. These redox potentials were calculated for pH = 7 and assuming activities of 10^{-5} for the dissolved metals (Mn^{2+} or Fe^{2+}), 10^{-4} for dissolved sulfate, and 10^{-5} for dissolved phosphate or arsenate. Data from Robie and Hemingway (1995), Navrotsky et al. (2008), Majzlan (2011), Lemire et al. (2011).The selected potentials relevant to bacterial metabolism were taken from Thauer et al. (1977). These potentials were calculated for pH = 7 but all other species involved in the reactions are in their standard states

Redox pair	E' (mV)	Redox pair	E' (mV)
O_2/H_2O	+818	Lepidocrocite/Fe^{2+}	−67
Birnessite/Mn^{2+}	+612	Akaganeite/Fe^{2+}	−83
Pyrolusite/Mn^{2+}	+549	Maghemite/Fe^{2+}	−92
Manganite/Mn^{2+}	+277	Magnetite/Fe^{2+}	−135
Bixbyite/Mn^{2+}	+269	Goethite/Fe^{2+}	−167
NO^{3-}/NO^{2-}	+350	P–HFO/Fe^{2+}	−171
Fumarate/succinate	+33	Hematite/Fe^{2+}	−177
Ferrihydrite/Fe^{2+}	+24	Pyruvate/lactate	−190
Schwertmannite/Fe^{2}	+14	CO_2/CH_4	−244
Glycine/acetate + NH_4	−10	S^0/HS^-	−270
As–HFO/Fe^{2+}	−27	CO_2/acetate	−290
Feroxyhyte/Fe^{2+}	−46	CO_2/formate	−432

several iron oxides and reported the value of −274 mV for the reduction of goethite at pH = 7 and $[Fe^{2+}] = 10$ μM.

The reaction considered for the reductive dissolution of goethite is

$$\alpha - \mathrm{FeOOH} + 3\mathrm{H}^+ + \mathrm{e}^- = \mathrm{Fe}^{2+} + 2\mathrm{H_2O}.$$

For the calculation of the Gibbs free energy of this reaction ($\Delta_r G^o$), we adopted the formation Gibbs free energies in kJ/mol: $\Delta_f G^o$(goethite) = −489.8 (Majzlan et al. 2003), $\Delta_f G^o$(liquid water) = −237.14 (Lemire et al. 2011), $\Delta_f G^o\left(\mathrm{Fe}^{2+}, \mathrm{aq}\right) = -90.719$ (Lemire et al. 2011). The result of the calculation is

$$\begin{aligned}\Delta_r G^\circ &= \Delta_f G^\circ\left(\mathrm{Fe}^{2+}, \mathrm{aq}\right) + 2\Delta_f G^\circ(\text{liquid water}) - \Delta_f G^\circ(\text{goethite}) \\ &= -75.199\,\mathrm{kJ/mol}.\end{aligned}$$

Using the equation

$$\mathrm{E}^\circ = -\Delta_r \mathrm{E}^\circ / n\mathrm{F}$$

where n is the number of electrons exchanged and F is the Faraday constant (96485.3 C/mol), we obtain E^o = +779 mV. The standard state conditions, even if they were not hypothetical, were of little relevance to most natural systems as the aqueous ion concentrations are fixed at very high values. The redox potential at chosen, perhaps more realistic conditions, can be calculated as

$$\mathrm{E'} = \mathrm{E}^\circ - (\mathrm{RT} \ln 10 / n\mathrm{F}) * \log \mathrm{Q}$$

where R is the universal gas constant, T is the thermodynamic temperature, and Q is the reaction quotient. For the dissolution reaction above, this equation is

$$E' = E^{o} - (RT\ln 10/nF) * \log\left[\frac{\left(\{Fe^{2+}\}\{H_2O\}^2\right)}{\left(\{\alpha - FeOOH\}\{H^+\}^3\right)}\right]$$

where the curly brackets denote activity. Assuming that the activities of goethite and liquid water are 1.0, the equation reduces to

$$E' = E^0 - \left(\frac{RT\ln 10}{nF}\right) * \log\left(\frac{\{Fe^{2+}\}}{\{H^+\}^3}\right), \text{ or}$$

$$E' = E^0 - \left(\frac{RT\ln 10}{nF}\right) 3pH - \left(\frac{RT\ln 10}{nF}\right) \log\{Fe^{2+}\}$$

Since $n = 1$ (one electron exchanged), the factor (RTln10/nF) reduces to 0.059156. Therefore, the redox potential for this reaction at pH = 7 and all other phases and species in their standard state is −463 mV. The redox potential for this reaction at pH = 7 and $\{Fe^{2+}\} = 10^{-5}$ M is −167 mV. The differences in the three redox potentials listed in this section (+779, −463, −167 mV) underscore the need to specify carefully the conditions for which the E' value was calculated. The redox potentials calculated from the available data for minerals of Mn and Fe are listed in Table 2.

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Energetic and Molecular Constraints on the Mechanism of Environmental Fe(III) Reduction by *Geobacter*

C. E. Levar, J. B. Rollefson and D. R. Bond

Abstract This review aims to discuss how *Geobacter* and its relatives are shaped by the nature of their electron donor and acceptor, where electrons liberated during complete cytoplasmic oxidation of organics must travel far beyond the cell to reduce extracellular metals without the aid of soluble shuttles. This sequence of reactions must often occur in permanently anoxic habitats where reactant concentrations lower the ΔG to only tens of kJ/mol, severely limiting the energy available for protein synthesis. Extracellular Fe(III) reduction is additionally challenging, from a bioenergetic perspective, as oxidation of organic matter (releasing protons and electrons) occurs in the cell interior, but only the negatively charged electrons are transferred outside the cell. Finally, the low amount of energy available from metals in direct contact with a cell predicts that *Geobacter* must organize electron transfer proteins to extend outward, to take advantage of the Fe(III) in the volume available a few microns beyond its outer membrane. This review will discuss these thermodynamic constraints on environmental metal reduction, and briefly mention recently described aspects of the molecular mechanism of electron transfer by *Geobacter* spp. when viewed through this lens.

1 Introduction

Representatives of multiple ∂-Proteobacterial genera are (1) consistently isolated from Fe(III)-reducing subsurface habitats (see "Metal Reducers and Reduction Targets. A Short Survey About the Distribution of Dissimilatory Metal Reducers

C. E. Levar · J. B. Rollefson · D. R. Bond (✉)
140 Gortner Laboratory, Department of Microbiology and BioTechnology Institute, University of Minnesota, 1479 Gortner Avenue, St. Paul, Minneapolis, MN 55108, USA
e-mail: dbond@umn.edu

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and the Multitude of Terminal Electron Acceptors") (Coates et al. 1995, 1996, 1998, 2001; Lin et al. 2007; Lonergan et al. 1996; Nevin et al. 2005; Straub et al. 1998), (2) found to be significant members of communities in molecular studies of stimulated Fe(III)-reducing zones and bioremediation sites (Anderson and Lovley 1997, 1999; Callister et al. 2010; Chang et al. 2005; Elifantz et al. 2010; Lovley and Anderson 2000; Petrie et al. 2003; Rooney-Varga et al. 1999; Snoeyenbos-West et al. 2000; Vrionis et al. 2005; Wilkins et al. 2011; Yun et al. 2011), and (3) are regularly enriched on electrodes poised as electron acceptors (Bond et al. 2002; Chae et al. 2009; de Cárcer et al. 2011; Finkelstein et al. 2006; Ha et al. 2008; Holmes et al. 2004; Jung and Regan 2007; Kiely et al. 2011; Williams et al. 2010; Xing et al. 2009). These bacteria are primarily known for their ability to couple complete oxidative metabolism to respiratory growth with Fe(III) (oxyhydr)oxide, and are represented by isolates from the genera *Desulfuromonas, Geobacter, Desulfuromusa, Malonomonas, Trichlorobacter, Geopsychrobacter,* and *Geothermobacter*. The available genomes of metal-reducing *Geobacter* and *Desulfuromonas* strains all contain a conserved core of genes enabling complete acetate oxidation, accompanied by hundreds of poorly conserved multiheme *c*-type cytochromes, most of which are predicted to be localized to the outer membrane or beyond the outer surface (Aklujkar et al. 2009, 2010; Butler et al. 2010; Holmes 2009; Lovley 2003; Methe et al. 2003; Nagarajan et al. 2010; Tran et al. 2008). Based on these observations, these bacteria are considered to have evolved to compete in anoxic habitats where simple fermentation end products are the electron donors, and the electron acceptors are primarily available outside the cell.

Gene phylogenies suggest that significant divergence within this group has occurred to take advantage of different environments. Marine habitats typically contain bacteria related to *Desulfuromonas* and *Desulfuromusa*, while *Geobacter* spp. are normally found in freshwater environments (Butler et al. 2010; Holmes et al. 2004). The *Geobacter* genus forms at least three distinct clades that also appear to correlate with habitat; relatives of *G. metallireducens* and *G. sulfurreducens* are associated with surficial sediments, and relatives of the more recently isolated *Geobacter psychrophilus* and *Geobacter uraniireducens* each represent separate clades usually found in subsurface aquifers (Holmes et al. 2004, 2007). An extreme example of specialization are the non-metal-reducing *Pelobacter* isolates, which share a common genus name due to their fermentative physiology, but are phylogenetically scattered throughout the ∂-Proteobacteria, with some related to *Geobacter* and others being close relatives of *Desulfuromonas* (Butler et al. 2009). This pattern suggests multiple independent evolutionary events have occurred in which metal reduction inherited from the common ancestor was lost (Butler et al. 2009).

Such diversity means that this collection describes a group which diverges over 10 % at the 16S rRNA level, demonstrates growth between 4 and 65 °C (Holmes et al. 2004; Kashefi et al. 2003; Nevin et al. 2005), and shows high variability in salt tolerance, substrate utilization range, and ability to transfer electrons to various acceptors in the laboratory. Given this diversity, it is perhaps no surprise that genomic and genetic analyses have failed to identify well-conserved cytochromes

or putative metal-reducing proteins by comparing the genomes of these metal-reducing bacteria. However, this lack of an obvious conserved electron transfer system is in contrast to the solution recently described for the γ-proteobacterial genus *Shewanella*, which encompasses isolates obtained from a range of ocean sediments, toxic, and fermentative environments. Despite the fact that *Shewanella* strains also display high phylogenetic and phenotypic diversity, they only retain a single conserved cytochrome conduit for electron transfer out of the cell, and largely depend on soluble flavins to move electrons beyond the cell surface (see "The Biochemistry of Dissimilatory Ferric Iron and Manganese Reduction in Shewanella oneidensis" and "On the Role of Endogenous Electron Shuttles in Extracellular Electron Transfer"; (Coursolle et al. 2010; Coursolle and Gralnick 2010; Hartshorne et al. 2009; Rodrigues et al. 2011).

This review aims to discuss how *Geobacter* and its relatives are shaped by the nature of their electron donor and acceptor, where electrons liberated during complete cytoplasmic oxidation of organics must travel far beyond the cell to reduce extracellular metals without the aid of soluble shuttles. This sequence of reactions must occur in permanently anoxic habitats where reactant concentrations lower the ΔG of respiration to only tens of kJ/mol, severely limiting the energy available. This review will discuss the thermodynamic constraints on environmental metal reduction, and briefly mention aspects of the molecular mechanism of electron transfer by *Geobacter* spp. when viewed through this lens.

2 The Energetic Challenge of Coupling Complete Oxidation to Fe(III) Reduction

The importance of the acetate oxidation phenotype is underscored by the enrichment of the first *Desulfuromonas* by Pfennig and Biebl (1976). While numerous sulfur- and sulfate-reducing bacteria capable of incomplete lactate oxidation were already known, anaerobic sulfate- or sulfur-reducing bacteria able to completely oxidize the copious amounts of acetate produced by incomplete oxidizers were lacking. *Desulfuromonas acetoxidans* provided the first answer to this mystery. Subsequent biochemical tests revealed that *D. acetoxidans* used the citric acid cycle for acetate oxidation when sulfur was the electron acceptor. This was surprising, considering the fact that the formal potentials of some steps in the citric acid cycle (such as fumarate/succinate, $E^{\circ\prime} = -32$ mV) have $E^{\circ\prime}$ values slightly more positive than reduction of menaquinone ($E^{\circ\prime} = -74$ mV), and much more positive than the terminal electron acceptor (S^0/H_2S $E^{\circ\prime} = -240$ mV) (Thauer et al. 1989). While changes in intracellular concentrations of reactants could help solve some of these issues, subsequent bioenergetic experiments showed the need for membrane potential to drive 'uphill' succinate oxidation, consistent with inward flux of protons being used during some steps to catalyze complete oxidation (Paulsen et al. 1986). Such reverse electron transport reduces the total

amount of energy remaining for bacterial ATP synthesis, but ensures unfavorable reactions operate in the oxidative direction (Pfennig and Widdel 1982; Schmitz et al. 1990).

The poor $\Delta G^{\circ\prime}$ of acetate/sulfur respiration (approximately −39 kJ/mol acetate, under standard conditions), coupled with this price of reverse electron transport and the need to use at least one ATP equivalent in activation of acetate to acetyl-CoA, leaves little free energy for respiratory ATP generation. Consistent with these findings, when committed to acetate oxidation, *D. acetoxidans* achieves less than 0.5 ATP per acetate oxidized, and respires nearly 95 % of acetate to CO_2 to generate enough ATP to produce biomass from this two-carbon precursor (Gebhardt et al. 1985; Mahadevan et al. 2006; Widdel and Pfennig 1992). Despite the low apparent value of acetate under such conditions, both calculations and sediment labeling studies have shown that nearly 70 % of anaerobic organic matter oxidation in sediments ultimately proceeds via anaerobic oxidation of acetate (King et al. 1983; Lovley and Klug 1982; Novelli et al. 1988; Thauer et al. 1989).

The reduction of Fe(III) presents a thermodynamic challenge similar to that of the reduction of S°. While the redox potential of freshly precipitated Fe(III), such as ferrihydrite, is estimated to be in the range of −100 to +100 mV (see "Minerals and Aqueous Species of Iron and Manganese as Reactants and Products of Microbial Metal Respiration") (Straub et al. 2001), this window represents a best-case upper boundary of the energy available to Fe(III)-reducing organisms. More crystalline Fe(III) forms such as goethite, lepidocrocite, and hematite will have much lower formal redox potentials. With this in mind, one of the most valuable findings from recent electrochemical measurements with *Geobacter* spp., is the observation that acetate oxidation can proceed down to an electron acceptor potential of approximately −220 mV (Marsili et al. 2008, 2010). This value reveals that *Geobacter* conserves very little energy, around 6 kJ per electron respired, when using Fe(III) as an external electron acceptor. The advantage of such a strategy is that, in taking so little for itself, *Geobacter* guarantees that electron transfer from the cell surface will always be downhill, even to more crystalline minerals or in environments where acetate concentrations are low (sub-μM).

The final consideration that makes extracellular Fe(III) reduction difficult, from a bioenergetic perspective, is the need to perform the oxidation of organic matter (releasing protons and electrons) in the cell interior, but transfer only the negatively charged electrons to the outside of the cell. The net effect of this reaction is accumulation of protons (and positive charge) inside the cell, acidifying the interior and canceling out many of the later proton-pumping events occurring during respiration (Mahadevan et al. 2006, 2011). This additional cost of Fe(III) reduction appears to diminish the yield of *Geobacter* more than 50 % compared to what would be predicted from standard ΔG calculations. An illustration of this phenomenon is the comparison of growth with fumarate versus growth with Fe(III) as the terminal electron acceptor (Mahadevan et al. 2006, 2011); when expressed as biomass per electron respired, *G. sulfurreducens* produces nearly three times more cells when grown with the intracellular acceptor fumarate ($E^{\circ\prime} = -32$ mV) compared to growth with the extracellular acceptor Fe(III)-citrate ($E^{\circ\prime} = +350$ mV),

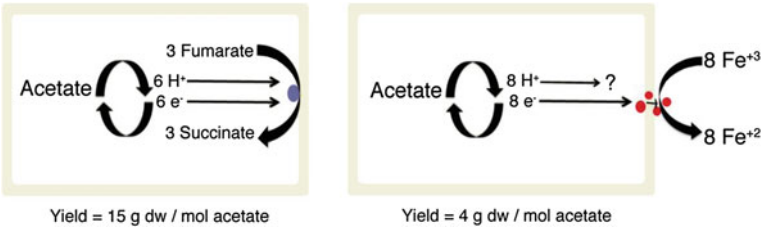

Fig. 1 Illustration of the difference between intracellular and extracellular electron acceptors. Intracellular reduction of fumarate consumes both protons and electrons produced during acetate oxidation, and all electron transfer can be devoted to proton translocation driving subsequent ATP synthesis (estimated at ~1.5 ATP/acetate). Extracellular reduction of electron acceptors consumes only electrons, which leave the cell, leading to accumulation of positive charge inside the cell which dissipates the proton motive force. From observed biomass yields and in silico modeling, subsequent energy-dependent disposal of proton equivalents decreases the net ATP production to ~0.5 ATP/acetate (Magnuson et al. 2001; Mahadevan et al. 2006)

even though fumarate supplies less potential energy according to standard calculations (Fig. 1). Similar yields have been found for *Geobacter* grown with high-potential Fe(III)-citrate acceptors as with lower potential electrode acceptors ($E^{\circ\prime} = 0$ to +200 mV), and there is no evidence *Geobacter* is able to modify the amount of ATP captured from external electron acceptors based on potential. The implications of this very low energy yield impose important constraints on the possible mechanisms of metal reduction.

3 Moving Electrons Beyond the Cell Must Require Multiple Attachment and Redox Proteins

Once electrons are released from the quinone pool to the periplasm, all energy generation steps have been completed. However, electrons must still overcome multiple independent barriers to escape. Electrons first cross the insulating outer membrane, then hop across a protein-mineral interface to the terminal electron acceptor. Decades of work with electron transfer proteins has shown that electrons require a continuous path of redox centers or sites for multistep tunneling, which must be not more than 15–18 Å apart (Gray and Winkler 2009, 2010). While a bacterium can ensure tight protein–protein interactions within membranes, the surface of a metal (oxyhydr)oxide electron acceptor is highly variable and uncontrollable in terms of charge, shape, and crystal structure. A single protein complex can achieve rapid and predictable transmembrane electron flow within or across a membrane, but should we expect a single protein to exist which is able to interface with all environmental metal acceptor surfaces?

An elegant illustration of this ‘surface interfacing’ problem was shown in molecular simulations by Kerisit et al. (2007), who found that electron transfer

rates from a cytochrome to a hematite surface could vary by over six orders of magnitude, simply depending on the orientation of the exposed heme colliding with the hematite surface. Although it may be theoretically simple to occasionally bring redox centers close enough to make physical contact with a particle, even tiny differences at the interface, or defects in the attachment process can mean a ten- to 100-fold difference in interfacial transfer rates. Given the variability in environmental metal oxides, this argues for some diversity in the extracellular redox proteins of non-shuttle producing bacteria.

The discovery that many Fe(III)-reducing bacteria will also attach to electrodes poised to act as electron acceptors has provided a new tool for their study, as electrochemistry can probe the relationship between interfacial electron transfer rate and driving force under highly controlled conditions (Jain et al. 2011; Marsili et al. 2008, 2010; Richter et al. 2009; Srikanth et al. 2008; Yi et al. 2009). In particular, electrochemistry has solidified three key aspects of the *Geobacter* electron transfer phenotype; First, there have been no soluble electron shuttles reported to be secreted by these bacteria. Removing the medium surrounding active *Geobacter* biofilms growing on electrodes has no effect on the rate of electron transfer at any stage of growth. Second, the interfacial electron transfer reaction, from cell surfaces to electrodes, is not rate limiting. *Geobacter* cultures using electrodes as electron acceptors double as fast on electrodes (approximately every 6 h) as they do with dissolved Fe(III)-citrate as electron acceptors, and electrode respiration is not accelerated by addition of dissolved redox shuttles. A more formal derivation of the argument for interfacial electron transfer being non-limiting can be found in the electrochemical modeling of Strycharz et al. (2011). Interestingly, growth with Fe(III) oxides is always slower (doubling times $\sim$12–24 h), but can be accelerated by dissolved electron shuttles, suggesting that a rate-limiting step with more environmentally relevant Fe(III) acceptors is related to the availability of a nearby electron acceptor surface, or traveling to the new surface, not electron transfer per se. Third, the unlimited nature of the electrode electron acceptor enables growth of thick biofilms, which has provided the proof that many *Geobacter* strains possess a between-cell conductivity able to transfer electrons between cells over distances as great as 10–20 μm.

4 Cytochromes and Pili: Often More Questions than Answers

If a list of proteins implicated in *Geobacter* metal reduction is made, over 15 *c*-type cytochromes (Afkar et al. 2005; Kim et al. 2005, 2008; Kim and Lovley 2008; Leang et al. 2003, 2005; Leang and Lovley 2005; Lloyd et al. 2003; Mehta et al. 2005; Shelobolina et al. 2007), as well as pili (Juarez et al. 2009; Richter et al. 2009), multicopper proteins (Holmes et al. 2008; Mehta et al. 2006; Qian et al. 2007), porins (Afkar et al. 2005), secretion systems (Mehta et al. 2006), and polysaccharide synthesis enzymes (Rollefson et al. 2009, 2011) could be described. This has led to some confusion, and an array of sometimes conflicting

hypotheses aimed at describing electron transfer. The source of this confusion is likely twofold; as mentioned previously, there is little conservation of cytochromes or other redox proteins across *Geobacter* genomes. High diversity in cytochromes involved in extracellular metal respiration has also been reported in the genomes of natural Fe(II)-oxidizing communities (Denef et al. 2010a, b), suggesting that proteins at the interface between bacteria and metals are under constant selection in response to metal structure or potential. Thus, any discussion of data derived from the most commonly studied strain (*G. sulfurreducens*) may not necessarily apply to members of other *Geobacter* clades.

The second consideration is that, for an organism not producing a soluble shuttle, there are many discrete electron transfer challenges, related to proteins bringing electrons to the outer membrane versus those required to interface with surfaces. The different proteins implicated in metal reduction do not need to all be involved in electron transfer, but could contribute via adhesion, localization, or secretion.

4.1 Escaping the Cell: The Example of OmcB

The best example of this confusion, and the need for caution when conducting deletion experiments, is the outer membrane dodecaheme *c*-type cytochrome OmcB. First identified via biochemical enrichment of outer membrane proteins (Magnuson et al. 2000, 2001), immunogold labeling has confirmed that OmcB is tightly associated with the outer membrane (Qian et al. 2007). Genetic experiments showed an *ΔomcB* mutant was unable to reduce both soluble and insoluble Fe(III) (Leang et al. 2003; Qian et al. 2007). Expression of *OmcB* increases when Fe(III) is the electron acceptor, especially under Fe(III)-limiting conditions (Chin et al. 2004; Yang et al. 2010), and when cells are grown in current-producing biofilms (Nevin et al. 2009).

The location of OmcB, its expression pattern, and the initial behavior of a deletion mutant is consistent with this cytochrome playing a key role in electron transfer at the outer membrane. What makes interpretation of these experiments difficult, however, is the fact that an Δ*omcB* mutant is able to easily adapt to grow using soluble Fe(III), via outgrowth of suppressor strains that appear to express homologs (such as a paralogous dodecaheme *omc*C located downstream), or alternate cytochromes encoded on the genome (Leang et al. 2005; Leang and Lovley 2005). Experiments such as these show that while OmcB is important, there also may be parallel pathways, or cryptic cytochromes not normally expressed under laboratory conditions which are easily selected for in mutants.

Another example of complexity is provided by the diheme peroxidase MacA (Butler et al. 2004; Kim and Lovley 2008; Nunez et al. 2006; Shelobolina et al. 2007). Deletion of this protein was reported to severely decrease the ability of *Geobacter* to reduce soluble and insoluble Fe(III), leading to its inclusion in some models of electron transfer out of the cytoplasmic membrane. However, later

studies found that transcript and protein levels of OmcB were also diminished in a Δ*macA* strain, and expression of *omcB in trans* restored Fe(III) reduction to a *macA*-deficient mutant (Kim and Lovley 2008). Thus, MacA was not critical for Fe(III) reduction in an electron carrying capacity, but was rather intertwined with some mechanism of *omcB* expression. Recent work has confirmed that MacA has all the characteristics of a classic diheme peroxidase, and is unlikely to be involved in electron transfer, although it is still drawn in some cartoons of *Geobacter* respiration (Seidel 2012).

OmcB expression, translation, or post-translational stability is further influenced by at least four other proteins. Deletion of the small monoheme cytochrome OmcF eliminates the ability of *G. sulfurreducens* to reduce Fe(III), but also prevents expression of *omcB* (Kim et al. 2005; Kim et al. 2008). Like the MacA mutant, Δ*omcF* mutants quickly evolve to select strains in which the expression of other compensatory *c*-type cytochromes is increased, showing that OmcF is not essential. Furthermore, when two homologous cytochromes, OmcG and OmcH are deleted in tandem, soluble Fe(III) reduction is again inhibited even though *omcB* mRNA is still detected (Kim et al. 2006). However, OmcB protein levels are depleted in this strain, indicating translational or post-translational regulatory mechanisms have been disrupted (Kim et al. 2006). Finally, a mutant lacking the abundant porin OmpJ shows significantly decreased rates of Fe(III) reduction, but also has a 50 % reduction in heme content, and lacks high molecular-weight membrane-associated cytochromes such as OmcB (Afkar et al. 2005).

Thus, many phenotypes ascribed to single proteins in *Geobacter* are now known to be due to downstream effects on OmcB. In addition, the high redundancy of cytochromes in *G. sulfurreducens* often means mutants can quickly evolve to obscure the Δ*omc*B phenotype. These factors should be taken into consideration when evaluating any disruption in electron transfer proteins in *Geobacter.*

4.2 Interfacing with External Acceptors: The Examples of OmcS Versus OmcZ

Two other cytochromes, OmcS and OmcZ, warrant mention as they have consistently been linked to reduction of insoluble metals or electrodes, respectively. The hexaheme cytochrome OmcS was originally discovered by shearing of cells (Mehta et al. 2005), an observation later explained by immunogold labeling that found at least some OmcS to be arranged along pili, which are also removed by shearing approaches (Leang et al. 2010). Deletion of OmcS eliminates reduction of insoluble Fe(III), with little effect on soluble Fe(III) reduction, further suggesting it is involved in processes beyond the cell membrane (Mehta et al. 2005). Proteomic studies also found OmcS to be more abundant in cells grown with insoluble Fe(III) compared to cells grown with soluble Fe(III) (Ding et al. 2008; Ding et al. 2006). However, it is less clear if OmcS is essential for growth on electrodes, as Δ*omcS*

mutants are still able to colonize electrodes and use them as electron acceptors, but are initially defective in development of thicker biofilms requiring between-cell conductivity (Nevin et al. 2009; Richter et al. 2009).

In contrast, the octoheme cytochrome OmcZ (Inoue et al. 2010) is more highly abundant when cells are grown as biofilms on electrodes, and an *omcZ*-deficient mutant is unable to transfer electrons to electrodes (Nevin et al. 2009; Richter et al. 2009). The OmcZ protein is not pili associated, but has been found distributed throughout a polymeric matrix between cells, and especially near the electrode in biofilms (Inoue et al. 2011). Also, Δ*omcZ* mutants are not severely impacted in their ability to reduce Fe(III) (Nevin et al. 2009). Data such as these support the hypothesis that different extracellular electron acceptors (Fe(III) oxides vs. electrodes) and/or modes of growth (suspended Fe(III) particles vs. attached as biofilms) may require different cytochromes, further indicating that there is no one master pathway that will emerge to explain all extracellular electron transfer by *G. sulfurreducens*.

4.3 Other Matrix Components: For Attachment or Cell–Cell Electron Transfer?

Because filaments sheared from the surface of *G. sulfurreducens* were shown to possess conductivity across their width when probed by conducting atomic force microscopy, and such filaments could not be found in a mutant lacking the Type IV pilin protein PilA, a hypothesis emerged that pili were involved in carrying electrons to electrode surfaces and other acceptors (Reguera et al. 2005, 2006). In addition, more recent measurements of conductivity through *Geobacter* biofilms placed across gaps in gold electrodes have provided support for unique conductivity between cells, which has again been attributed to pili.

In support of this theory, a *ΔpilA* mutant is partially defective in Fe(III) oxide reduction, and can barely attach to electrodes. Confounding this result, however, is the fact that pili are also involved in the attachment of cells to all surfaces, and to each other (Reguera et al. 2005, 2006). For example, *ΔpilA* mutants cannot form robust biofilms on glass, Fe(III)-oxide-coated surfaces, or electrodes, even in the presence of additional dissolved energy sources such as fumarate (Klimes et al. 2010; Krushkal et al. 2010; Reguera et al. 2005, 2006, 2009). Mutants lacking PilA also lack the ability to bind to each other in cell–cell agglutination assays. These defects in attachment and biofilm formation mean that, to study issues such as conductivity of biofilms, reactors must be incubated for up to 2 months to accumulate enough cells to perform measurements.

The pili of *Geobacter* have also proven difficult to solubilize and study via traditional biochemical techniques, leading to additional uncertainty in terms of amounts present outside the cell (Cologgi et al. 2011). As measurements have not been made on purified pili from Δ*omcS* strains, where pili-associated OmcS could

not participate in conductivity, it is not yet known if the retractable Type IV *Geobacter* pili are actively involved in electron transfer per se, if they serve as scaffolds for other proteins, if they mediate attachment, or are essential for bringing cells in close enough contact for robust electron transfer. More recent work has shown that Δ*pil*A mutants show defects in cytochrome secretion, which is not surprising, as Type IV pili are evolved from the Type II secretion mechanism (Richter 2012). Type IV pili have been shown to be required for the secretion of extracellular proteins in a number of other bacteria (Hager et al. 2006).

Similar to the role of pili in aspects of surface binding and cytochrome function, mutants in production of cell-surface polysaccharides are defective in attachment and cytochrome localization (Rollefson et al. 2009, 2011). Mutants in a locus encoding a series of glycosyl transferases and sugar exporters demonstrate decreased affinity for Fe(III) oxides and electrode surfaces, lowering Fe(III) reduction rates and eliminating electrode biofilm formation. These mutants also possess significantly lower amounts of cytochromes outside the cell, particularly OmcZ, which is known to be involved in electrode colonization (Rollefson et al. 2009, 2011). These results are consistent with labeling studies showing OmcZ to be located on polymers distant from the cell.

As with cytochromes, many single mutations in pili or polysaccharides show a pattern of more broadly affecting *Geobacter's* surface charge, extracellular sugar content, and secretion of cytochromes, producing an external surface very different from the wild type (Richter 2012). As the Type IV pili system is known to be used in secretion of extracellular proteins by other bacteria (Hager et al. 2006), attention should be paid to how the extracellular matrix of *Geobacter* is assembled, and if a cascade of downstream effects result from mutations in pili or pili-like structures. Mutations which manifest as the failure to attach to a surface are difficult to use as evidence for, or against, the larger concept of conductivity between cells.

5 A Final Word: Energetic Constraints for Accessing Fe(III) Beyond the Cell

The laboratory demonstration of *Geobacter* cells producing 20–50 μm thick biofilms on electrodes suggests that *Geobacter* may form multicellular biofilms on Fe(III) oxide crusts which precipitate on sand grains. In the environment, could cells be surrounded by such dense suspensions of freshly precipitated Fe(III) oxide that they need to form thick microcolonies of cells connected by conductive pathways? The fact that extracellular attachment structures such as pili and polysaccharides, as well as cytochromes distributed between cells, are needed for efficient metal reduction, reinforces the idea that somewhere in nature, cells are growing as interconnected colonies. However, basic energetic calculations do not support this model. Instead, the low ATP yield of Fe(III) reduction, coupled with the high cost of protein synthesis, provides clues as to why *Geobacter* may possess strategies for moving electrons beyond the cell membrane.

The yield of *Geobacter*, in both Fe(III)-reducing chemostats and on electrodes, shows that acetate-oxidizing cells require at least 3.33 mol electrons to synthesize a gram of cell protein (Esteve-Nunez et al. 2005; Mahadevan et al. 2006; Marsili et al. 2010; Sun et al. 2009). Based on an estimated value of 1×10^{-13} g protein/cell (a range also consistent with chemostat measurements of *E. coli* cell doubling at similarly slow rates), 3.3×10^{-13} mol electrons are needed to produce a cell. From this basic yield value, one can ask the question: if *G. sulfurreducens*, which is not motile in laboratory experiments, finds itself surrounded by Fe(III) oxyhydroxide particles that occupy 50 % of the volume in all dimensions (using values from goethite, which has a MW of 88.8 g/mol and density of 4.26 g/cm^3), how many electrons can it transfer to the Fe(III) in contact with the cell membrane (i.e. forming a skin a few nm thick around the cell)? The answer is, perhaps, surprising; this Fe(III) would not support synthesis of even a few percent of a new cell. In fact, we need to expand the volume a cell has access to outward in all dimensions to satisfy the needs of a single cell. Again, assuming 50 % of the space around a cell is occupied with an Fe(III) oxyhydroxide, it would need to reduce all Fe(III) available in the space extending 2–4 μm *in all directions* beyond the outer membrane to access enough acceptor to even approach the ATP requirement for a single cell doubling (Fig. 2).

In other words, the layer of Fe(III) that can make contact with the outer membrane of *Geobacter* is not sufficient to support growth, nor is the Fe(III) extending a cell length away. Instead, cells must access a space at least equal to 25–50 times their own biovolume in order to replicate, depending on the dimensions of the cell. Even if yield assumptions, or Fe(III) densities are off by a factor of two, there is no way to imagine dense microcolonies sitting still, reducing the Fe(III) they can access a few microns away, as a productive strategy.

Another way to approach this challenge is to imagine a cell residing on a sand grain, which is covered with a crust of Fe(III) oxide. If a *Geobacter* cell is able to use only what it can directly touch beneath itself, effectively drilling a hole 1 μm in diameter, it needs to reduce into a crust over 10 μm deep in order to support a single doubling of itself. If that same cell sitting on a sand grain was able to also access all Fe(III) extending 2 μm in all directions on that same surface, enlarging its own 'footprint' and drilling a hole 5 μm in diameter, it could produce enough energy to double by dissolving down into less than 1 μm of crust. While this would not produce a thick biofilm, it is at least in the realm of possibility for doubling.

Thus, in both planktonic and surface-attached situations, these calculations suggest the only viable strategy for Fe(III) reduction coupled to acetate oxidation is one in which a cell has access to the environment many microns beyond what would be considered 'direct contact' by surface-exposed, outer membrane embedded cytochromes.

Shuttle-producing bacteria (or bacteria using naturally present shuttles such as humic acids), partially solve this issue by secreting redox-active molecules at nanomolar concentrations that allow access to Fe(III) on the micron scale, as evidenced by stimulation of both current production and Fe(III) reduction by flavins in *Shewanella* incubations (Coursolle et al. 2010; Marsili et al. 2008; Ross et al. 2009; von Canstein et al. 2008). However, bacteria such as *Shewanella,*

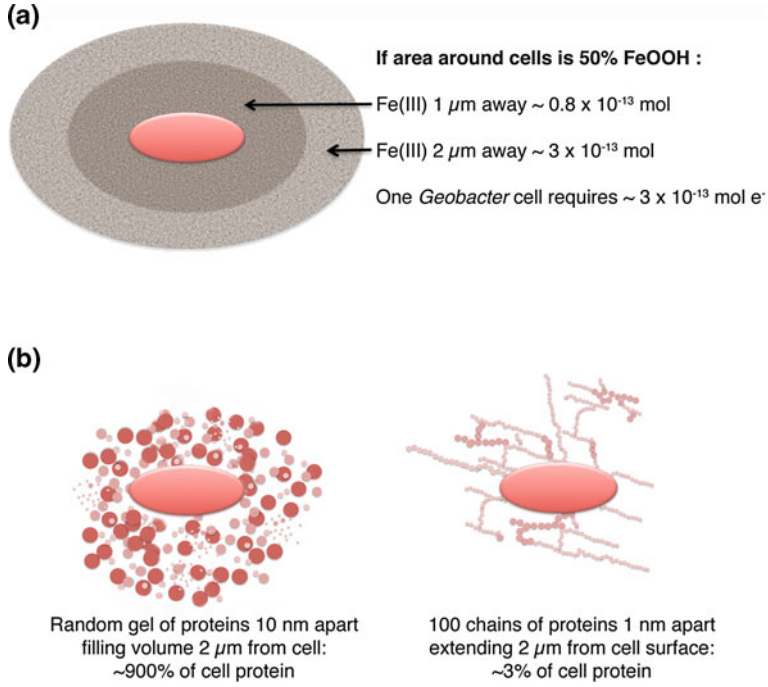

Fig. 2 **a** Illustration of the amount of energy available to a cell in a dense (50 % by volume) Fe(III) (oxyhydr)oxide environment. If *Geobacter* could reduce all Fe(III) 1 μm away from its cell surface, it could not produce enough energy to make a second cell. The volume represented by extending outward 2 μm beyond the cell surface contains enough electrons to support one doubling, but daughter cells would have to move to a new location to find enough Fe(III) to continue respiration. In general, this shows growth in multicellular biofilms is unlikely when Fe(III) oxides are the electron acceptor. **b** Comparison of two strategies for secreting proteins into the extracellular space. Producing a conductive hydrogel of randomly oriented proteins, even when spaced as wide as 10 nm apart on average, would consume nearly 900 % of a cell's protein. However, if proteins are organized in chains or clusters, 100 such organized structures could be produced, extending outward in all directions, for less than 3 % of the cell's protein budget

which partially oxidize lactate, obtain a three- to sixfold higher yield of ATP/electron, meaning they do not need to access as much Fe(III) to grow or recover the cost of shuttle production. Motility can also partially address this issue of accessing nearby Fe(III), although it also comes at a cost, and again, eliminates the need for conductive biofilms.

Geobacter's 'mediators' that provide access to the Fe(III) beyond the cell membrane, or that provide conductivity between cells are not soluble, but are entrapped by structural proteins and polysaccharides. There are many ways to envision a conductive network of proteins outside the cell. For example, if redox or electron transfer proteins were randomly anchored outside the cell, creating a gel extending 2 μm in all dimensions from the outer membrane, they would need to be at a concentration high enough to randomly collide often enough to create

conductivity. For a 50 kDa protein [which has a diameter of about 4.8 nm, (Erickson 2009)], filling a gel where each protein is on average 10 nm apart would require $\sim 0.7 \times 10^{-13}$ g protein, or over 70 % of a cell's total protein! As rapid electron transfer requires proteins to be much closer than this, a highly conductive gel of proteins spaced 2 nm apart approaches 900 % of a cell's total protein. Such calculations show that, while hydrogels containing high concentrations of randomly oriented redox-active mediators may work for enzyme electrodes, such 3D randomness is prohibitively expensive for a single cell.

However, if these same 50 kDa proteins are imagined as being aligned in aggregates or chains, with an average distance of only 1 nm between each protein (a distance facilitating the conductivity observed in redox proteins) (Strycharz-Glaven et al. 2011), roughly 345 proteins end-to-end would extend twice the cell's length (2 μm). A cell could construct 100 such chains to extend in 100 different directions, for a cost of <3 % of the cell's total protein (Fig. 2). Visualized differently, if proteins were arrayed akin to netting, with proteins spaced 1 nm from each other and intersecting every ten proteins on average, a cell could produce over 20 μm^2 of conductive material for a similar cost. If other proteins are used to anchor or build these networks, the protein use could increase, but as polysaccharides cost about 25 % as much as protein to produce, a conductive matrix extending widely in all directions, rather than a random gel, remains the only thermodynamically feasible approach.

In all permutations of these calculations, two facts become clear. First, no form of Fe(III) (oxyhydr)oxide appears to contain enough energy for an acetate-oxidizing *Geobacter* to form a classical, multilayer biofilm, just by touching it. This creates a requirement that cells are able to 'reach out and touch' Fe(III) in a dense suspension or crust over ~2–4 μm away *in all directions,* just to have a chance at making another cell. Lacking a dissolved shuttle, this rewards a single cell if it manufactures long-distance pathways which have the capacity to carry electrons, even if that cell is motile. Second, the enormous volume reaching 2 μm beyond the cell membrane (about 15–25 μm^3, depending on the cell size and shape) is prohibitively expensive to fill with randomly oriented proteins. Regardless of the actual mechanism, any strategy must be organized in 2D, as this volume is much too big to fill randomly. Chains, nets, sheets, and aggregations of proteins are very reasonable ways to solve this issue, and already existing extracellular structures may have been adapted to solve the challenge of Fe(III)'s low energy value.

Thus, the ability of cells to form conductive multicellular networks on electrodes may not be due to growth as Fe(III)-reducing biofilms in the environment. Rather, conductivity on the outside of the cell may be a response to the need to reach beyond the cell membrane just to obtain enough energy while in planktonic mode. Alternatively, conductive pathways may also reward cells growing syntrophically, where electrons are continuously shared between some cells able to oxidize a unique electron donor, and cells able to reduce soluble non-Fe(III) electron acceptor (Butler et al. 2009; Morita 2011; Summers et al. 2010).

In this light, consider the observation that some proteins essential for Fe(III) reduction (such as OmcS) are not needed for direct electrode reduction, but are required for thicker biofilms. In contrast, some proteins required for direct

electrode reduction (such as OmcZ) are not required for Fe(III) reduction. This further underscores the difference between reducing an acceptor that can reach the outer membrane, versus building a conductive pathway to another cell or a distant Fe(III) particle. Polysaccharide fibrils, nonconductive proteins, and pili could be essential components in metal reduction because of their ability to organize electron transfer proteins in 2D efficiently.

From these calculations, it also emerges that planktonic growth of *Geobacter* may actually be a sign of active metal reduction, since there is so little to gain from forming a biofilm on a single particle, and little evidence there is enough energy to support biofilm growth on particulate Fe(III). In every case, these energetic constraints show that the delicate, highly inconsistent space beyond the cell remains an important, relatively unexplored compartment. As it represents the crucial link between cells and their energy source, how this challenge is overcome in response to varying surfaces and electron acceptors may ultimately be what controls the competitiveness of *Geobacter* in the environment.

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The Biochemistry of Dissimilatory Ferric Iron and Manganese Reduction in *Shewanella oneidensis*

Clemens Bücking, Marcus Schicklberger and Johannes Gescher

Abstract More than two decades ago, Kenneth Nealson and Charles Myers published a seminal manuscript, describing an organism that can couple growth to the respiratory reduction of manganese oxide, an extracellular electron acceptor. This was the starting point of research aiming to elucidate mechanisms of extracellular respiration in a γ-proteobacterium named *Shewanella oneidensis*. This research is manifested in a nearly confusing multitude of publications that are sometimes even contradictory. It is the aim of this chapter to give a thorough overview of our knowledge about the biochemistry of metal respiration in *S. oneidensis*. This chapter starts off with a technological survey describing the molecular toolbox we have in our hands to genetically modify *S. oneidensis*. Thereafter, the path of electrons from the cytoplasmic membrane to the cell surface is followed, and thereby potential proteins for this electron transport and the transfer onto terminal metallic electron acceptors are brought to the reader's attention. Moreover, the potential role of further proteins is analyzed that are not necessarily involved in the electron transport chain to ferric iron or manganese oxides per se but still seem to provide a selective advantage for the organism. Throughout the text it will become clear that the list of open questions concerning *S. oneidensis* physiology is still, even after decades of research and although it is the best studied dissimilatory metal reducer, extensive, and that there is room for more fascinating questions that can be addressed using the system *S. oneidensis*.

C. Bücking · M. Schicklberger · J. Gescher (✉)
Abteilung Angewandte Biologie, Karlsruher Institut für Technologie, Fritz-Haber-Weg 2, 76131 Karlsruhe, Germany
e-mail: johannes.gescher@kit.edu

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1 *Shewanella oneidensis*: A Novel Model Organism

S. oneidensis is a gram-negative γ-proteobacterium originally isolated from Oneida Lake in Upstate New York, USA, due to its capability of reducing manganese (IV) oxides (Myers and Nealson 1988; Venkateswaran et al. 1999). In some older publications it is referred to as *Shewanella putrefaciens* (all changes in names are reviewed in (Gralnick et al. 2006)). *S. oneidensis* is an obligate respirer and can use a variety of compounds as electron acceptor for anaerobic respiration such as ferric iron, manganese dioxide, and uranium (VI) as well as nitrate, nitrite, sulfur, thiosulfate, fumarate, sulfite, dimethylsulfoxide, and trimethylamine-N-oxide (Burns and DiChristina 2009; Cruz-Garcia et al. 2007; Gralnick et al. 2006; Myers and Nealson 1988; Schwalb et al. 2002, 2003; Shirodkar et al. 2011). *S. oneidensis* can further grow aerobically with oxygen as terminal electron acceptor. The organism is believed to live in stratified environments at the oxic/anoxic interface where a multitude of electron acceptors could be available (Venkateswaran et al. 1999). *S. oneidensis* uses fermentation end products (lactate, formate, and H_2) and N-acetylglucosamine (the chitin monomer) as carbon and electron sources (Scott and Nealson 1994; Yang et al. 2006).

The interest in *S. oneidensis* and organisms belonging to the genus *Shewanella* has greatly increased during the last two decades. A good surrogate for this growing interest is the number of publications dealing with *Shewanella*, which increased from almost zero in the period from 1988 to 1990 to more than 1,500 between 2009 and the mid of 2011 (Fig. 1).

Currently, this "boom" for metal reducing bacteria like *S. oneidensis* is to a large extent caused by potential applications in biotechnology, mainly remediation of soils and aquifers contaminated with radionuclides, and the utilization of these bacteria as biocatalysts in microbial fuel cells for electricity production. Both aspects will be reviewed in later chapters of this book (see "Bioremediation via Microbial Metal Reduction" and "Dissimilatory Metal Reducers Producing Electricity: Microbial Fuel Cells"). The benefits of using *Shewanella* species to study these processes led to genome sequencing projects for 23 *Shewanella* species so far, mostly funded by the department of energy (DOE) of the USA.

The available genome sequences as well as several proteomic and transcriptomic studies of *S. oneidensis* lead to a good understanding of systems biology of the organism under several conditions. In 2005, a *Shewanella* knowledgebase was established to organize available data and integrate experimental as well as in silico approaches to describe the "system" *Shewanella* (Karpinets et al. 2010). The physiology of *S. oneidensis* is certainly the reason why researcher compiled an enormous amount of data so far, but researchers used especially this dissimilatory iron reducer since it is fairly easy to handle in the laboratory. The next short paragraph will give a quick survey about different ways to grow and manipulate *S. oneidensis*.

Shewanella oneidensis MR-1 in the laboratory. The cultivation and manipulation of *S. oneidensis* MR-1 under laboratory conditions is very easy compared to

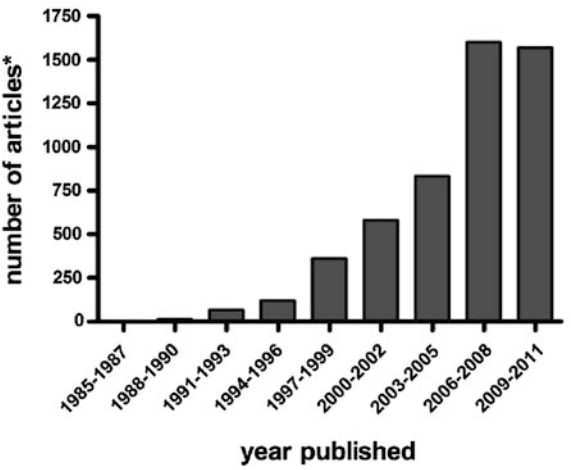

Fig. 1 Number of publications with topic "*Shewanella*" retrieved from Web of Knowledge. *Articles dealing with health and food issues were excluded

Table 1 Plasmids and antibiotics used in *S. oneidensis*

Plasmid name	Resistance marker	Typical concentration of antibiotic in μg ml^{-1}	Publication which uses this plasmid in *S. oneidensis*
pME6031	Tetracycline	20	Thormann et al. (2005)
PACYC184	Tetracycline	8	Myers and Myers (1997c)
pJQ200KS	Gentamycin	10	Myers and Myers (2002b)
pJBC1	Chloramphenicol	30	Bouhenni et al. (2010)
pRLSK4	Streptomycin	50	Schwalb et al. (2003)
pVK100 (Cosmid)	Kanamycin	50	Myers and Myers (2001)
pBAD202/D-TOPO (Invitrogen)	Kanamycin	50	Gescher et al. (2008)

other metal reducing organisms. It can be cultivated aerobically in LB medium and on agar plates where it grows at a temperature optimum of 30 °C and a doubling time of approximately 40 min. Genetic systems for *S. oneidensis* are well established. Scientist can take advantage of several types of plasmids and antibiotic selection markers. Table 1 gives an overview about plasmids that are routinely used for *Shewanella* research and typical concentrations of antibiotics that have to be added for plasmid maintenance.

The most common technique for *DNA transfer* in *Shewanella* is *conjugation* with an *Escherichia coli* donor strain. The usual donor strains are *E. coli* S17-1 (Thormann et al. 2005) and *E. coli* WM3064 (Saltikov and Newman 2003). The latter one, *E. coli* WM3064 is a diaminopimelic acid (DAP) auxotrophic strain that has to be grown in LB medium supplemented with 0.3 mM DAP. This strain allows for a rapid selection of transconjugants since plating on LB agar without DAP can eliminate the donor strain.

Besides conjugation, standard protocols for *electroporation* according to (Sambrook et al. 1989) using ice-cold water can be utilized. Transformation efficiency is low compared to *E. coli* but can be increased by prolonged phenotypic expression (up to 12 h) on SOC medium (Bücking et al. 2010). Myers et al. reported the use of a sorbitol treatment of the cells prior to electroporation (Myers and Myers 1997c).

Many different suicide plasmids have been used to perform directed *mutagenesis* based on R6K ori and SacB counterselection (Myers and Myers 2001). In our studies, a yeast cloning-based vector system has turned out to be particularly useful (Bücking et al. 2010; Shanks et al. 2006). Additionally, a Cre lox gene excision system has been successfully adapted to *Shewanella* (Learman et al. 2009). For random mutagenesis several groups have applied transposon tools based on Tn5, Tn10, and mariner transposons (Beliaev and Saffarini 1998; Bouhenni et al. 2005; Newman and Kolter 2000). Site-directed integration of DNA can also be accomplished using a TN7 platform (Thormann et al. 2004).

In summary, genetic work with at least *S. oneidensis* MR-1 but potentially also with other members of the genus is almost as easy and fast as working with *E. coli*. This certainly encouraged several groups to work with *S. oneidensis* and accelerated the process of identifying how this organism can grow with ferric iron and other terminal extracellular electron acceptors.

2 Components of the Electron Transport Chain to Ferric Iron and Manganese in *S. oneidensis*

S. oneidensis evolved an extended respiratory chain to the cell surface. This allows the organism to transport the electrons to sparsely soluble electron acceptors like ferric or manganese (oxyhydr)oxides (Fig. 2).

Generally, *c*-type cytochromes seem to play an important role in the respiratory electron transport chains to the cell surface. The sheer number of 41 *c*-type cytochrome encoding genes (9 corresponding proteins putatively localized in the cytoplasmic membrane, 5 in the outer membrane, 27 in the periplasm) in the genome of *S. oneidensis* is a first indication for this. As a comparison, *E. coli* has only seven genes encoding *c*-type cytochromes (*yhjA, nrfA, nrfB, napB, napC, torC, torY*) (Blattner et al. 1997; Meyer et al. 2004; Romine et al. 2008). Cells of *Shewanella* cultivated under anoxic conditions appear dark red in color due to the expression of a multitude of heme containing proteins. These proteins are crucial for dissimilatory metal reduction since mutants in the cytochrome maturation machinery of *S. oneidensis* (encoded by the *ccm*-genes) are unable to respire on extracellular electron acceptors like ferric iron or manganese oxides (Bouhenni et al. 2005; Carpentier et al. 2005).

The number of *c*-type cytochromes seems to be a selective advantage for the organism but leads to a number of complications when researchers try to outline

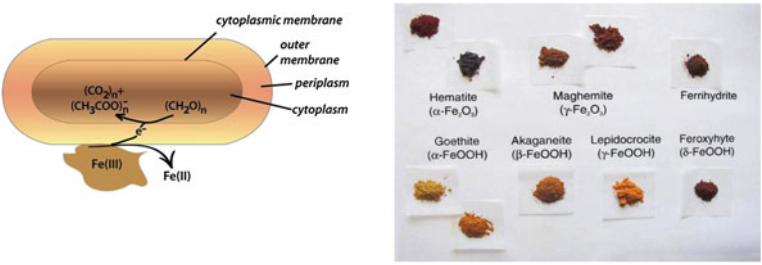

Fig. 2 **a** Connection between oxidation of organic carbon sources and the reduction of iron minerals in *S. oneidensis*. **b** Typical poorly soluble iron(III) (oxyhydr)oxides. Photograph by Prof. Majzlan, inserted with permission

a path of electron transfer. Multiple *c*-type cytochromes are coexpressed under anoxic conditions. Transcriptome studies showed that in a large number of cases the availability of oxygen rather than the available anaerobic terminal electron acceptor seems to be the trigger for *c*-type cytochrome expression (Beliaev et al. 2005). Hence, here seems to be a fundamental difference to *E. coli* physiology. *E. coli* regulates expression of respiratory enzymes according to the potential free energy that is available from the reduction (Gunsalus 1992; Unden and Bongaerts 1997). In *Shewanella,* multiple respiratory pathways seem to be coexpressed under iron reducing conditions. It is therefore very well conceivable that electron transfer pathways in *Shewanella* are much more intertwined than in *E. coli*. We recently proposed such a kind of interconnection between different respiratory pathways regarding the function of the soluble fumarate reductase FccA in iron reduction (see below) (Schuetz et al. 2009). The complication for researchers arises from the fact, that *c*-type cytochromes do not seem to catalyze specific electron transfer reactions in most cases (Bretschger et al. 2007; Gao et al. 2010; Myers and Myers 2003b). Consequently, single mutants in *S. oneidensis* cytochromes frequently do not show a clear phenotype, since multiple cytochromes are coexpressed (Myers and Myers 2003b). Bretschger et al. published a study that underlines the redundancy driven complexity of the electron transport chains in *S. oneidensis* (Bretschger et al. 2007). The reduction potential of 34 different cytochrome mutants toward poorly soluble iron and manganese was compared. Only deletion of one cytochrome (MtrA, metal reducing protein A) resulted in the inability to reduce iron- or manganese-oxides, while several mutants showed only minor defects. It will become evident in further sections that this cytochrome MtrA has a dual function, which could very well be the reason for this defined phenotype.

The following considerations regarding the electron transport chain to Fe(III) or Mn(IV) will be separated in three parts following the electron flow from the cytoplasmic membrane through the periplasm and to the outer membrane in *S. oneidensis*.

2.1 Electron Transfer from the Cytoplasmic Membrane to the Periplasm

CymA (SO_4591). The standard reduction potential of the Fe^{3+}(aq)/Fe^{2+}(aq) couple (at pH 0) is 770 mV, and therefore almost as high as the oxygen/water couple with 820 mV. However, and as outlined by Prof. Majzlan in "Minerals and Aqueous Species of Iron and Manganese As Reactants and Products of Microbial Metal Respiration", this high redox potential is relevant only for acidophilic iron reduction. At neutral pH, the redox potential of ferric (oxyhydr)oxides varies between –177 and +24 mV (Majzlan "Minerals and Aqueous Species of Iron and Manganese As Reactants and Products of Microbial Metal Respiration"). Due to this low redox potential ubiquinone ($E^{o\prime} = +66$ mV) is not suitable as electron carrier in the cytoplasmic membrane and instead menaquinone ($E^{o\prime} = -74$ mV) is necessary. Consequently, mutants in the menaquinone synthesis pathway are unable to grow with ferric iron as terminal electron acceptor (Saffarini et al. 2002). It is well established that electrons are transported from the menaquinol pool to the periplasm by the catalytic activity of a tetraheme *c*-type cytochrome protein called cytoplasmic membrane protein *A* (CymA). As a member of the NapC/NirT protein family, the putative role of CymA is to direct electron flow from the menaquinol pool to several oxidoreductases located in the periplasm (Fig. 3; (Myers and Myers 1997b; Schwalb et al. 2003)). Interestingly, menaquinol was recently shown to be not only a substrate for CymA but also a cofactor for the enzyme (McMillan et al. 2012).

Deletion of the *cymA* gene from the chromosome leads to a mutant that is unable to use not only ferric iron as terminal electron acceptor, but also nitrate, nitrite, fumarate, and DMSO (Gao et al. 2009; Myers and Myers 1997b; Schwalb et al. 2003). A *cymA* deletion mutant is furthermore affected in its ability to use manganese oxides as electron acceptors (Bretschger et al. 2007). In the arsenate respiring strain *Shewanella sp.* strain ANA-3, CymA is furthermore electron donor for the periplasmic arsenate reductase (Murphy and Saltikov 2007).

Interestingly, the apparent midpoint potential of CymA (revealed using protein film voltammetry) is with –200 mV below the potential of the menaquinol/menaquinone couple. It was therefore hypothesized that a predominantly reduced menaquinole pool is necessary to enable electron transfer (Firer-Sherwood et al. 2008; Hartshorne et al. 2007). Of note, in terms of bioenergetics, the energy conservation associated with respiration of ferric iron is most likely associated with electron input into the menaquinone pool. CymA and all further proteins that build the electron transport chain to ferric iron serve therefore most probably only by means of menaquinone recycling and are not involved in proton pumping. Therefore, minor differences in the redox potentials of the cytochromes involved in electron transport to ferric iron might be sufficient since they exclusively serve as a wire that connects the menaquinole pool to ferric iron. Interestingly, CymA expression is necessary and sufficient to convert *E. coli* into a dissimilatory iron reducer if nitrilotriacetic acid (NTA) chelated ferric iron is added as electron

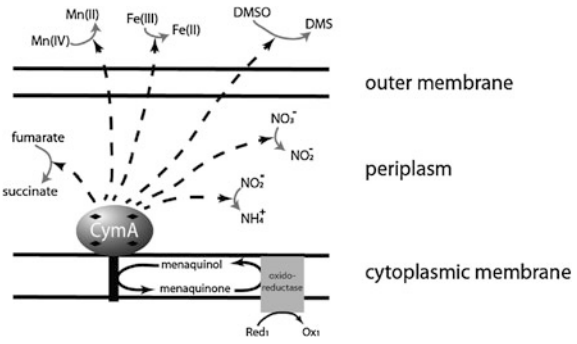

Fig. 3 Connection between CymA and periplasmic as well as extracellular respiratory chains. Heme cofactors are indicated by a diamond

acceptor. We could show in further experiments that at least in *E. coli* ferric NTA can pass the outer membrane and that CymA is the terminal reductase in this strain. Although a conductive connection to the surface of *E. coli* was not established, these experiments allowed concluding that iron reduction in gram-negative bacteria seems to be primarily limited by the lack of access of the terminal electron acceptor to a ferric reductase and that ferric iron reduction is not necessarily a process that demands a specific ferric iron reductase. Instead, *c*-type cytochromes in general can reduce a wide variety of ferric iron species and other organic and inorganic electron acceptors as well (Gescher et al. 2008).

2.2 Periplasmic Electron Transfer Reactions

Isolated periplasmic fractions from *S. oneidensis* cells have a bright to dark red color, which is due to the multitude of *c*-type cytochromes that are localized to the periplasm. This multitude can be easily separated and visualized using SDS polyacrylamide gels and subsequent heme activity staining (Fig. 4). Several research groups used heme containing protein bands excised from SDS gels or whole protein pools of the cell to conduct mass spectrometry (MS) in order to reveal the proteins that correlate to the detected heme containing protein bands. An overview of the results is presented in Table 2. While information about the function of most of these proteins is sparse, at least some evidence for the function of nine of the detected periplasmic *c*-type cytochromes is available. Hence, we will now direct our attention to the potential physiological function of these proteins.

FccA (SO_0970). FccA is the respiratory fumarate reductase of *S. oneidensis*. Its structure was solved by Leys et al. (1999). This enzyme can be distinguished from other respiratory fumarate reductases by two characteristics. First, it is a soluble, monomeric periplasmic protein while most other fumarate reductases consist of more than one subunit and are bound to the cytoplasmic membrane (Thauer et al. 1977). Second, FccA contains an N-terminal tetraheme domain which is not present in other fumarate reductases (Morris et al. 1994). The flavin domain contains a noncovalently bound FAD close to the active site (Pessanha et al. 2009).

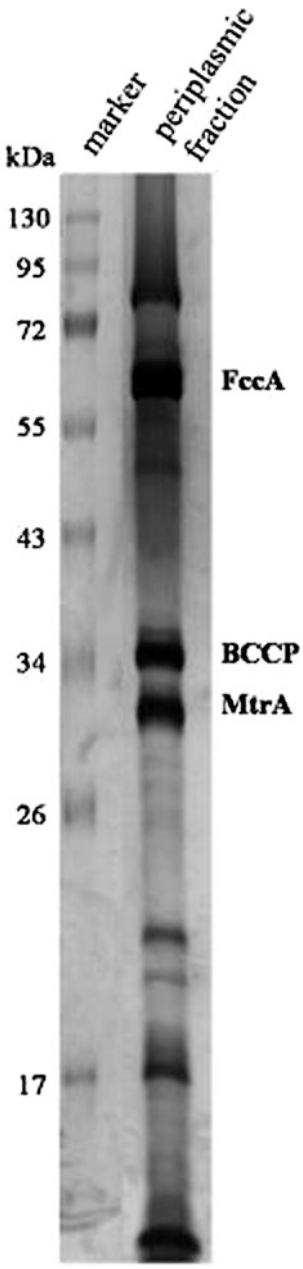

Fig. 4 Heme stain of a periplasmic protein fraction derived from *S. oneidensis* cells grown under ferric iron reducing conditions (Schuetz et al. 2009). Copyright © American Society for Microbiology

Surprisingly, FccA is the most prominent cytochrome in the periplasm of cells that were grown under ferric iron reducing conditions (Fig. 4). Due to the metabolic burden that is accompanied by the massive production of an enzyme having four heme and one FAD cofactor, it seems likely that FccA might have a function even

Table 2 Periplasmic *c*-type cytochromes and their detection in mass spectrometry studies

Protein-number	Predicted molecular weight in Da	Name	(Zhang et al. 2011)	(Sturm and Gescher, In preparation)	(Meyer et al. 2004)	(Brown et al. 2010)	(Elias et al. 2005)	(Elias et al. 2007)
SO_0264	10423.07	ScyA Monoheme c5	x	x	x	x		
SO_0479	79256.03	SirA octaheme c						
SO_0714	11294.13	Monoheme c4						
SO_0716	14277.38	Hypothetical Monoheme c						
SO_0717	12123.05	Monoheme c4						
SO_0845	15431.64	NapB Diheme c						
SO_0939	28710.52	Split-Soret Diheme c						
SO_0970	62447.76	FccA Tetraheme flavocyt	x	x	x	x	x	
SO_1413	13307.08	Split Tetraheme flavocyt						
SO_1421	63024.62	IfcA-1 Tetraheme flavocyt		x				
SO_1427	34656.96	DmsE Decaheme c						
SO_1777	36058.67	MtrA Decaheme c		x		x	x	x
SO_1782	33355.31	MtrD Decaheme c	x					
SO_2178	36200.37	CcpA Diheme c	x	x	x	x	x	
SO_2727	12147.57	STC Tetraheme c (also CctA)	x	x	x		x	
SO_2930	97515.91	Hypothetical Diheme c						
SO_3056	14233.20	Split Tetraheme flavocyt						
SO_3300	14099.24	Split Tetraheme flavocyt						
SO_3420	16282.57	Monoheme c9		x		x		
SO_3980	52333.77	NrfA Pentaheme c	x	x		x		
SO_4047	38364.35	SoxA-like Diheme c		x		x		
SO_4048	21913.12	Diheme c4		x		x		
SO_4142	11895.47	Hypothetical Monoheme c						

(continued)

Table 2 (continued)

Protein-number	Predicted molecular weight in Da	Name	(Zhang et al. 2011)	(Sturm and Gescher, In preparation)	(Meyer et al. 2004)	(Brown et al. 2010)	(Elias et al. 2005)	(Elias et al. 2007)
SO_4144	51399.81	Otr Octaheme c	x					
SO_4360	33171.30	MtrAD-like Decaheme c						
SO_4484	15892.94	SHP Monoheme c						
SO_4485	20360.82	Diheme c	x	x				
SO_4666	21917.14	Diheme c4	x	x	x	x		

under ferric iron reducing conditions. In vitro and in vivo experiments revealed that FccA can be directly reduced by the membrane bound cytochrome CymA (Schuetz et al. 2009; Schwalb et al. 2002). An *fccA* deletion mutant in *S. oneidensis* shows an interesting phenotype when analyzed carefully (Myers and Myers 1997a; Schuetz et al. 2009): the initial ferric iron reduction rate is faster when compared to the wild type, but the increase in colony forming units is not as high as in wild type cells (Fig. 5a; (Schuetz et al. 2009)). This small difference in growth rate becomes more prominent in competitive growth experiments (Fig. 5b, unpublished results). Since, the slower rate of ferric iron reduction in the wild type is not coupled to a decrease in cell growth or fitness, it was proposed that FccA could act as a transient electron storage protein or capacitor that is filled with electrons via reaction kinetics that are faster than electron transfer to an extracellular electron acceptor. This electron storage protein could allow for an initial growth rate higher than that of the Δ*fccA* mutant. For a certain time, this electron uptake could ensure that the respiratory chain is not stalled due to the limiting terminal electron transfer reactions and the subsequently reduced electron transfer proteins. This strategy would also allow *Shewanella* cells to use a carbon and electron source even if no terminal electron acceptor is present, since the transient electron storage would act as an intermediate electron acceptor. A recent publication supports this idea since it reports on electron storage within *Shewanella* biofilms measured with an electrode of a microbial fuel cell (Uria et al. 2011).

A similar hypothesis, but concerning periplasmic *c*-type cytochromes in general, was raised by Esteve-Nunez et al. (2008) for *Geobacter metallireducens* and by Rodrigues et al. (2006) for *Desulfovibrio vulgaris*.

If FccA is a transient electron storage protein, then it has to be connected to the catabolic electron flow to ferric iron. Evidence for a function in the electron transfer chain to ferric iron is derived from an in vitro experiment with membrane fractions from *S. oneidensis* cells. It was recently demonstrated that the outer membrane of *S. oneidensis* contains a protein complex that consists of the periplasmic cytochrome MtrA, the *β*-barrel protein MtrB, and the outer membrane decaheme cytochrome MtrC (also called OmcB in older studies). This complex will be discussed in more detail later. For now it is relevant to know that the complex can catalyze electron transfer across a membrane and that the decaheme cytochrome MtrC can directly reduce ferrihydrite. When purified and dithionite reduced FccA is added in the presence of ferrihydrite to catalytic amounts of *Shewanella* membranes (containing this complex) an oxidation of FccA coupled to ferric iron reduction can be detected ((Schuetz et al. 2009); Fig. 6). This redox process is most probably due to the MtrABC complex since the addition of membranes derived from a Δ*mtrA* strain did not lead to FccA driven ferrihydrite reduction (Schuetz et al. 2009).

Taken together, FccA could likely fulfill a dual function: first as a fumarate reductase and second as an electron storage protein in the periplasm that is directly connected to CymA at the cytoplasmic membrane and MtrA (in form of the MtrABC complex) at the outer membrane.

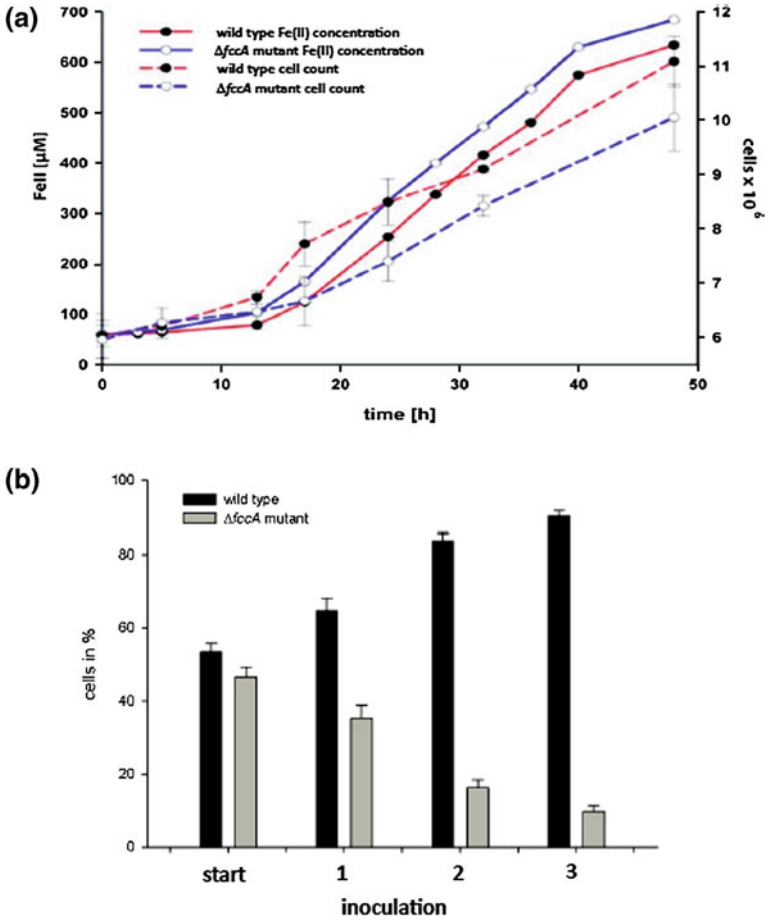

Fig. 5 **a** Growth and iron reduction curves of *S. oneidensis* wild type and Δ*fccA* mutant strain (Schuetz et al. 2009). The growth medium contained 10 mM hydrous ferric oxide and 5 mM lactate. Error bars indicate standard deviations. Copyright © American Society for Microbiology. **b** Competitive growth experiments with *S. oneidensis* wild type and Δ*fccA*. *S. oneidensis* wild type and Δ*fccA* cells were mixed in a 1:1 ratio and subsequently added to medium containing 50 mM ferric citrate and 25 mM lactate. An aliquot of the sample was spread on LB agar and the relative amount of wild type and Δ*fccA* cells was determined using PCR. Subsequently, 1 % of the culture was transferred to fresh medium when 35 mM ferric citrate were reduced and the relative amount of cells was determined. This procedure was repeated twice. All experiments were conducted in independent triplicates (unpublished data)

MtrA (SO_1777). MtrA is a 32 kDa monomeric decaheme *c*-type cytochrome. It was one of the first proteins identified to be necessary for iron reduction (Beliaev and Saffarini 1998). This central function for metal reduction was confirmed by numerous groups (Bretschger et al. 2007; Hartshorne et al. 2009; Schicklberger et al. 2010) while in one publication no growth deficiency of an *ΔmtrA* strain was detected (Gao et al. 2010). It is not clear why the mutant used by Gao et al.

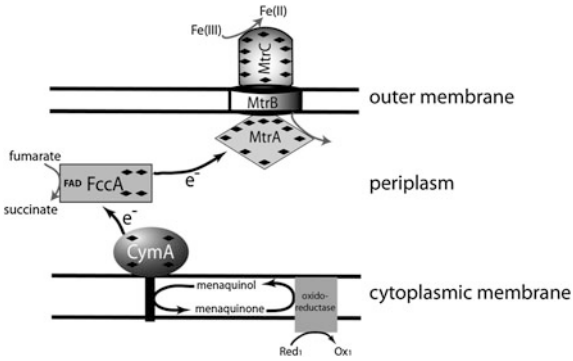

Fig. 6 Connection of FccA to the respiratory electron transport chain to ferric iron. Heme cofactors are indicated by a diamond. The FAD cofactor of FccA is accentuated

behaved that differently, but in our eyes the authors might have selected for a suppressor mutant that could overcome the impact of the *mtrA* deletion.

The cellular localization of MtrA is also under debate: until now, three publications have examined the localization of MtrA under ferric iron reducing conditions. Pitts et al. (2003 and Schuetz et al. (2009) detected MtrA (via SDS-PAGE and heme staining or MS analysis) associated to membranes and in a soluble state in the periplasm. Ross and coworkers (2007) detected MtrA only in the membrane fraction. These contradicting results might be due to the different methods that were used for the isolation of periplasmic fractions. Pitts et al. and Schuetz et al. used polymyxin B to insert holes in the outer membrane, while Ross et al. used an osmotic shock method accompanied with the destabilization of the outer membrane using EDTA.

Further evidence for a function in periplasmic electron transfer is derived from connection of in vitro and heterologous expression experiments. In vitro, MtrA can be directly reduced by CymA, which is bound to the cytoplasmic membrane. The rate of electron transfer is slightly higher when compared to FccA reduction. It is furthermore known that MtrA similar to CymA is a reductase acting on chelated ferric iron forms (Pitts et al. 2003). *E. coli* cells that express MtrA and CymA together respire Fe(III)-NTA faster than *E. coli* cells that express CymA alone (Schuetz et al. 2009). It was therefore hypothesized that the higher reduction rate in CymA and MtrA expressing cells is due to expression of a second ferric NTA reduction site that is directly connected to CymA (Fig. 7).

It is so far unknown whether this periplasmic MtrA can serve as electron donor for MtrA that is localized to the outer membrane within the MtrABC complex. Nevertheless, it was shown that purified reduced MtrA could serve as electron donor for FccA catalyzed fumarate reduction (Fig. 8). Therefore, MtrA and FccA can exchange electrons and the electron flow might solely be directed by the availability of the electron acceptor.

MtrD (SO_1782). MtrD is a decaheme *c*-type cytochrome, which is very similar to MtrA. Only one MS study identified peptides from this protein so far (Zhang et al. 2011). The *mtrD* gene is transcribed in an operon with *mtrD* and *mtrF*, which are believed to be involved in metal detoxification (Gao et al. 2010; McLean et al. 2008).

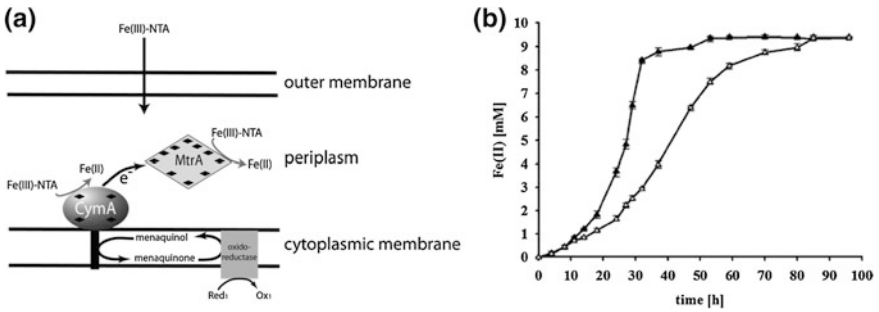

Fig. 7 Fe(III)-NTA reduction of the *E. coli* strain expressing CymA and MtrA. **a** Scheme of the electron transfer reactions in the periplasm of the constructed *E. coli* strain. Heme cofactors are indicated by a diamond. **b** Ferric iron reduction by the constructed strain expressing CymA and MtrA (▲), and the strain expressing CymA only (△) (Schuetz et al. 2009). 10 mM ferric NTA were added to mineral media as the sole electron acceptor, while glycerol (50 mM) was used as carbon and electron source. Both strains were induced with 0.43 mM anhydrotetracycline. Copyright © American Society for Microbiology

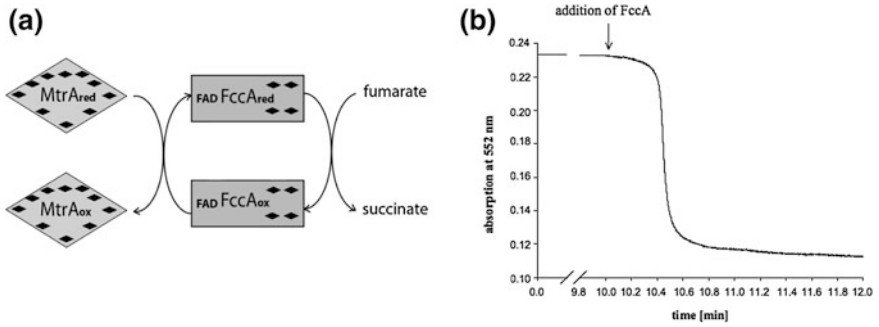

Fig. 8 FccA mediated electron transfer from MtrA to fumarate **a** Scheme of the tested electron transfer reaction. Heme cofactors are indicated by a diamond. **b** Redox status of initially reduced MtrA in the presence of fumarate and FccA (Schuetz et al. 2009). 1.2 µmol MtrA were reduced with dithionite and incubated for 10 min with fumarate. Addition of 120 pmol FccA (indicated with an *arrow*) caused a rapid reoxidation of MtrA as visualized using the absorption maximum of reduced *c*-type cytochromes at 552 nm. Copyright © American Society for Microbiology

The role of MtrD in iron reduction is controversial: a *ΔmtrD* strain had a strong negative effect in a competition study by Gao et al. while a similar mutant had an insignificant effect in a study by Bretschger et al. (2007); (Gao et al. 2010).

CcpA (SO_2178). CcpA has strong similarities to proteins of the *c*-type cytochrome peroxidase (CCP) family, which can be found in yeast and bacteria. In bacteria CCPs are diheme proteins with two *c*-type heme groups (Cabiscol et al. 2000). They seem to protect the bacterial cell from oxidative damage caused by hydrogen peroxide or the peroxide-based formation of hydroxyl radicals via the Fenton reaction (Atack and Kelly 2007; Pauleta et al. 2004). Exposure of bacterial cells to excess oxygen increases the amount of hydrogen peroxide but

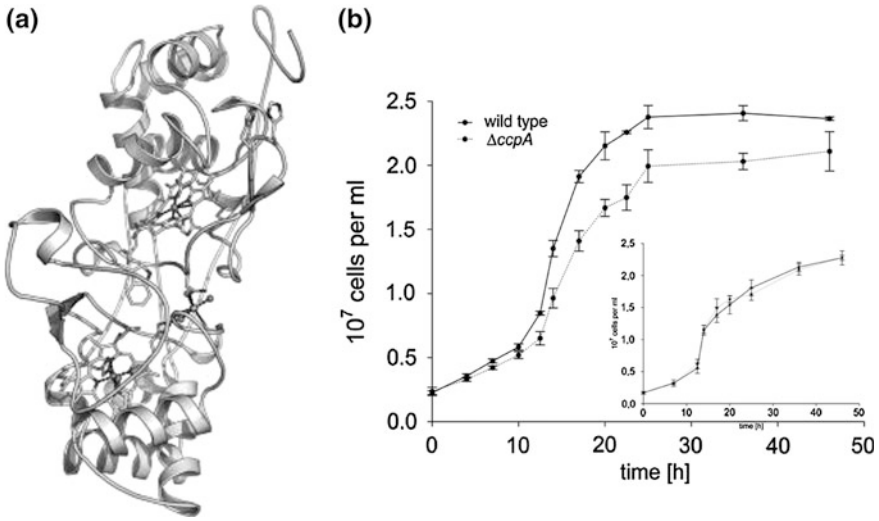

Fig. 9 **a** Three-dimensional structure of CcpA from *S. oneidensis*. The structure of CcpAHis was solved by X-ray crystallography to a resolution of 1.8 Å. **b** Growth of *S. oneidensis* wild type and Δ*ccpA* mutant. Cells were grown in independent triplicates under microoxic conditions in medium containing 50 μM oxygen, 50 mM ferric citrate and 15 mM lactate. Growth was determined via the quantification of colony forming units on LB-agar plates. The inset shows a control experiment performed in media that was further reduced with 1.5 mM titanium citrate (Schütz et al. 2011). Copyright © American Society for Microbiology

paradoxically bacterial CCPs are often up-regulated under microoxic or anoxic conditions (Atack and Kelly 2007).

All bacterial CCP proteins investigated so far have a conserved tertiary structure. They are two domain proteins, with a single heme group per domain covalently bound to the cysteines of the general binding motif of *c*-type cytochromes, CXXCH. As expected, CcpA of *S. oneidensis* has this typical three-dimensional structure and in vitro tests revealed the typical catalytic activity (Schütz et al. 2011); Fig. 9a). Furthermore, a deletion of the *ccpA* gene from the *S. oneidensis* chromosome resulted in a decreased capability to remove hydrogen peroxide from the medium and a decreased growth rate when cells were grown under microoxic conditions with ferric iron as terminal electron acceptor ((Schütz et al. 2011); Fig. 9b).

Electron transfer from CymA to CcpA was detectable only in the presence of the small monoheme *c*-type cytochrome ScyA (see below). This is interesting, since electron transfer between *c*-type cytochromes was so far regarded as being rather unspecific. The interplay between ScyA and CcpA provides proof that this does not necessarily have to be the case. Although CcpA is not directly connected to CymA, hydrogen peroxide could theoretically function as terminal electron acceptor under anoxic conditions with an electron transport chain that potentially consists of CymA, ScyA, and CcpA (Fig. 10). But more importantly, the anaerobic respiratory chain can provide electrons for the detoxification of reactive oxygen

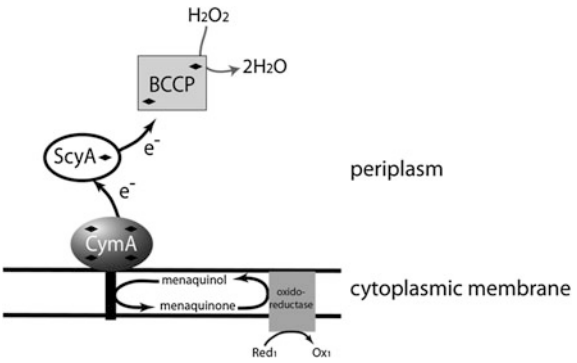

Fig. 10 Electron transport chain to hydrogen peroxide. Experimentally established electron transport connections are indicated by an *arrow*. Heme cofactors are shown by a *diamond*

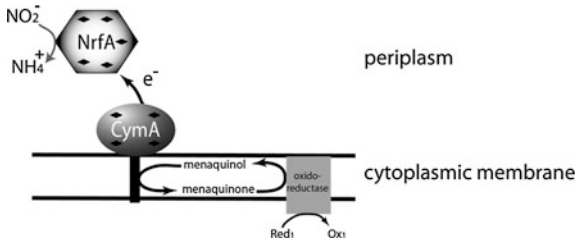

Fig. 11 Electron transport chain to nitrite. Experimentally established electron transport connections are indicated by an *arrow*. Heme cofactors are shown by a *diamond*

species. It might be beneficial to fuel peroxidases using anaerobic electron transport chains since this will guarantee a fast response to hydrogen peroxide evolution in case of oxygen inflow into so far anoxic growth environments.

ScyA (SO_0264). It was outlined above that ScyA (SO_0264) is a small monoheme cytochrome (8.3 kDa) that is necessary for the reduction of the diheme peroxidase CcpA. ScyA can be directly reduced by CymA (Schütz et al. 2011). Interestingly, we and others were unable to construct a markerless *scyA* deletion mutant. Hence, we had to use a conditional mutant to study the *ΔscyA* phenotype. It is so far not known why this *scyA* locus might be necessary for the cell.

NrfA (SO_3980). NrfA is a 50 kDa pentaheme protein that is catalyzing nitrite reduction in *S. oneidensis*. NrfA is upregulated under nitrate reducing conditions (Beliaev et al. 2002). Transcriptional and mutational analyses suggest that CymA (instead of NrfH in other γ-protebacteria) is the electron-donating enzyme to NrfA (Gao et al. 2009) (Fig. 11). A biochemical analysis of probable further electron donating and accepting enzymes for NrfA was so far not conducted. A *nrfA* mutant reduced similar amounts of ferric iron compared to the wild type within 24 h (Bretschger et al. 2007).

IfcA-1 (SO_1421). IfcA is a tetraheme *c*-type cytochrome that was only described in *S. frigidimarina* so far (Dobbin et al. 1999). In this strain, it was specifically induced under iron reducing conditions. The purified protein has fumarate reductase activity in vitro but seems not to be operating under fumarate reducing conditions (Dobbin et al. 1999).

STC (also called CctA) (SO_2727). STC is a small tetraheme *c*-type cytochrome. It has been extensively characterized structurally and electrochemically (Fonseca et al. 2009; Leys et al. 2002; Paquete et al. 2010; Qian et al. 2011a). The exact role in the physiology of *Shewanella* remains unclear. Coursolle and Gralnick (2010) propose a role in periplasmic electron transfer as rather unspecific electron shuttling protein, eventually even between different respiratory pathways.

Otr (SO_4144). Otr is an octaheme *c*-type cytochrome that has $S_4O_6^{2-}$, NO_2, and NH_2OH reduction activities in vitro (Atkinson et al. 2007; Mowat et al. 2004). Still, the physiological role remains to be explored.

Other periplasmic cytochromes. As it was listed above, a number of further periplasmic cytochromes were detected using mass spectrometry (Table 2). So far, these cytochromes were not studied in more detail and are currently not connected to a physiological function in *S. oneidensis*. Bretschger et al. (2007) tested mutants in these cytochromes for a putative defect in ferric iron reduction but the amount of ferric iron reduced after 24 h was in most cases even above the level reached by the wild type. In a recent study, Gao et al. (2010) were using mainly competitive growth experiments to assess the fitness of several mutants. There study revealed that most of the constructed mutants in *c*-type cytochromes were negatively affected in fitness as compared to the wild type (Gao et al. 2010). Still, it is so far not possible to assign specific functions to these proteins and the ecological reason for their expression has to be studied in the future. Nevertheless, one hypothesis regarding their function could be that they expand the transient electron storage capacity and might link electron transfer to further potential electron acceptors, and hence widen the potential electron transfer network in the periplasm of *S. oneidensis,* as will be discussed in the following paragraphs.

A highly dynamic network formed by periplasmic c-type cytochromes could promote a rapid dispatch of transiently stored electrons to various electron acceptors. For instance, when cells are in contact with extracellular Fe(III) (oxyhydr)oxides, electrons derived from CymA are transferred via MtrA to MtrC, which is oxidized by the Fe(III) (oxyhydr)oxide or extracellular electron shuttles. In the absence of such a poorly soluble electron acceptor, oxidation of MtrC, and consequently of MtrA, is blocked, and electrons are transferred from CymA either directly or indirectly via MtrA to the transiently electron-storing FccA. FccA can be rapidly reoxidized by MtrA when MtrA is oxidized by outer membrane MtrC once cells are contacting Fe(III) (oxyhydr)oxides again. In the presence of fumarate, a direct oxidation of FccA by fumarate would bypass the outer membrane electron transfer.

Depending on mineral identity, ferric iron can have the lowest redox potential of all usable respiratory electron acceptors of *S. oneidensis*. Therefore, the midpoint redox potentials of proteins involved in the electron transport chain to ferric iron should theoretically also allow electron transfer to other periplasmic or surface exposed terminal reductases of *S. oneidensis*. Hence, the respiratory chain to ferric iron could be furthermore connected to electron transport chains to electron acceptors such as nitrate, nitrite, DMSO, or manganese oxides (Fig. 12). Of note, one of the other periplasmic cytochromes that are not as highly expressed

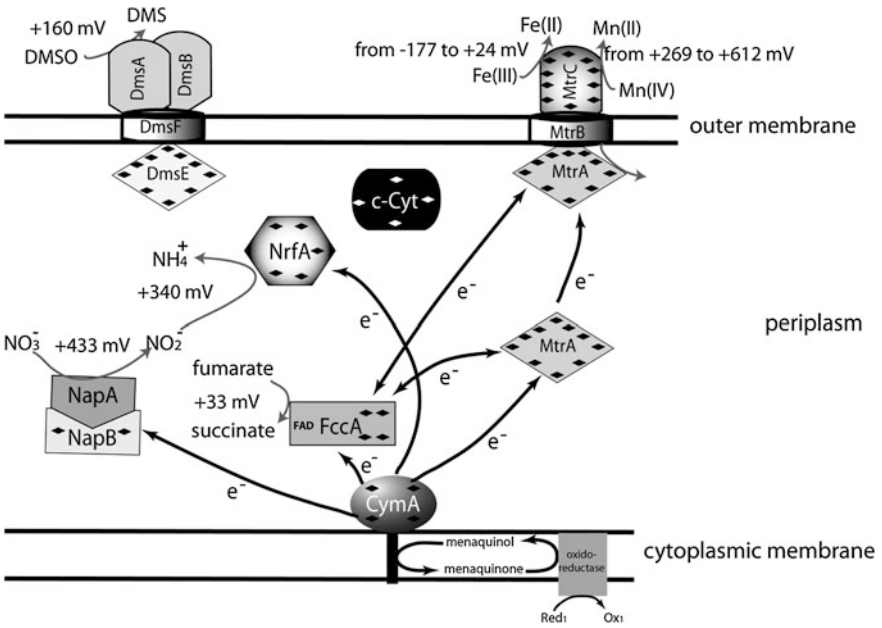

Fig. 12 Potential components of a periplasmic and outer membrane electron transfer network. Indicated are terminal reductases, that were detected using mass spectrometry and that might be involved in the network. Already elucidated electron transfer connections are indicated by an *arrow*. Redox potentials of the reactions are given E_0' in mV (Thauer et al. 1977) (Majzlan "Minerals and Aqueous Species of Iron and Manganese As Reactants and Products of Microbial Metal Respiration"). The possible function of other *c*-type cytochromes (*c*-Cyt) is indicated

as FccA or MtrA is the nitrite reductase NrfA. Furthermore, we detected (using MS-analysis) the terminal nitrate reductase NapA and the terminal DMSO reductase DmsA and B in cells grown with ferric iron as terminal electron acceptor. In addition, MtrC, the potential final reductase of the electron transport chain to ferric iron, has also manganese oxide reducing activity. Hence, if all these pathways are connected like and together with MtrA and FccA, then the availability of nitrate, nitrite, manganese oxide, or DMSO could furthermore result in a dispatch of electrons derived from reduced MtrA and FccA.

The existence of a periplasmic electron transfer network could also explain the partial or general lack of phenotypes observed in mutants defective in a number of periplasmic *c*-type cytochromes. It is furthermore in line with data recorded by Firer-Sherwood and coworkers (2008). In their study, the authors subjected the multiheme cytochromes CymA, STC, MtrA, OmcA, and MtrC of *S. oneidensis* to protein film voltammetry and determined not only apparent midpoint potentials but also windows of redox potentials that allow for electron uptake and abstraction. The widths of the individual redox potential windows have a large overlapping region, which potentially allows for rapid interprotein electron transfer (Table. 3; (Firer-Sherwood et al. 2008)).

Table 3 Apparent midpoint potentials (E_m) and width of potential windows at 4 °C and pH 6 (Firer-Sherwood et al 2008)

Protein	E_m (mV)	Window width (mV)
MtrC	−138	275
OmcA	−175	300
MtrA	−100	300
STC	−160	250
CymA	−200	250

The electron transfer network and the ecological niche of S. oneidensis. In terms of respiratory energy generation, *S. oneidensis* does not seem to be a specialist but rather a generalist. The strain reduces a wide variety of possible electron acceptors but oxidizes carbon sources under anoxic conditions only to the level of acetate. The energy yield that can be gained via the respiratory chains is not fully exploited completely in some cases. For instance, the electron transport chain to nitrate goes via CymA, and hence possible further coupling sites that could be used (given the redox potential of the electron acceptor) are not exploited (Gao et al. 2009). Furthermore, regulation of the expression of terminal oxidoreductases is rather relaxed and a multitude of these enzymes is detectable in the periplasm (Beliaev et al. 2005). Moreover, it was pointed out that substrate level rather than oxidative phosphorylation is the primary energy source of *S. oneidensis* under anoxic conditions (Hunt et al. 2010). Therefore, it seems as if the ecological strategy of *S. oneidensis* is a rapid consumption of available carbon sources with the least number of possible reactions. This has to be connected with a rapid dispatch of electrons on available electron acceptors, which could very well be achieved by the realization of an electron transfer network in the periplasm.

2.3 Electron Transfer Reactions at the Outer Membrane

2.3.1 The Electron Conduit Through the Outer Membrane

The hydrophobic thickness of the outer membrane of gram-negative bacteria is approximately 25 Å (Lomize et al. 2006). Therefore, tunneling-based electron transfer through the membrane is not possible even if cytochromes are associated with the periplasmic and the extracellular site of the membrane. The electron conduit over the outer membrane of *S. oneidensis* is composed of three proteins that build a membrane-spanning complex. The β-barrel protein MtrB (SO_1776) is connected to the decaheme cytochrome MtrA (SO_1777) on the periplasmic site and the outer membrane cytochrome MtrC (SO_1778) at the outer surface. It was demonstrated in vitro that this complex has the capacity to transport electrons over a liposomal membrane (Hartshorne et al. 2009). Hence, the in vivo function of this complex is most likely outer membrane spanning electron transfer (Fig. 12).

Interestingly, a mutant in *mtrB* is unable to use ferric iron (ferric citrate or hydrous ferric oxide) or manganese oxide as electron acceptor (Beliaev and Saffarini 1998; Bretschger et al. 2007). Thereby, MtrB is besides MtrA the only protein that is definitely necessary for these respiratory processes (Beliaev and Saffarini 1998; Shyu et al. 2002). The impact of the mutation can certainly be explained by the integral function of MtrB for the MtrABC complex. But earlier publications nicely displayed that MtrB might influence ferric iron reduction also on further levels. Strikingly, deletion of *mtrB* causes mislocalization of outer membrane cytochromes into the cytoplasmic membrane or the periplasm (Myers and Myers 2002a). Hence, it seems possible that MtrB has an influence on the transport of these proteins through the periplasm and furthermore the assembly into the outer membrane.

Building the MtrAB complex. Surprisingly, it was found that MtrB is not detectable in a Δ*mtrA* mutant (Hartshorne et al. 2009; Schicklberger et al. 2010). Since the genes for MtrA and MtrB most likely form an operon, it was first sought for an effect of the *mtrA* deletion on *mtrB* transcription or RNA stability. Still, quantitative PCR excluded this possibility. Instead, MtrA seems to affect the periplasmic stability of MtrB, because a mutant in the periplasmic protease DegP (SO_3942) did not require MtrA for MtrB stability (Schicklberger et al. 2010). DegP is well known for its major role in the prevention of a toxic accumulation of unfolded outer membrane proteins in the periplasm in a variety of species including *E. coli* (Bos et al. 2007). The so far established pathway of β-barrel protein transport to the outer membrane necessitates activity of the periplasmic chaperones Skp and SurA that bind nascent β-barrel proteins and guide them through the periplasm (Bos et al. 2007; Sklar et al. 2007; Spiess et al. 1999). It was proposed that MtrA (besides its function in electron transport) is also a periplasmic chaperone or scaffold, which is specific for MtrB. However, the detailed mechanism of MtrABC complex formation and of the exact role of MtrA in the stability of MtrB still has to be shown experimentally.

Excursus—Distribution of MtrAB-like modules. Interestingly, modules similar to MtrAB are present in bacteria belonging to the α, β, γ, and δ group of the proteobacteria (Table 3). With the exception of *S. denitrificans*, 18 sequenced *Shewanella* strains contain up to nine copies of an MtrAB like module. *S. denitrificans* cannot grow with ferric iron as terminal electron acceptor which might explain the lack of a module similar to MtrAB. In *S. oneidensis*, it was shown that MtrAB like modules are involved in dissimilatory metal and DMSO reduction (Gralnick et al. 2006). The module involved in dissimilatory iron, manganese, and uranium reduction is in 3'direction flanked by the gene for the outer membrane cytochrome MtrC, whereas the module that is necessary for DMSO reduction is adjacent to the two 5' encoded DMSO reductase genes (*dmsA*, *dmsB*; Fig. 13). A number of *Shewanella* strains contain another MtrAB like module that is in 5'direction flanked by an outer membrane cytochrome (MtrF) that is highly similar to MtrC (*mtrDEF* cluster; Fig. 13; (Bücking et al. 2010).

Gene clusters similar to the *mtrCAB* cluster were found in a number of pathogenic *Vibrio* strains and in the known ferric iron reducer *Rhodoferax*

Table 4 Distribution of MtrAB modules in proteobacteria

Class of Proteo-bacteria	Strain	Number of AB moduls	Number of moduls similar to *S. oneidensis mtrCAB* cluster	Number of moduls similar to *S. oneidensis dmsEFAB* cluster	Number of moduls similar to *S. oneidensis mtrDEF* cluster	Number of moduls similar to *R. palustris pioABC*	Number of moduls with unknown function
α	*Magnetospirillum magnetotacticum*	1	0	0	0	0	1
	Rhodopseudomonas palustris	1	0	0	0	1	0
β	*Sideroxydans lithotrophicus*	1	0	0	0	0	1
	Rhodoferax ferrireducens	1	1	0	0	0	0
	Dechloromonas aromatica	1	0	0	0	0	1
γ	*Shewanella oneidensis MR1*	4	1	2	1	0	0
	S sp. MR4	3	1	1	1	0	0
	S. sp. MR7	3	1	1	1	0	0
	S. frigidimarina	3	1	2	0	0	0
	S. sp. ANA3	2	1	0	1	0	0
	S. baltica OS155	2	1	0	1	0	0
	S. loihica	2	1	0	1	0	0
	S. putrefaciens 200	3	1	2	0	0	0
	S. putrefaciens CN32	2	1	1	0	0	0
	S. baltica OS185	2	1	0	1	0	0
	S. sediminis HAW-EB3	9	1	6	1	0	1
	S. pealeana ATCC700345	*4*	1	2	1	0	0
	S. baltica OS199	*3*	1	1	1	0	0
	S. benthica	*3*	1	1	1	0	0
	S. halifaxensis	*3*	1	2	0	0	0
	S. woodyi	*2*	1	0	1	0	0
	S. piezotolerans	*2*	1	0	1	0	0
	S. baltica OS223	*2*	1	0	1	0	0
	Vibrio sp.[a]	*3*	1	2	0	0	0
	Aeromonas hydrophila	*2*	1	1	0	0	0
	Halorhdospira halophila	*2*	1	0	1	0	0
δ	*Geobacter sp.*	1	0	0	0	0	1
	Nitrosococcus oceani	1	0	0	0	0	1

[a] Genomes from nine different *Vibrio* strains contain MtrAB modules (*V. parahemolyticus* strains Pery4-66, AQ4037, AN5034, K5030, AQ3810; *V. sp.* EX25; *V. vulnificus* strains YJ016, CNCP6)

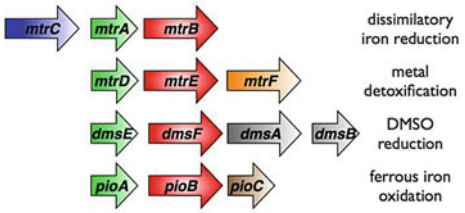

Fig. 13 Gene clusters containing an MtrAB like module that could have been connected to a physiological function. Genes similar to *mtrA* are drawn in *green*; genes similar to *mtrB* are drawn in *red*

ferrireducens. While the cluster might be important for metal oxide respiration in *R. ferrireducens*, in *Vibrio* it might be a way for iron acquisition, and hence a pathogenicity factor. Interestingly, it was discovered that proteins with a high similarity to MtrAB are necessary for the back reaction of iron reduction meaning the oxidation of Fe(II): In *Rhodopseudomonas palustris TIE-1* these proteins are necessary for phototrophic Fe(II) oxidation (*pioABC*; Fig. 13; (Jiao and Newman 2007) and in *Sideroxydans lithotrophicus ES-1* the module seems to be important for chemotrophic Fe(II) oxidation (*mtoA;* (Liu et al. 2012)). Modules similar to MtrAB were also detected in a number of other bacteria. Currently, we do not know what the function of these modules might be.

In summary, MtrAB like modules are widely distributed and seem to comprise a strategy for electron transfer over the outer membrane. Table 4 shows the distribution and putative functions of the modules in different classes of proteobacteria (representative examples of sequenced organisms are shown). While the outer membrane cytochrome MtrC is necessary for ferric iron reduction in *S. oneidensis,* other surface exposed modules seem furthermore connectable to complexes similar to MtrAB and might determine the terminal electron acceptor or the electron donor of the respiratory pathway.

2.3.2 Outer Membrane Cytochromes

Many studies on the role of outer membrane cytochromes (OMC) have been published to date. Surprisingly, it is still a matter of ongoing research to assign specific functions to the individual proteins. *S. oneidensis* contains the genetic information for five putative outer membrane cytochromes (Meyer et al. 2004). All of these are exposed to the cell surface when expressed in *S. oneidensis* from a plasmid-encoded copy of the gene (Bücking et al. 2010; Richter et al. 2010). The deduced amino acid sequences of OmcA, MtrC, MtrF, and SO_1659 indicate 10 putative heme attachment sites, while SO_2931 is expected to be a diheme *c*-type cytochrome Fig. 14.

OmcA and MtrC. The function of OmcA and MtrC has been studied best so far. They were shown to be lipoproteins exposed to the outer surface of the cell (Myers and Myers 2003a, 2004a). Both proteins form a high affinity complex in the outer membrane (Shi et al. 2006). Surprisingly, Reardon et al. (2010) outlined that the overall distribution of these cytochromes on the cell surface is not similar, which

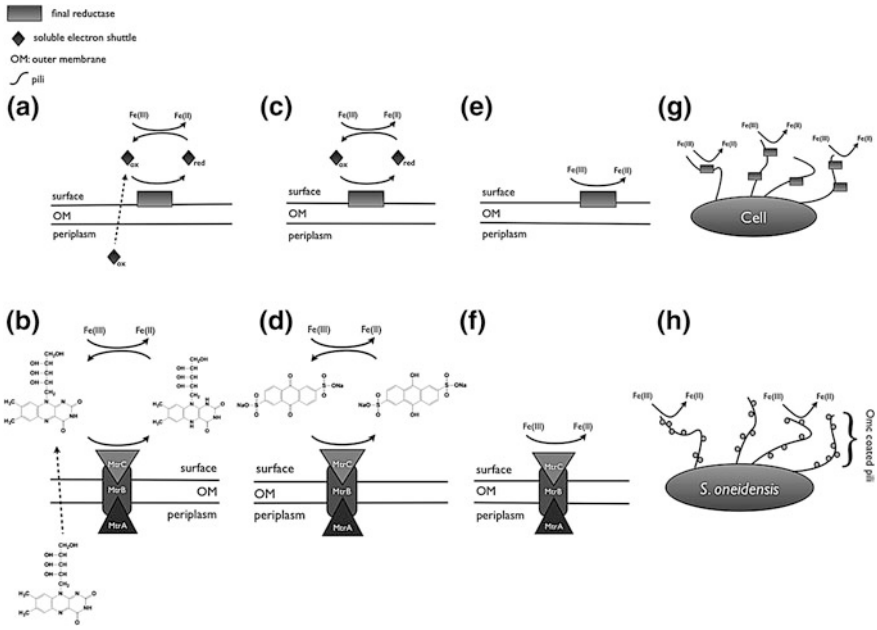

Fig. 14 The different modes of terminal extracellular electron transfer in *S. oneidensis*. Schematic overview of **a** indirect electron transfer to Fe(III) using an endogenous electron shuttle **c** indirect electron transfer to Fe(III) using an exogenous electron shuttle **e** direct electron transfer to Fe(III) **g** cell using pili coated with final reductases for Fe(III) reduction. **b d f** and **h** represent an example for the more general overview shown in **a**, **c**, **e**, and **g**, respectively

seems contradictory to the detection of a high affinity complex. When *S. oneidensis* cells were grown with ferrihydrite as electron acceptor, MtrC was shown to localize in close association to iron precipitates on the cell surface. In contrary, OmcA was distributed more diffusely on the cell exterior and was also detected outside of the cell in the layer of extracellular polymeric substances. Both proteins are capable of transferring electrons to various soluble and insoluble ferric iron species including ferrihydrite (Reardon et al. 2010). Furthermore, both proteins seem to contain hematite binding motifs as described by Lower et al. (2008) using phage display. Nevertheless, single gene deletion mutants in *omcA* or *mtrC* have differing phenotypes. In general, *mtrC* mutants show stronger growth deficiencies under dissimilatory metal reducing conditions (Myers and Myers 2001, 2002a). This might in part be due to the formation of the MtrABC complex that was mentioned before. Myers et al. showed that within 24 h an *mtrC* deletion mutant had only 10–15 % of the reducing activity toward manganese oxide, ferric citrate, or amorphous ferric oxide compared to the wild type (Myers and Myers 2002a). However, prolonged incubation seems to result in the evolution of suppressor mutations that compensate partly for the loss of outer membrane cytochromes. In the study of Myers et al., the amount of ferrous iron in mutant and wild type samples was almost equal after 4 days (Myers and Myers 2002a). In a recent study

from our lab, we could show that a strain deficient in all outer membrane cytochromes (later on referred to as ΔOMC), was able to regain the ability to reduce ferric iron due to the occurrence of a suppressor mutation (Bücking et al. 2012).

An *omcA* single deletion mutant was shown to possess lower MnO_2 reduction rates, whereas activity toward soluble electron acceptors and iron (oxyhydr)oxides remained unaffected or, in the case of Fe(III), mildly affected (Myers and Myers 2001) (Coursolle and Gralnik 2010). Expression of OmcA in the above mentioned ΔOMC strain did not complement the mutant for ferric iron reduction or growth in a microbial fuel cell but was surprisingly sufficient to allow for manganese oxide reduction, although on a lower level compared to the wild type or the MtrC expressing ΔOMC strain (Bücking et al. 2010).

MtrF. Deletion of the *mtrF* gene alone did not lead to decreased ferric citrate, ferrihydrite, MnO_2, or U(VI) reduction rates (Marshall et al. 2006; Myers and Myers 2002a). Surprisingly, even a pronounced stimulating effect of the mutation on ferrihydrite reduction has been reported (Bretschger et al. 2007). It seems that MtrF is not expressed under the tested iron reducing conditions, since experiments conducted with a ΔOMC strain expressing MtrF from a plasmid encoded copy revealed that the activity of MtrF is highly similar to MtrC. Consequently, MtrF expression can rescue the ΔOMC strain when ferric iron, manganese oxide or an anode is provided as terminal electron acceptor (Bücking et al. 2010). Hence, it can be further hypothesized that the similarity between MtrC and MtrF allows for the formation of a MtrABF complex into the outer membrane of *S. oneidensis* cells.

Recently, the crystal structure of MtrF was solved. Using this structural data, the authors aimed at elucidating the actual electron transfer mechanism from MtrF to a metal surface. MtrF shows a surface exposure of heme 5 and 10 of 250 and 300 $Å^2$, respectively (Clarke et al. 2011). Based on a previous report, the authors suggested that those terminal hemes have sufficient surface exposure such that they could be involved in direct contact with the iron surface (Clarke et al. 2011). This direct electron transfer would require a distance of less than 15 Å between the catalytic heme group and the poorly soluble metal oxide (Kerisit and Rosso 2007). Interestingly, the structure of MtrF shows besides the surface exposed heme containing domain, two domains that hold heme cofactors that are not surface exposed but solvent accessible. These domains furthermore hold β-barrel structures that are similar to flavin mononucleotide (FMN) binding domains. Still, FMN was not found in this region even after soaking the crystals in an FMN solution. Clark et al. suggest that these domains could interact with soluble electron shuttles, potentially FMN (see below and chapter 4).

Of note, MtrF is upregulated under oxic conditions in a specific medium that led to an autoaggregation of *S. oneidensis* cells. Therefore, McLean and coworkers (2008) hypothesized that MtrF might have a function in the aerobic reduction-based detoxification of metals like uranium or technetium that become sparsely soluble upon reduction. A hypothesis which points in the same direction was formulated by Gao et al. (2010) regarding chromium stress.

SO_2931 and SO_1659. Physiological expression of SO_2931 and SO_1659 has so far never been reported (Elias et al. 2006; Kolker et al. 2005). In line with these results, deletion of *SO_2931* did neither hamper current production in a microbial fuel cell nor reduction of ferric iron (Bretschger et al. 2007). Expression of these cytochromes in the ΔOMC strain did not result in a phenotypic alteration, although they were surface exposed (Bücking et al. 2010; Richter et al. 2010). Therefore, it is currently unknown if these proteins are involved in other reactions or are nonfunctional.

Additional necessary and supporting factors. DiChristina et al. (2002) showed that the type II secretion system (T2SS) of *S. oneidensis* is necessary for dissimilatory iron reduction. Mutants in important parts of the T2SS are defective in the secretion of OmcA and MtrC (Shi et al. 2008), in DMSO reduction (Gralnick et al. 2006) and in the production of conductive nanowires (see below (Gorby et al. 2006)). It seems most likely that the T2SS is necessary for the translocation of the terminal reductases across the outer membrane to the surface of the bacterial cell. Support for this hypothesis comes from a study by Shi et al. (2008) Here, the authors observed a proteinase K resistance of MtrC and OmcA in cells with a nonfunctional version of the T2SS, while a functional T2SS lead to outer membrane cytochrome degradation. Apparently, outer membrane cytochromes are only surface localized in the presence of a T2SS and are localized to the periplasmic site of the membrane in the absence of this export machinery.

Interestingly, Qian et al. provided evidence for the possible function of a protein similar to TonB-dependent receptor proteins in dissimilatory iron reduction in *S. oneidensis*. The authors could show that this protein, SO_2907, is capable of binding soluble Fe(III) forms and that a deletion mutant is affected in reduction of ferric citrate. It was therefore speculated that SO_2907 might be involved in iron transport to the periplasm which would represent another physiological strategy to bring ferric iron in contact with potential reductases (Qian et al. 2011b). Still, mutants in outer membrane cytochromes show strong phenotypes in ferric citrate reduction which underlines the fact that the cell surface is the major site for ferric iron reduction (Bücking et al. 2010). Nevertheless, it will be interesting to see in further experiments which role this potential ferric iron transport route might have in comparison to the established electron transport to ferric iron via outer membrane cytochromes.

3 Extending the Extended Respiratory Chain

For a long time, electron transfer to poorly soluble metal oxides was believed to be realized via direct electron transfer reactions catalyzed by cytochrome proteins on the cell surface. This hypothesis was challenged by Lies et al. (2005) using an innovative experiment. The authors synthesized ferric iron containing porous glass beads. This glass material prohibited direct contact of the ferric iron to the bacterial cell. Nevertheless, *S. oneidensis* was capable of reducing this trapped iron. This was evidence for a way of distributing electrons to poorly soluble

electron acceptors that can be micrometers away from a bacterial cell. Since then, several ideas have been proposed and several mechanisms have been elucidated how indirect electron transfer reactions could be realized by the cell itself or using exogenous electron shuttling components.

Endogenous electron shuttles. Endogenous electron shuttles could interact between a metal oxide surface and a bacterial cell and thereby enabling reduction at a distance. If this shuttling compound could be reused several times by the organism that secretes it, then the energy invested in the synthesis of that compound could be almost negligible.

A first hypothesis for an endogenous electron shuttle of *S. oneidensis* was raised by Diane Newman. She speculated about a possible function of quinones as endogenous electron shuttles (Newman and Kolter 2000), because she could show that menaquinone synthesis mutants, unable to respire on extracellular electron acceptors, could be rescued using menaquinone-related substances that were detected in the supernatant of wild type cells. Later, it became evident; that the secreted substances allowed the mutants to overcome the gap in the menaquinone synthesis pathway and that they were not involved in electron shuttling (Myers and Myers 2004b).

In 2008, two groups published reports on the role of flavins as electron shuttles. They were detected in the growth medium of *S. oneidensis* cells and in microbial fuel cells (Marsili et al. 2008; von Canstein et al. 2008). Interestingly, it was demonstrated that their presence strongly enhanced ferric iron and anode reducing activity of *S. oneidensis* cells (Marsili et al. 2008; Ross et al. 2009). Prof. Gralnick will discuss their role in metal reduction in more detail in "On the Role of Endogenous Electron Shuttles in Extracellular Electron Transfer" of this book. Furthermore, Prof. Kappler will introduce the reader to exogenous electron shuttles like humic substances that are ubiquitously distributed in a variety of habitats (see "Humic Substances and Extracellular Electron Transfer").

Nanowires. Pilus like cellular appendages that are conductive are termed nanowires. These nanowires can be several microns long, and hence enable a dissimilatory metal reducer to reach a distantly localized electron acceptor. They were first described in *Geobacter* (Reguera et al. 2005) and later also discovered in *S. oneidensis* (El-Naggar et al. 2010; Gorby et al. 2006). While they are necessary for the reduction of ferric oxides by *G. sulfurreducens*, *S. oneidensis* produces them under conditions of electron acceptor limitation. *S. oneidensis* nanowires seem to be decorated with outer membrane cytochromes that are necessary for electron transport along the pilus. Consequently, nonconductive but morphologically similar pili are produced in the absence of MtrC and OmcA. Therefore, it seems as if electrons are transported from outer membrane cytochrome to outer membrane cytochrome along the pilus which necessitates a distance of less than 14 Å between individual proteins (Gorby et al. 2006). Curiously, it is so far unknown what the structural component of these pili in *S. oneidensis* is. Moreover, conductivity was so far only shown for dried samples. Hence, a number of further experiments have to be conducted to find out the physiological function that these appendages might have for *S. oneidensis*.

Membrane vesicles. A number of publications report the production of outer membrane vesicles by gram-negative bacteria. Their production seems to be a stress response and they seem to be important tools for nutrient acquisition, biofilm development, as well as pathogenesis (Kulp and Kuehn 2010). Yuri Gorby reported that *Shewanella* strain CN32 produces such vesicles and that they can reduce ferric iron in the presence of hydrogen (Gorby et al. 2008). Hydrogen apparently reduces the outer membrane cytochromes localized to the membrane vesicles. Furthermore it seems likely, that these vesicles can be reduced by *Shewanella* itself and can then act as complex endogenous electron shuttles. Still, so far we are not able to assess the influence that these vesicles might have in environmental iron reduction.

Iron chelators. Iron assimilation depends for a number of strains on the production of siderophors that are able to chelate sparsely soluble ferric iron and to convert it thereby into organic ligand complexed, and thus dissolved Fe(III) (Braun and Hantke 2011). One major group of siderophors consists of derivatives of hydroxamic acid. *S. oneidensis* has the genetic information for hydroxamate biosynthesis. Furthermore, *S. oneidensis* produces soluble Fe(III) when grown under anoxic conditions either with ferrihydrite or with ferrihydrite plus fumarate as terminal electron acceptor (Jones et al. 2010). Surprisingly, hydroxamate biosynthesis is not involved in the solubilization of ferrihydrite under the tested conditions. Instead, a different yet uncharacterized organic chelator is produced as was shown by Jones et al. (2010). The authors could find a number of mutants that are apparently not able to produce this soluble iron, as detected using a novel microelectrode screening assay. Unfortunately, it is so far not known how this organic chelator is produced by *S. oneidensis* and what chemical structure it has. Still, the detected mutants that are not able to produce dissolved organic-Fe(III) complexes are furthermore unable to reduce ferric citrate or ferrihydrite.

4 Concluding Remarks

S. oneidensis shows an astonishing versatility in terms of electron acceptors that can be used under anoxic conditions. The versatility seems to be mostly due to a variety of *c*-type cytochromes that enable electron transport to terminal electron acceptors. There is growing evidence for a second function of *c*-type cytochromes as electron storage proteins. The presence of these capacitors seems to go along with a fitness benefit for the organism. Hence, the often-asked question why *Shewanella* produces numerous *c*-type cytochromes under anoxic conditions might have this function as an answer. Not the expression of certain singular electron transport pathways but the simultaneous expression of a multitude of pathways might result in a concentration of cytochromes that is high enough to enable this capacitor function. If we further follow this hypothesis, it seems also reasonable to assume that it is of a higher benefit to split the capacitor function into several partly independent pathways that can communicate with each other. Thereby, a

rapid oxidation of all cytochromes is guaranteed in response to the availability of a variety of electron acceptors. Still, further experiments are necessary to elucidate the redox-behavior of the *c*-type cytochrome pool. Furthermore, competition experiments between *Shewanella* as a versatile organisms and more specialized organisms like *Geobacter* would be helpful to understand if the network leads to a growth advantage for *Shewanella* species. Moreover, it seems necessary to dissect the ecological niche of *Shewanella* in more detail in the environment to test whether the proposed advantages of versatility and capacitor function of *c*-type cytochromes apply in the environment as well as under laboratory conditions. Last but not least, a further focus of future research should be to elucidate which role electron shuttles play in environmental metal reduction.

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On the Role of Endogenous Electron Shuttles in Extracellular Electron Transfer

Evan D. Brutinel and Jeffrey A. Gralnick

Abstract As a result of cellular metabolism, microbes dramatically alter the chemistry of environments in which they live. Microbes directly influence cycling of metals in the environment via respiratory redox transformations, often influencing solubility and toxicity of these metals by altering their redox state. Metal oxides and a number of other potential electron acceptors are inaccessible to most organisms due to poor solubility at neutral pH. Insoluble substrates cannot diffuse into the cell and therefore require specific electron transfer strategies. The primary focus of research in model organisms has been the mechanisms underlying electron transfer to insoluble, extracellular substrates. Two distinct mechanisms, which are not mutually exclusive, have been championed to explain how organisms transfer electrons from the surface of the cell to an extracellular substrate. In the first mechanism, electrons are transferred during direct contact between the insoluble substrate and redox active proteins associated with the cell surface. The second mechanism involves small redox active molecules termed 'endogenous electron shuttles' secreted by the organism. These molecules are reduced at the cell surface and react abiotically with the insoluble substrate in a cyclic fashion. In this chapter the discovery, characterization, and implications of endogenous electron shuttles are discussed with emphasis on the experimental evidence for shuttle-based electron transfer mechanisms. Model systems of *Shewanella oneidensis*, *Pseudomonas* sp., and *Geothrix fermentans* are examined in detail to illustrate the current state of the field.

E. D. Brutinel · J. A. Gralnick (✉)
BioTechnology Institute, University of Minnesota, 140 Gortner Lab,
1479 Gortner Ave, St. Paul, Minneapolis, MN 55108, USA
e-mail: gralnick@umn.edu

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1 Introduction

Many microorganisms possess the ability to respire a variety of electron acceptors in the absence of oxygen. The best understood anaerobic respiratory processes have traditionally been those of soluble compounds such as nitrate and fumarate. Typically, proteins associated with the cytoplasmic membrane catalyze the reduction of respiratory substrates using electrons from the quinone/menaquinone pool, thus regenerating key redox mediators in the electron transport chain. Like oxygen, nitrate, and fumarate are soluble in aqueous environments. Respiratory substrates with the ability to freely diffuse to the cytoplasmic membrane, past an outer membrane and/or peptidoglycan layer, do not require drastic modification of the electron transport chain. A host of electron acceptors are present in the environment that are unable to access the cytoplasmic membrane of Gram negative bacteria. Examples include large metal ions, rocks composed of oxidized metals that are poorly soluble at neutral pH (i.e. Fe(III) and Mn(IV) oxides), and large organic molecules such as humic and fulvic acids (see "Minerals and Aqueous Species of Iron and Manganese as Reactants and Products of Microbial Metal Respiration" and "Humic Substances and Extracellular Electron Transfer"). In order to utilize respiratory substrates spatially separated from the cytoplasmic membrane a system must be in place that is able to transfer electrons from the cytoplasmic membrane, through the periplasm, and across the outer membrane. The specific mechanism of electron transfer used by the model organism *Shewanella oneidensis* is described in detail in "The Biochemistry of Dissimilatory Ferric Iron and Manganese Reduction in *Shewanella oneidensis*" of this book and will be briefly reviewed later in this chapter.

Once associated with the extracellular face of the outer membrane, electrons must be transferred to extracellular electron acceptors to complete respiration. Two mechanisms have been envisioned to accomplish this final stage of the process. The first mechanism is the direct transfer of electrons from a reduced functional group in a biomolecule associated with the cell surface or extracellular matrix to an oxidized extracellular compound. Conceptually, this seems to be the most parsimonious solution and is thought to be the mechanism used by model *Geobacter* species (Lovley et al. 2004; "Energetic and Molecular Constraints on the Mechanism of Environmental Fe(III) Reduction by Geobacter"). The second mechanism, and the focus of this chapter, is the utilization of a soluble redox active compound, reduced at the cell surface, able to diffuse into the environment and reduce extracellular substrates. These molecules, termed "electron shuttles", are hypothesized to undergo multiple oxidation-reduction cycles. One advantage of an electron shuttle-based respiratory mechanism is the ability to reduce a substrate that the cell is not in direct contact with. Reduction at a distance may have advantages in some environments, advantages with certain extracellular substrates, and/or increased reduction kinetics when a dissolved mediator between cell and substrate is used.

Two distinct types of shuttles, exogenous and endogenous, are utilized by microorganisms. Exogenous shuttles are those present in the environment and are reviewed in "Humic Substances and Extracellular Electron Transfer" in this book.

Organisms may use an exogenous shuttle, with little or no energy invested, but are at the mercy of the natural processes which produce and/or cycle them. Endogenous shuttles are produced and secreted by the organism for the explicit purpose of electron transfer. While biosynthesis of shuttling compounds represents an energetic cost, organisms can tailor production to need, perhaps expanding the number of environments in which the organism can be successful. An important distinction must be made between a shuttling respiratory mechanism and the secretion of compounds which solubilize or chelate poorly soluble metals. In the later scenario the complexed, and thus solubilized, metal is now able to freely diffuse to the surface of the cell where it can be reduced by a direct contact mechanism. So, while a compound is being secreted which facilitates extracellular electron transport, this is not considered a true electron shuttle.

The efficacy of secreted electron shuttles remains a topic of some debate. Synthesis of redox active organic compounds which are secreted into the environment is a substantial investment, both in energy and organic carbon, without guaranteed return. Implicit in the term electron "shuttle" is the ability to undergo multiple reduction-oxidation cycles. In natural systems, it is thought that only a catalytic amount of electron shuttle would be produced to mitigate the biosynthetic cost (Lovley et al. 2004). The benefit of using a shuttling mechanism, i.e., the ability to more rapidly respire an electron acceptor inaccessible to other bacteria, clearly must outweigh the energetic cost of synthesis and secretion of electron shuttles. Organisms that utilize shuttling-based respiratory mechanisms may have sacrificed energy efficiency for speed, an observation that seems to fit with the anaerobic energy strategy of *S. oneidensis* (Hunt et al. 2010; "The Biochemistry of Dissimilatory Ferric Iron and Manganese Reduction in *Shewanella oneidensis*").

Cell density likely plays a significant role in the viability of an electron shuttle-based respiration. The usefulness of secreted shuttles may be limited in environments with low cell density or high flow rates due to diffusion, however, shuttle secretion may be mutually beneficial to a community with relatively high cell densities (Lovley et al. 2004). The biofilm growth mode may be a strategy to increase the efficiency of shuttle secretion by concentrating the shuttle secretion efforts of many cells into a small area. In agreement with this hypothesis, the organisms that will be discussed later in this chapter as specific examples of electron shuttle producers are all known to form biofilms.

2 Methods for Detection of Shuttling-Based Extracellular Electron Transfer

Traditionally, the reduction of extracellular substrates has been studied using two different terminal electron acceptor classes. The first are metal oxides (i.e. Fe(III) (oxyhydr)oxides, Mn(IV) oxides, etc.) which, while representing a physiologically relevant electron sink in the environment, present a number of technical challenges

in the laboratory. Production of chemically homogenous metal oxides can be difficult and variability between batches as well as mineral aging and transformation during storage can influence experimental results. Toxicity of substrates and/or products can also be a major issue if batch cultures are used and reduction of metal oxides often drastically changes solubility (see "Minerals and Aqueous Species of Iron and Manganese as Reactants and Products of Microbial Metal Respiration" and "Metal Reducers and Reduction Targets. A Short Survey About the Distribution of Dissimilatory Metal Reducers and the Multitude of Terminal Electron Acceptors"). The second commonly used substrate is the electrode (anode) of a microbial fuel cell (MFC) or a three-electrode bioreactor/potentiostat system (hereafter referred to as a bioreactor). For a more in-depth explanation of the design and function of MFCs and bioreactors see "Dissimilatory Metal Reducers Producing Electricity: Microbial Fuel Cells". Electrodes, clearly not the natural substrate for extracellular electron transfer, do offer some advantages; (1) reduction does not solubilize the substrate, (2) toxicity of the substrate and/or product is not an issue, and (3) quantitative measurements of electron transfer and reduction potentials can be taken. Additionally, reduction of an anode by a bacterial culture results in electrical current, garnering interest from the bioenergy community.

Electron shuttles are only one of the strategies used by microorganisms to reduce extracellular electron acceptors. Organisms which utilize a direct contact mechanism for extracellular electron transfer do not require electron shuttles for the reduction of metal oxides or electrodes. In fact, model organisms such as *Geobacter* sp., which use a direct contact mechanism, reduce both metal oxides and electrodes quite well in the absence of any detectable soluble shuttling compound (Bond and Lovley 2003; Nevin and Lovley 2000; Srikanth et al. 2008). In contrast, the reduction of metal oxides or electrodes by *Shewanella* and *Pseudomonas* sp. is strongly influenced by endogenous electron shuttles (Baron et al. 2009; Marsili et al. 2008; von Canstein et al. 2008; Hernandez et al. 2004; Rabaey et al. 2005; Wang et al. 2010). It can be difficult to determine if reduction of extracellular substrates is mediated by a direct contact and/or shuttling-based mechanism and the remainder of this section will discuss the various techniques that have been used.

2.1 Reduction at a Distance

Extracellular electron transport using a shuttle-based mechanism should eliminate the need for direct contact with the substrate, and it is this property that researchers first tried to detect as an indication of electron shuttling. A conceptually simple test is the separation of bacteria and substrate by a semipermeable barrier which allows diffusion of dissolved shuttles, although the effective range of secreted electron shuttles can be problematic. Using simple geometry, one can visualize why, if an organism is secreting a dissolved shuttle able to diffuse into the environment,

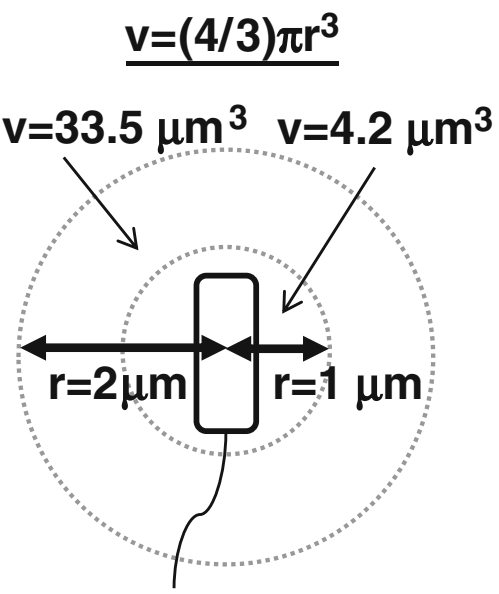

Fig. 1 Illustration of how diffusion limits the effectiveness of secreted electron shuttles. As the linear distance from the organism increases, the volume of the extracellular medium increases exponentially, resulting in an exponential increase in the amount of secreted shuttle needed to maintain a given concentration. The volumes given are those of a sphere with the provided radii

the effective range of the shuttle is so small. It is helpful to imagine the three-dimensional cloud of secreted shuttles surrounding an organism as a sphere with the highest concentrations near the surface of the cell. As the linear distance from the cell increases the volume of the sphere increases by a factor of three ($v = (4/3)\pi r^3$; where v is the volume and r is the radius). Therefore, the local concentration of electron shuttle drops exponentially as the distance from the cell increases (Fig. 1). The primary consequence of this limitation is that while electron shuttles confer certain advantages to the organisms that use them, the effective range is probably very limited.

The first technique that was successfully used to detect reduction at a distance is similar in principle to a dialysis membrane but on a much smaller scale. In these experiments, Fe(III) oxide was precipitated within the interior of either alginate beads or nanoporous glass beads (Nevin and Lovley 2002a, b; Lies et al. 2005). In both cases, only a small fraction of the Fe(III) oxide associated with the beads is accessible to direct cell contact, with the bulk of the Fe(III) oxide precluded within the bead itself. The effective pore size of the alginate beads (12 kDa molecular weight cutoff [MWCO]) and the average pore size of the glass beads (50 nM average diameter) are insufficient for bacteria to access the interior, necessitating a dissolved mediator for electron transfer. *Geobacter* sp. incubated with the Fe(III) oxide impregnated alginate beads readily reduced the Fe(III) oxide on the exterior of the beads but were unable to reduce Fe(III) oxide within the beads (Nevin and Lovley 2000). The inability to access electron acceptor within the beads is consistent with the observation that *Geobacter* sp. require direct contact to carry out extracellular electron transfer (Lovley et al. 2004). If a bacterium uses a shuttling mechanism for

extracellular electron transfer, incubation with the beads should result in significant reduction of the precipitated and immobilized Fe(III) oxide from the interior of the beads, as was seen with *Shewanella* (discussed in detail below) (Nevin and Lovley 2002a; Lies et al. 2005). Careful controls must be implemented to detect a chelator or other factor resulting in solubilization of the Fe(III) oxide.

Electrodes can also be used to detect reduction at a distance and one such experiment will be outlined here. An insulating layer, in one of two configurations, is fabricated over nanoelectrodes to control access of bacteria to the electrode surface (Jiang et al. 2010). The first configuration has a window (12 μm^2) that allows direct contact between bacteria and electrode. The second configuration has only nanoholes ($\sim$280 nm^2), which preclude direct contact by bacteria, but allowed diffusion of dissolved compounds to the electrode surface. Importantly, the total exposed electrode surface area (12 μm^2) is identical between the two electrode configurations. If the current produced by bacteria using either electrode as the sole electron acceptor is similar, regardless of the configuration of the insulating layer, a dissolved molecule is likely mediating electrode reduction (discussed in detail below). As is the case for the Fe(III) oxide bead assays the insulated electrode experiment provides strong evidence for a shuttle-based extracellular reduction strategy but does not reveal the identity of the shuttle.

2.2 Bioreactor Media Exchange and Chronoamperometry

The use of electrodes to study extracellular electron shuttling compounds has proven to be an invaluable addition to the field. The contribution of electron shuttling to the ability of a biofilm to reduce an electrode surface can be evaluated using chronoamperometry, which is the measurement of current over time. Exchanging the culture medium surrounding an electrode grown biofilm with fresh medium removes secreted dissolved compounds, including electron shuttles (this method is known as 'media exchange'). While medium exchange should have little effect on the current produced by bacteria directly reducing an electrode, current produced by bacteria dependent on a shuttling mechanism should be significantly reduced until the appropriate concentration of shuttle can be regenerated (Fig. 2). When media exchange experiments are performed on model *Geobacter* sp. current production is virtually unaffected (<5 %), consistent with a direct contact mechanism (Bond et al. 2002; Bond and Lovley 2003; Srikanth et al. 2008). Media exchange experiments performed with bacteria that use a shuttle-based mechanism should result in a dramatic drop in current followed by a slow recovery to the original level of current production, corresponding to the regeneration of relevant concentrations of electron shuttles (Fig. 2). When the medium surrounding electrode grown bacteria is centrifuged and returned, current should immediately returned to the pre-exchange levels, suggesting that; (1) the loss of planktonic cells is not responsible for the drop in current and (2) a dissolved compound in the medium is mediating the observed electron transfer (i.e. current produced).

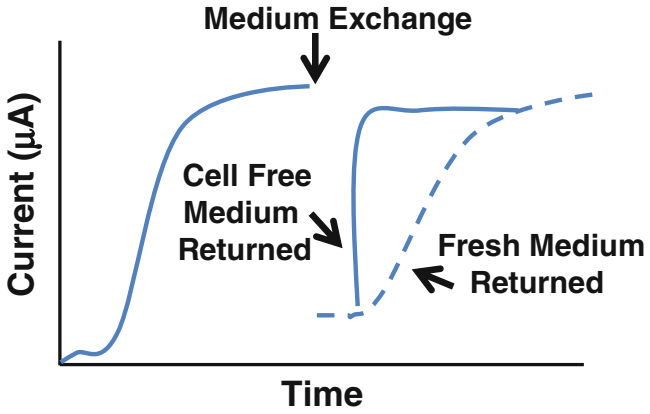

Fig. 2 Idealized illustration of a typical medium exchange experiment in a three-electrode bioreactor adapted from experimental data (Marsili et al. 2008). Data is plotted as current over time (chronoamperometry). The *solid line* represents the current generated by a shuttle utilizing organism at the anode of a bioreactor, before medium exchange and after return of cell free culture media. The *dotted line* illustrates the slow return to pre-exchange current levels when fresh anoxic medium is returned in the exchange

An observed reduction in measured current in media exchange experiments is a strong indication of a shuttle-based mechanism for extracellular reduction. While the argument could be made that the exchange of media resulted in the disruption of direct cell–electrode contacts, it is difficult to imagine how these contacts would be immediately restored by the reintroduction of that same media. The drop in current observed can vary from as little as 50 % to as much as 95 %, dependent on the organism and the MFC/bioreactor system being used (Biffinger et al. 2007; Jiang et al. 2010; Marsili et al. 2008; Ringeisen et al. 2006; Velasquez-Orta et al. 2010). The source of residual current immediately following medium exchange cannot be determined, however, because it could result from incomplete purging of soluble components or a direct contact mechanism.

2.3 Cyclic Voltammetry

Chronoamperometry measures current over time but gives very little information as to the reduction potential of the redox active species, except that it is more electronegative than the potential of the electrode. When using cyclic voltammetry the electrode potential is ramped linearly versus time, and when the midpoint potential of a redox active compound able to transfer electrons to the electrode is reached, electrons flow into the electrode generating a measurable current. The ramp is then reversed and when the midpoint potential is again reached, electrons flow from the electrode back into the redox active compound (Fig. 3).

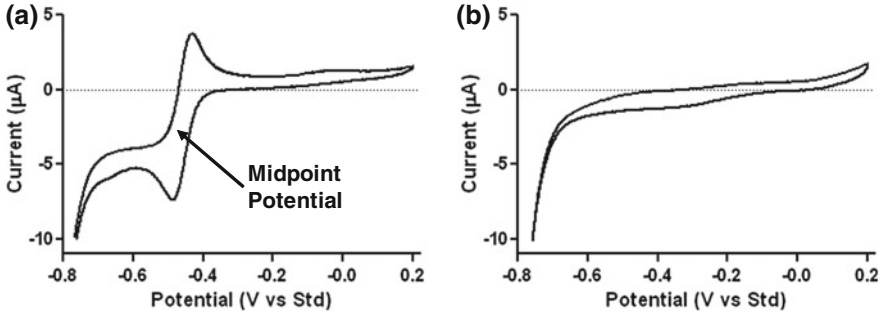

Fig. 3 Idealized cyclic voltammograms generated **a** in the presence and **b** in the absence of a redox active compound. When the potential of the working electrode is ramped up (*X*-axis; more electropositive) electrons flow from a redox active compound into the electrode when the midpoint potential of the redox active compound is reached (*upper peak* in panel A). The potential is then ramped back down (more electronegative) and electrons flow from the electrode back to a redox active compound when the midpoint potential is again crossed (*lower valley* in panel A). Panel B shows a *cyclic voltammogram* generated in the absence of a redox active compound

Cyclic voltammetry is useful because midpoint potential measurements can identify a redox active molecule by comparing measured potentials to known values. Additionally, cyclic voltammetry can discriminate between potential electron donors/acceptors with different reduction potentials in the same solution.

3 Early Attempt to Identify Extracellular Electron Shuttles

Conceptually, endogenous electron shuttles are an elegant mechanism for catalyzing extracellular electron transfer, however, demonstrating that an organism actively produces shuttling compounds at physiologically relevant concentrations is not a trivial task. The concentrations at which shuttles are produced make detection and identification problematic, and experimental artifacts can be a recurrent issue. The following two examples can be viewed as cautionary tales.

The first endogenous electron shuttle to be purposed was a small *c*-type cytochrome present in *Geobacter sulfurreducens* culture supernatants (Seeliger et al. 1998). Intuitively this was an attractive candidate for an extracellular electron shuttle. Seeliger et al. determined that purified preparations of the 9.6-kDa cytochrome could reduce Fe(III) and Mn(IV) oxides, colloidal sulfur, 2,6-anthraquinone disulfonate (AQDS), FMN, and humic acids. Additionally, cultures of *G. sulfurreducens* with excess electron donor were able to reduce the 9.6-kDa cytochrome. These data led researchers to conclude that the 9.6-kDa cytochrome was an extracellular electron shuttle utilized by G. *sulfurreducens* (Seeliger et al. 1998). Unfortunately, the critical experiment, linking reduction of the 9.6-kDa

cytochrome by G. *sulfurreducens* and the reduction of Fe(III) oxide by the 9.6-kDa cytochrome, was not performed. Subsequently, it was demonstrated that the 9.6-kDa cytochrome could not serve as an electron shuttle between *G. sulfurreducens* and Fe(III) oxide, the hallmark of a shuttling molecule (Lloyd et al. 1999; Straub and Schink 2003). The physiological relevance of the 9.6-kDa cytochrome in the extracellular environment was called into question as well when it was shown that media components had a profound effect on the presence and/or amount of 9.6-kDa cytochrome released from the cells (Lloyd et al. 1999). The conclusion that this 9.6-kDa cytochrome could mediate extracellular electron transfer for *G. sulfurreducens* appears to have been based on insufficient data and highlights the importance of testing whole systems to augment biochemical approaches.

A quinone-like molecule produced by *S. oneidensis* was the next candidate molecule thought to serve as an extracellular electron shuttle (Newman and Kolter 2000). *S. oneidensis* mutants which cannot synthesize menaquinone are unable to respire extracellular electron acceptors. A quinone-like compound found in the supernatant of wild type cultures was shown to restore the ability to reduce extracellular electron acceptors to a menaquinone biosynthesis mutant. The authors concluded that, while the quinone-like compound may simply allow the mutant stains to complete menaquinone biosynthesis, it could be an endogenous extracellular electron shuttle (Newman and Kolter 2000). A follow-up study determined that the quinone-like compound was, in fact, simply complementing the defect in menaquinone biosynthesis (Myers and Myers 2004).

The preceding two examples illustrate the importance of careful methodology and controlled experimentation when determining if a molecule found in a culture supernatant is truly a physiologically relevant extracellular electron shuttle. A large number of redox active compounds are present in growing cells and the release of these compounds at some level into the surrounding environment by cell lysis or another mechanism is unavoidable. However, the presence of a redox active compound able to reduce an extracellular substrate does not demonstrate that the organism releasing them is able to link the reduction to the electron transport chain in a physiologically relevant way.

4 *Shewanella* sp. and Flavins

Members of the genus *Shewanella* comprise a diverse group of facultative anaerobes found in both marine and freshwater environments. *Shewanella* sp. are capable of respiring a wide array of substrates including inorganic and organic compounds, toxic metals, and electrodes (Hau and Gralnick 2007). A number of metal oxides such as Fe(III) and Mn(IV) are poorly soluble at neutral pH, and therefore are inaccessible electron acceptors for most microorganisms. Dissimilatory metal reducing bacteria (DMRB), which includes most of the members of the *Shewanella* genus, can utilize extracellular electron acceptors under anoxic conditions, coupling the oxidation of organic and/or inorganic compounds to the

reduction of extracellular metals (Lovley et al. 2004). DMRB are thought to play a significant role in biogeochemical cycling of metals. The fact that the redox state of many metal ions drastically affects solubility has generated interest in using *Shewanella* sp. for bioremediation of contaminated groundwater sites (see "Metal Reducers and Reduction Targets. A Short Survey About the Distribution of Dissimilatory Metal Reducers and the Multitude of Terminal Electron Acceptors" and "Bioremediation Via Microbial Metal Reduction"). *Shewanella* sp. are also able to generate electrical current from anaerobic respiration of electrodes (Lovley 2008). Of great interest is how DMRB are able to transfer electrons to a substrate that cannot freely diffuse to the cytoplasmic membrane/cytoplasm.

In *S. oneidensis*, the Mtr pathway forms an electron conduit able to move electrons from the menaquinone pool, through the periplasm, and across the outer membrane (Beliaev and Saffarini 1998; Beliaev et al. 2001; Hartshorne et al. 2009; Ross et al. 2007; Shi et al. 2007). The biochemistry of each of the components of the Mtr pathway is covered extensively elsewhere in this book ("The Biochemistry of Dissimilatory Ferric Iron and Manganese Reduction in *Shewanella oneidensis*") and so, for brevity, will only be briefly outlined here. The Mtr pathway consists of three multiheme cytochromes (CymA, MtrA, and MtrC [formerly OmcB]) and an integral outer-membrane protein (MtrB). Once electrons have reached MtrC, transfer to an extracellular electron acceptor occurs (Shi et al. 2007). It is at this step that an extracellular electron shuttle could be reduced.

A clarifying note is required for further discussions of extracellular electron transfer in *S. oneidensis*. The primary source of ATP for *S. oneidensis* comes from substrate-level phosphorylation when growing under anoxic conditions with oxidative phosphorylation, sometimes thought of as the hallmark of respiration, making little contribution to ATP synthesis (Hunt et al. 2010). Regardless, a terminal electron acceptor, whether soluble or insoluble, is still required to regenerate the reducing equivalents used to oxidize the carbon and energy source. This justifies the use of the term "respiration", and throughout this chapter the terms "respiration" and "electron transfer" are used interchangeably.

4.1 Evidence for Extracellular Electron Transfer in the Absence of Direct Contact

S. oneidensis strain MR-1 was isolated in 1987, and in the following years numerous studies have been published examining both the kinetics of extracellular electron transfer to a variety of substrates and the genes/proteins of the Mtr pathway. It was not until 2005, however, that Lies et al. demonstrated that *S. oneidensis* was able to carry out extracellular electron transfer without direct contact, using Fe(III) oxide precipitated within nanoporous glass beads (discussed in detail above) (Lies et al. 2005). In this study, the Mtr pathway was found to be required for reduction of Fe(III) oxide within the bead interior, solidifying the role

of the Mtr electron conduit in a shuttle-based mechanism. It should be noted that Nevin et al. demonstrated reduction at a distance by *Shewanella alga* using an alginate bead system 3 years earlier in 2002; however, no further studies regarding extracellular electron transfer by *S. alga* have been published (Nevin and Lovley 2002a).

The ability of *S. oneidensis* to reduce an extracellular substrate in the absence of direct contact was also demonstrated using specialized electrodes (Jiang et al. 2010). Insulating layers fabricated onto the surface of nanoelectrodes controlled the ability of *S. oneidensis* to access the electrode surface. Current generated by *S. oneidensis* growing on nanoelectrodes with an insulating layer that allowed diffusion of small molecules to the electrode surface, but not direct contact by bacteria, was similar to electrodes that allowed for direct contact (discussed in detail above). The ability of *S. oneidensis* to reduce the electrode in the absence of direct contact is consistent with a shuttle-based extracellular respiratory mechanism (Jiang et al. 2010). Additionally, the fact that a higher level of current was not observed when bacteria were able to directly contact the electrode surface indicates a negligible contribution by a direct transfer mechanism, at least in this experimental setup (Jiang et al. 2010). As is the case for the Fe(III) oxide bead assays the insulated electrode experiment provides strong evidence for a shuttle-based extracellular reduction strategy, but does not reveal the identity of the shuttle.

4.2 *Shewanella sp. Accumulate Flavins in Culture Supernatants*

Following the study by Lies and coworkers, the identification of secreted riboflavin, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) (hereafter collectively referred to as flavins) in the culture supernatants of *Shewanella* sp. occurred simultaneously by two groups. von Canstein et al. tested supernatant fractions from *Shewanella* cultures for the ability to decolorize a redox active dye (von Canstein et al. 2008). Two fractions, corresponding to riboflavin and FMN, were identified. Eleven phylogenically distinct *Shewanella* sp. were examined and in every case, micromolar quantities of flavins were observed in cultures grown with and without oxygen suggesting a role for flavins across the *Shewanella* genus. The levels of flavin observed were considered to be high relative to other γ-proteobacteria (*Escherichia coli* and *Pseudomonas* sp.) used as controls. These data led to the conclusion that redox active flavins are the electron shuttle used by *Shewanella* sp. (von Canstein et al. 2008). Marsili and coworkers came to the same conclusion based on media exchange experiments using bioreactors (Marsili et al. 2008). The reductive current produced by *S. oneidensis* (strain MR-1 and MR-4) growing on a carbon electrode dramatically declined to $\sim$25 % of pre-exchange current when the surrounding medium was exchanged with fresh

anoxic medium, followed by a slow recovery to pre-exchange levels over 72 h. If the original medium was returned after removal of planktonic cells, current immediately returned to ~95 % of pre-exchange levels. These data suggested that ≥75 % of the current produced by *S. oneidensis* growing on a carbon electrode was dependent on a soluble factor; however, the actual contribution of extracellular flavins could be higher (Marsili et al. 2008). Measurements taken using cyclic voltammetry suggested that the removal of extracellular flavins was not complete and some flavins were retained by the electrode-associated biofilm. In agreement with previous results, mass spectrometry identified riboflavin (250–500 nM) in bioreactor supernatants (Marsili et al. 2008). The media exchange phenomenon was also observed by a different group using nanoelectrodes where current generated by electrode grown *S. oneidensis* declined to ~5 % of pre-exchange current when the surrounding medium was exchanged (Jiang et al. 2010).

The detection of electron shuttling compounds in culture supernatants should be viewed with a certain measure of skepticism until cell lysis has be eliminated as an explanation. In the case of *Shewanella* sp., it is reasonable to suppose that flavins detected in the supernatant could have come from cell lysis, as FMN and FAD are essential cofactors present in the cytoplasm. Three lines of evidence strongly indicate that flavin secretion is an active process. First, while the dominant flavin species found in the intracellular fraction is FAD, the flavin profile of culture supernatants is dominated by FMN (von Canstein et al. 2008). Second, FMN secretion correlated with cell growth, and ceased when cultures reached stationary phase, the opposite result one would expect from cell lysis (von Canstein et al. 2008). Lastly, the discovery of mechanisms for the transport and processing of FAD indicate an active process (Covington et al. 2010). This example highlights the importance of careful investigation of the origin of putative electron shuttling compounds for a given organism.

4.3 Reduction of Extracellular Substrates by Flavins

The finding that *Shewanella* sp. accumulate flavins in culture supernatants does not by itself demonstrate that flavins are able to act as extracellular electron shuttles. Even media exchange experiments with electrodes suggest only that a dissolved mediator is present, not that it is flavin. A critical piece of the puzzle is the ability of flavins to reduce the extracellular electron acceptors utilized by *Shewanella* sp. In support of the role of flavins as extracellular electron shuttles the addition of purified riboflavin or FMN (1) abiotically reduces poorly soluble Fe(III) oxide, (2) increases the rate of Fe(III) oxide reduction by *S. oneidensis*, and (3) enhances the growth yield of *S. oneidensis* growing on lactate with Fe(III) oxide as the sole electron acceptor (von Canstein et al. 2008). The enhancement of growth yield by the addition of exogenous flavins likely results from an enhanced rate of extracellular respiration, consistent with electron shuttling as a key limiting step in electron transfer. Moreover, addition of flavins to the medium of electrode grown

cells dramatically enhances the production of current in bioreactors (Marsili et al. 2008).

Media exchange experiments have demonstrated that *S. oneidensis* cells attached to the electrode in bioreactors are responsible for the lions share of the observed current, with little contribution from planktonic cells present in the medium (Marsili et al. 2008). A requirement for attachment makes it difficult to determine if a soluble flavin is the redox molecule that is actually reducing the electrode, however, clever electrochemistry can be used to determine the midpoint reduction potential of the molecule(s) generating the reductive current. Measurements using cyclic voltammetry have been taken demonstrating that flavins are responsible for the majority of reduction measured from carbon electrodes in *S. oneidensis* bioreactors (Marsili et al. 2008). Midpoint potential measurements for *S. oneidensis* using the electrode as the sole electron acceptor are similar to those observed for sterile electrodes incubated with riboflavin. Consistent with in vitro experiments using purified outer-membrane cytochromes and Fe(III) oxide, a higher midpoint potential is observed for direct electron transfer from outer-membrane cytochromes to the electrode surface in the absence of flavins (Baron et al. 2009; Ross et al. 2009). Although outer-membrane cytochromes play an essential role in the reduction of extracellular substrates (see below), these data strongly suggest that despite the fact that *S. oneidensis* biofilms are attached to the electrode in bioreactors, flavins are the redox species reducing the electrode.

Reduction of extracellular electron acceptors by *Shewanella* appears to only occur over a very short distance necessitating the elegant experimental setups described above. The introduction of this chapter addressed one possible reason, the problem of local concentration. When respiring solid terminal electron acceptors *Shewanella* forms a thin biofilm consisting of a monolayer of cells (Marsili et al. 2008; Baron et al. 2009; Coursolle et al. 2010), likely due to the relatively short range at which electron shuttles appear to function effectively. These observations explain why researchers do not observe thick (>10 μM) biofilms with *Shewanella* on electrodes under strictly anoxic conditions.

In addition to being redox active, flavins also weakly chelate metals that could facilitate extracellular respiration by binding to, and solubilizing, metal oxides (Albert 1950; Furia 1972). Two pieces of data suggest that chelation does not significantly contribute to the reduction/increased rate of reduction catalyzed by flavins. The addition of a known chelator with 1,000-fold higher affinity for Fe(III) than that of flavins, at concentrations comparable to those reached by flavins in culture supernatants, has little effect on the rates of Fe(III) oxide reduction by *S. oneidensis* (Coursolle et al. 2010). Moreover, carbon electrodes are not solubilized when reduced and cannot be chelated, yet flavins readily reduce electrodes and increase current output of *Shewanella* sp. grown on electrodes (Velasquez-Orta et al. 2010; Marsili et al. 2008).

4.4 Shewanella Reduces Extracellular Flavins

Strong experimental evidence has been published demonstrating that (1) *Shewanella* sp. accumulate physiologically relevant concentrations of flavins in culture supernatants, (2) flavins can reduce poorly soluble Fe(III) oxide and electrodes, and (3) the addition of exogenous flavins accelerates the reduction of Fe(III) oxide and electrodes. The last piece of the puzzle is the ability of *Shewanella* sp. to reduce extracellular flavins, crucial for an electron shuttle-based mechanism.

The Mtr pathway is responsible for the flow of electrons from the menaquinone pool to the extracellular face of the outer membrane and deletion of genes in the Mtr pathway severely inhibits the ability of *S. oneidensis* to respire extracellular substrates including Fe(III) oxides, Mn(IV) oxides, and carbon electrodes (Shi et al. 2007). Before the discovery of flavins in *Shewanella* culture supernatants it was known that the Mtr pathway was involved in the reduction of extracellular shuttles. A study examining the ability of *S. oneidensis* to reduce AQDS, an analog of redox active moieties of humic acids, found that strains harboring mutations of Mtr genes were deficient in AQDS reduction (Lies et al. 2005).

The first evidence that the Mtr pathway directly reduces flavins came from kinetic studies of Fe(III) oxide reduction using purified Mtr proteins. Ross et al. examined the rate at which the outer-membrane cytochromes MtrC and OmcA reduced dissolved Fe(III) and poorly soluble Fe(III) oxide (Ross et al. 2009). Using purified proteins, reduction of Fe(III) oxide was an order of magnitude slower than with whole cells containing a comparable concentration of cytochromes. The addition of exogenous flavins to the purified system increased the rate of Fe(III) oxide to that of whole cells (Ross et al. 2009).

Genetic analysis has since confirmed the role of the Mtr pathway in the reduction of extracellular flavins. Wild type *S. oneidensis* is able to couple the oxidation of lactate to the reduction of exogenously added oxidized flavins under anoxic conditions, and strains with gene deletion mutations in the Mtr pathway are impaired for reduction of flavins (Coursolle et al. 2010, Coursolle and Gralnick 2010). Strains carrying mutations in the Mtr pathway are also impaired for reduction of carbon electrodes, even when provided with an identical concentration of flavins (Coursolle et al. 2010).

4.5 Crystal Structure of an Outer-Membrane Cytochrome

Ample evidence has been collected demonstrating the role of flavins in extracellular electron transfer by *Shewanella* sp. Additionally, the Mtr electron conduit linking the menaquinone pool to the outer membrane has been described and is required for the reduction of extracellular flavins (Coursolle et al. 2010; Shi et al. 2007). The recently solved crystal structure of MtrF, a paralog of MtrC in *S. oneidensis*, illustrates the mechanism by which electrons flowing through the Mtr pathway may reduce flavins outside the cell (Clarke et al. 2011). MtrF is a

decaheme *c*-type cytochrome predicted to localize to the extracellular side of the outer membrane, able to functionally complement *S. oneidensis* strains lacking other outer-membrane decaheme *c*-type cytochromes for iron reduction (Coursolle and Gralnick 2010). The crystal structure clearly shows that the heme groups of MtrF are close enough to allow electron transfer through the protein, forming a branched path for electron flow that has been termed a "staggered cross." Hemes 10 and 5 are solvent exposed, positioned at opposing poles of the protein in such a way to accept electrons from a periplasmic electron carrier (MtrA) and donate electrons to an extracellular electron acceptor, respectively. On either side of the protein, hemes 2 and 7 are located near the β-barrel of extended Greek-key split barrel domains commonly found in flavin binding proteins. The remaining heme groups allow electron flow through the protein. Electrons entering MtrF at heme 10 effectively have three avenues for electron egress and, given the location and adjacent protein domains, it is likely that electrons that travel to heme 5 directly reduce extracellular substrates and hemes 2 and 7 reduce flavins (Clarke et al. 2011). Unfortunately, flavin binding was not directly observed despite efforts to resolve a flavin in either of the proposed binding sites. However, the inability to capture a crystal with bound flavins may be indicative of the fact that a shuttling mechanism would require the rapid reduction and release of flavins, predicting that the interaction of MtrF with flavins would be transient and likely redox dependent.

5 *Pseudomonas* sp. and Phenazines

Phenazines are a large family of secondary metabolites based on a common heterocyclic compound, with more than 100 phenazine derivatives having been described in nature. Phenazines are one of the most intensely studied group of bacterial secondary metabolites due to broad-spectrum antibiotic properties and a major role in the physiology and/or virulence of organisms which secrete them (Pierson and Pierson 2010). Phenazines have also been shown to play a role in cell–cell signaling pathways (Dietrich et al. 2008). The most highly studied phenazine producers are the *Pseudomonads* which secrete phenazines derivatives at relatively high concentration into the extracellular environment. More recently, the capacity of phenazines to mediate the transfer of electrons from cellular processes to extracellular electron acceptors has been appreciated and a number of different functions have been attributed to this ability. The remainder of this section will focus on the evidence for phenazines as endogenous extracellular electron shuttles in *Pseudomonas* sp.

5.1 Phenazine Mediated Reduction of Fe(III) Oxide

Until recently, the primary focus of research concerning phenazines secreted by soil bacteria has been a crucial role in the suppression of fungal plant pathogens,

largely thought the production of oxygen radicals (Pierson and Pierson 2010). The realization that microbes such as S. *oneidensis* can catalyze the dissimilatory reduction of Fe(III) oxides led researchers to examine if secreted phenazines could mediate the reduction of insoluble Fe(III) oxide as well. *Pseudomonas chlororaphis*, but not an isogenic phenazine biosynthesis mutant, readily reduced Fe(III) oxide added to liquid cultures (Hernandez et al. 2004). The addition of purified phenazine carboxylic acid (PCA; a naturally secreted phenazine by *P. chlororaphis*) restored the ability of the mutant to reduce Fe(III) oxide. Reduced PCA is known to abiotically reduce Fe(III) oxide and the exogenous addition of purified PCA stimulated Fe(III) oxide reduction by diverse γ-proteobacteria, including *S. oneidensis* (Hernandez et al. 2004). The PCA mediated stimulation of Fe(III) oxide reduction by *S. oneidensis* was similar to that of an identical concentration of AQDS, a prototypical extracellular electron shuttle. The reduction of Fe(III) by phenazines is thermodynamically favorable under physiologically relevant conditions, suggesting that phenazine mediated mineral reduction can occur in natural environments (Hernandez et al. 2004).

5.2 Fe Acquisition and Biofilm Formation

The physiological role of extracellular Fe(III) oxide reduction by *Pseudomonas* sp. in the environment is somewhat undefined, however, a strong case for a role in biofilm development has been made. Biofilms play an important role both in environmental and pathogenic settings and Fe levels higher than that essential for planktonic growth are required to signal the transition to a biofilms growth state (Banin et al. 2005). The available literature strongly indicates that phenazines play a critical role in Fe acquisition from biologically unavailable sources, promoting biofilm formation. Researchers demonstrated that phenazines promoted biofilm formation by *P. aeruginosa* strain PA14 under iron limiting conditions in the presence of insoluble Fe(III) oxide (Wang et al. 2011). Experiments using *P. aeruginosa* strains deficient in siderophore production demonstrated that phenazine-mediated Fe reduction could bypass the need for chelators to acquire Fe from the environment. The proposed mechanism for the stimulation of biofilm formation is the reduction of Fe(III) to Fe(II), mediated by phenazines, followed by cellular uptake of Fe(II) via the ferrous iron transport system (Feo) (Wang et al. 2011).

Bioavailable Fe(III) is thought to be extremely scarce in aerobic environments, including many mucose membranes and tissues of animals. Host secreted Fe(III) chelators sequester Fe(III), creating an important barrier to microbial colonization and pathogenesis. The extracellular reduction of Fe(III) by phenazines has been shown to play a role in Fe acquisition from host factors (Wang et al. 2011). Wang et al. demonstrated that *P. aeruginosa* stain PA14 forms biofilms in the presence of inhibitory concentrations of the Fe(III) chelator conalbumin. Conalbumin specifically binds Fe(III), but not Fe(II). The authors hypothesized that phenazines reduced Fe(III) to Fe(II) within conalbumin, thereby liberating Fe(II) for uptake by

the *P. aeruginosa* Feo system. The role of phenazines in pathogenesis was traditionally thought to be limited to the generation of oxygen radicals, promoting pathogenesis through cellular damage. The above experiments reveal other possible roles for the secreted redox mediators.

5.3 Biofilm Formation and Viability

Phenazines play a role in biofilm development independent of Fe acquisition (Wang et al. 2011; Ramos et al. 2010; Dietrich et al. 2008; Pierson and Pierson 2010). *P. chlororaphis* phenazine biosynthetic mutants are deficient in attachment to surfaces and biofilm formation in flow cell biofilms (Maddula et al. 2006, 2008). *P. aeruginosa* phenazine biosynthetic mutants on the other hand are able to attach to surfaces; however, subsequent biofilms have a lower biomass and higher surface-to-volume ratio (Ramos et al. 2010; Dietrich et al. 2008). The mechanism(s) by which phenazines affect biofilm formation/architecture is not fully understood. Experimental evidence suggests that the viability of cells deep within a colony or surface attached biofilm is enhanced by phenazine mediated extracellular electron transfer (Wang et al. 2010; Okegbe et al. 2012). Phenazine secretion-based cellular redox homeostasis may represent a way for cells in the anoxic interior to balance the cytoplasmic redox state (Price-Whelan et al. 2007). In support of this hypothesis, the survival of *P. aeruginosa* biofilms attached to electrodes serving as the sole electron acceptor is enhanced by phenazines. While growth was not observed on the electrode, cells producing phenazines survived for seven days while phenazine biosynthesis mutants started dying after only 3 days (Wang et al. 2010).

5.4 Phenazines and Electrodes

Shewanella sp. generate anodic current when grown in MFCs or bioreactors stemming primarily from flavin mediated extracellular electron transfer (Marsili et al. 2008; Baron et al. 2009). Phenazines are also able to mediate transfer of electrons from redox reactions within the cell to an electrode. *Pseudomonads* can generate anodic current in MFCs, dependent on secreted phenazines (Luo et al. 2009). It is difficult to compare current generation or phenazine production to *Shewanella* experiments since the conditions and methods are so varied. The addition of purified phenazines to an MFC containing *Brevibacillus* sp., which do not normally secrete phenazines, resulted in a twofold increase in the rate of electron transfer (Rabaey et al. 2005; Pham et al. 2008a). Interestingly, the addition of purified phenazines or a phenazine producing strain of *P. clororaphis* to a mixed community MFC resulted in higher rates of electron transfer (Pham et al. 2008b). Clearly, a greater understanding of the role of phenazines in MFCs/bioreactors has potential to increase efficiency and produce more power.

5.5 Evidence for Shuttling

While a preponderance of data overwhelmingly suggests that phenazines secreted by *Pseudomonas* sp. act as extracellular electron shuttles, reduction at a distance has not been definitively shown. The evidence supporting a shuttling mechanism for phenazines is as follows. (1) *Pseudomonas* sp. secrete phenazines into the extracellular environment, (2) reduced phenazines abiotically reduce extracellular electron acceptors such as Fe(III) oxide and electrodes, (3) phenazines accumulated in culture supernatants undergo multiple redox cycles, and (4) *Pseudomonas* sp. can reduce oxidized phenazines (Wang et al. 2010, 2011; Pierson and Pierson 2010; Hernandez et al. 2004). The missing piece of the puzzle is an experiment demonstrating *Pseudomonas* sp. can reduce extracellular substrates in the absence of direct contact, in a phenazine dependent manner. Such experiments have been performed for other bacterial species discussed in this chapter and will surely be performed with phenazines in the near future.

6 Geothrix Fermentans

The use of endogenous electron shuttles in the reduction of extracellular substrates does not appear to be restricted to the γ-proteobacteria. The DMRB *Geothrix fermentans* is a member of the Acidobacteria, phylogenetically distinct from *Shewanella* and *Pseudomonas*. *G. fermentans* is an obligate anaerobe able to couple the complete oxidation of organic acids to the reduction of a number of terminal electron acceptors, including Fe(III) oxide, humic acids, and electrodes. *G. fermentans* was originally noticed as a constituent of sediment communities in which Fe(III) was an important electron acceptor, typically overshadowed by other DMRB such as *Geobacter* sp., and the use of electron shuttles may represent a competitive strategy (Lovley et al. 2004; Coates et al. 1999).

The first evidence that *G. fermentans* uses a shuttling electron transfer mechanism came from studies of Fe(III) oxide reduction in the absence of direct contact. Using the alginate bead system described above for *S. alga*, researchers observed reduction of Fe(III) oxide precipitated within alginate beads (MWCO 12 kDa) when incubated with *G. fermentans* (Nevin and Lovley 2002b). Importantly, Fe(II) generated from the reduction of Fe(III) oxide remained within the alginate beads indicative of a shuttling mechanism. With a chelator-based mechanism, Fe(II) would have been expected to be found primarily in the growth medium with some fraction perhaps diffusing back into the bead. Media exchange experiments performed on electrode grown cells were also indicative of a shuttle-based extracellular reduction mechanism. When the medium surrounding *G. fermentans* bioreactors was exchanged with fresh anoxic medium, an immediate 50 % reduction in current was observed which slowly returned to pre-exchange levels over the course of 10 days (Bond and Lovley 2005). In media

exchange experiments with *S. oneidensis*, replacement of bioreactor medium with cell-free culture filtrates from electrode grown cells resulted in an immediate increase in current to pre-exchange levels (Marsili et al. 2008). Unfortunately, a similar experiment has not been performed using *G. fermentans*, however, cell-free media from electrode grown cultures was shown to stimulate Fe(III) oxide reduction by *Geobacter sulfurreducens* (Bond and Lovley 2005).

While the electron shuttling compound used by *G. fermentans* was never identified the following characteristics are known; the molecule(s) is/are (1) water soluble, (2) able to diffuse into an alginate bead with an MWCO of 12 kDa, (3) unaffected by protease treatment, and (4) accumulates in the supernatant only when *G. fermentans* is grown on a poorly soluble electron acceptor (Nevin and Lovley 2002b; Bond and Lovley 2005). Selective accumulation is in marked contrast to the flavin shuttle from *Shewanella* sp., which accumulates under all conditions tested thus far (Marsili et al. 2008; von Canstein et al. 2008; Coursolle et al. 2010). Additionally, thin layer chromatography experiments designed to isolate water soluble quinone-like molecules from *G. fermentans* culture filtrates repeatedly detected a fluorescent molecule not present in the culture filtrates of *Geobacter metallireducens* (Nevin and Lovley 2002b). The above evidence points to a flavin or flavin like molecule secreted by *G. fermentans* as an extracellular electron shuttle; however, this hypothesis has not been experimentally validated. Interestingly, the genomes of four members of the Acidobacteria have been sequenced and high-identity homologs of the Mtr system appear to be absent. If flavins truly are the extracellular electron shuttle used by *G. fermentans*, the mechanism of flavin reduction will likely be novel.

7 The Energetics of a Shuttle-Based Extracellular Reduction Strategy

It has often been assumed that secreted redox mediators could not be a viable strategy for extracellular electron transfer by microorganisms in natural environments. The two arguments most commonly used are as follows. (1) The biosynthetic cost to obtain useful concentrations of redox mediators outside the cell would be too great and (2) secreted shuttles would be utilized/consumed by ‘cheaters’ and/or other organisms. The fact that shuttles are used by microorganisms provides strong evidence that the advantages of a shuttle-based extracellular reduction strategy outweigh the disadvantages. The synthesis/secretion of an extracellular electron shuttle represents a biosynthetic cost which must be outweighed by the benefits, i.e., the ability to utilize an alternative electron acceptor or solubilize a metal ion inaccessible to other bacteria. Implicit in the term ‘shuttle’ is the repeated oxidation and reduction of a given molecule, mitigating biosynthetic cost, and it is thought that only a catalytic amount of an electron shuttle would be produced, followed by multiple redox cycles (Lovley et al. 2004).

Redox cycling of phenazines has been measured under a number of different culture conditions where, depending on the strain of *Pseudomonas* and growth condition, phenazine concentrations ranging from 5–300 μM have been observed (Hernandez et al. 2004; Price-Whelan et al. 2007). Phenazines are secondary metabolites, and therefore by definition not essential for growth under all conditions. Phenazine biosynthesis mutants have made it possible to precisely determine the contribution of phenazines to reduction of extracellular substrates. Researchers examining the reduction of Fe(III) oxide by *P. chlororaphis* found that, when the final concentrations of Fe(II) and phenazines were taken into account, phenazines must have been cycled ~60 times over the course of 4 days (Hernandez et al. 2004). Phenazine cycling has also been estimated for *P. aeruginosa* biofilms attached to electrodes. In this scenario, the cells do not grow but the ability to reduce the electrode promotes survival and is dependent on secreted phenazines. Researchers determined the number of redox cycles for each of the phenazines normally produced by *P. aeruginosa* required to generate the measured current. Over the course of the 7-day experiment pyocyanin, phenazines-1-carboxylic acid, and 1-hydroxyphenazine were cycled 31, 22, and 14 times, respectively (Wang et al. 2010).

It is difficult to estimate the number of times flavin molecules are cycled in *S. oneidensis* cultures. Unlike phenazines, flavins are essential cofactors for many enzymes in a cell and biosynthesis cannot be disrupted. In addition, the absolute contributions of shuttling versus direct contact cannot be determined because a *Shewanella* strain that does not secrete flavins has yet to be isolated. In lieu of definitive experimental evidence, careful calculations have been performed addressing the efficacy of flavin shuttles secreted by *S. oneidensis* as a strategy for extracellular electron transfer (Marsili et al. 2008). As many as 25 molecules of ATP are consumed in the de novo biosynthesis of one molecule of riboflavin, and using this figure <0.1 % of the cells ATP budget is required to generate the concentrations of secreted flavin observed in electrode grown *S. oneidensis* cultures (250–500 nM). Importantly, the observed concentration of riboflavin increased the rate of electron transfer by 370 % (Marsili et al. 2008). *Shewanella* sp. that utilize flavin shuttles have a clear competitive advantage when reduction of an extracellular electron acceptor is required.

8 Concluding Remarks

Extracellular electron shuttles represent a powerful strategy for organisms, allowing the utilization of extracellular electron acceptors inaccessible to competitors. To date *Shewanella* sp., *Pseudomonas* sp., and *Geothrix fermentans* are the only organisms with direct evidence for a shuttle-based extracellular reduction strategy. Surely, other phylogenetically distinct organisms might possess some form of this strategy as well. Importantly, the search for extracellular electron shuttles should not be limited to those involved in respiration/redox homeostasis. Data from experiments with *Pseudomonas* sp. suggest multiple roles for secreted phenazines in addition to redox homeostasis and survival within a biofilm.

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Humic Substances and Extracellular Electron Transfer

Annette Piepenbrock and Andreas Kappler

Abstract Humic substances (HS) are redox-active organic molecules that are present in virtually all environments. A wide variety of bacteria including Fe(III)-reducers, sulfate reducers, methanogens, and fermenting bacteria can reduce HS and in a second, abiotic step, the reduced HS can transfer their electrons to terminal electron acceptors such as poorly soluble Fe(III) minerals, in summary a process called humic substance electron shuttling. Electron shuttling between HS-reducing bacteria and Fe(III) minerals can increase the rate of Fe(III) reduction compared to direct Fe(III) reduction and, furthermore, enables the indirect reduction of Fe(III) minerals by some bacterial groups that are not able to reduce the Fe(III) minerals directly. This chapter will first summarize the knowledge about the redox properties of humic substances including a discussion of their redox-active functional groups. We then focus on the mechanism of electron shuttling and evaluate the advantages and disadvantages of electron shuttling versus direct contact Fe(III) mineral reduction. The role of solid-phase humics and other extracellular electron shuttles is discussed as well as the environmental consequences for long-range electron transfer via humic substances. The chapter concludes by illustrating some remaining open questions and by providing suggestions for future research.

A. Piepenbrock · A. Kappler (✉)
Geomicrobiology, Center for Applied Geosciences, University of Tübingen,
Sigwartstrasse 10, D-72076 Tübingen, Germany
e-mail: andreas.kappler@uni-tuebingen.de

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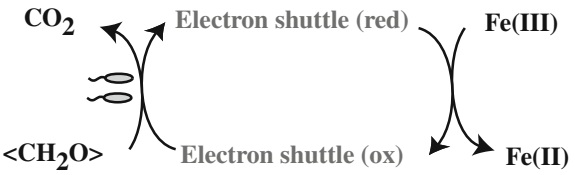

Fig. 1 Schematic illustration of electron shuttling between microbial cells and Fe(III) by dissolved electron shuttles, e.g., humic substances

1 Electron Shuttling in Metal Respiration

Fe(III) is an important electron acceptor for microbial respiration in natural soils and sediments (chapter "Energetic and Molecular Constraints on the Mechanism of Environmental Fe(III) Reduction by *Geobacter*", and "Biochemistry of Extracellular Respiration in *Shewanella oneidensis*", this book; Kappler and Straub 2005; Konhauser et al. 2011; Weber et al. 2006) due to its abundance in these environments (see chapter "Minerals and Aqueous Species of Iron and Manganese as Reactants and Products of Microbial Metal Respiration"). At circumneutral pH the solubility of Fe(III) is very low and it is therefore mainly present as poorly crystalline and crystalline Fe(III) (oxyhydr)oxides. Unlike other electron acceptors such as O_2, particulate Fe(III) is more difficult for microbes to access since it cannot easily be taken up into the cells. Instead, bacteria must transfer electrons out of the cells and onto a solid Fe(III) mineral using outer membrane cytochromes. However, this electron transfer requires direct contact between the cells and the Fe(III) mineral. Therefore, microbial Fe(III) reduction is often limited by available mineral surface area (Kappler and Straub 2005; Weber et al. 2006). There are several strategies bacteria can use to overcome the limitations of a non-dissolved electron acceptor and restricted access to a mineral surface. Reguera et al. (2005) suggested that conductive cell appendages, so-called "nano-wires", can facilitate electron transfer to non-dissolved electron acceptors. Bacteria can also excrete chelators that complex and thereby dissolve the Fe(III), which is subsequently taken up into the cell and reduced (Lovley et al. 1994). Finally, it has been shown that microbial Fe(III) reduction can be facilitated by electron shuttling (Fig. 1) (Lovley et al. 1996). Dissolved, redox-active molecules serve as electron shuttles, which are reduced by the bacteria and, in a second step, transfer electrons to the Fe(III) mineral. The electron shuttle is re-oxidized by the Fe(III) mineral in the process and can again accept electrons from the cells (Fig. 1). Thus, the same electron shuttle molecule can be recycled many times, transferring multiple generations of electrons from bacteria to the Fe(III) mineral.

Some microorganisms produce and excrete electron shuttles, e.g., *Shewanella* species (Marsili et al. 2008; von Canstein et al. 2008), and endogenous electron shuttles are discussed in the chapter "On the Role of Endogenous Electron Shuttles in Extracellular Electron Transfer". There are, however, redox-active compounds naturally present in the environment that can function as electron shuttles for bacteria. This chapter will focus on these exogenous electron shuttles, especially on humic substances.

2 Humic Substances: Composition and Properties

Humic substances (HS) are chemically heterogeneous polyfunctional organic molecules and constitute operationally defined fractions of organic matter (OM) that is present at varying concentrations in almost all natural environments (Stevenson 1994). Concentrations of OM in the environment are typically quantified as organic carbon (OC), which represents approximately half of the OM. Soils contain up to 5–10 % (w/w) OC (Stevenson 1994), whereas natural surface waters and ground waters commonly contain up to 30 and 10 mg dissolved OC/L, respectively (Aiken et al. 1985). However, OM values much higher than these are found in selected natural waters and soils (D. Macalady, "personal communication"). Depending on the environment, HS originate primarily from the degradation of senescent plant organic matter, and can also contain various amounts of animal and microbial remains. Consequently, HS can contain fragments of aromatic lignin derivatives, peptides, carbohydrates, and aliphatic molecules. HS are thought to be largely recalcitrant and rather inert to chemical and microbial degradation (Stevenson 1994), contain a wide variety of reactive functional groups, absorb strongly to mineral surfaces, have the ability to complex metal ions, and have long been known to be redox-active (Aiken et al. 1985; Stevenson 1994; Visser 1964). Natural HS are isolated first by extraction from soils and waters using alkaline solvents, then partial precipitation with a strong acid followed by separation over columns filled with resins that have different affinity to different HS fractions (different XAD materials, i.e., hydrophobic cross-linked polystyrene copolymer resins). In particular, the treatment with strong base and acid is known to cause some chemical changes in the molecules.

Due to differences in origin and diagenesis, the chemical composition and structure of HS in nature vary significantly among different HS samples and are impossible to describe accurately by chemical formulas. For a long time, HS were thought to be large macromolecules consisting of complex aromatic and aliphatic structures containing functional groups such as hydroxyl, carboxyl, amino, and phenolic groups (Stevenson 1994). Due to its varying chemical structure, natural OM has been operationally classified according to solubility and molecular weight criteria. HS are classified as fulvic acids, which have lower molecular weights (0.5–5 kDa) and are soluble at all pH values, or humic acids, which are bigger (20–100 kDa) and are soluble at alkaline pH only. Humins, the largest OM fraction in soils, are insoluble at all pH values (Aiken et al. 1985; Stevenson 1994).

In contrast to attempts to characterize the macromolecular structure of HS, Piccolo (2001) and Sutton and Sposito (2005) described a new model for the structure of HS. According to this model, HS do not consist of single macromolecules but rather of associations of smaller organic molecules of different kinds held together by hydrophobic effects and hydrogen bonds. Upon changes in geochemical conditions, these bonds are strengthened or loosened, causing structural changes, that may even lead to the separation of single small organic molecules from the associations (Sutton and Sposito 2005). The molecules contributing to HS can be all kinds of organic

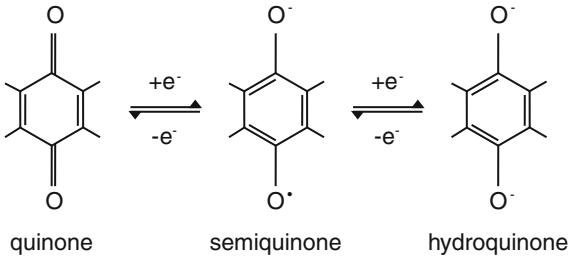

Fig. 2 Two-step reduction and oxidation of quinones and quinoid functional groups via the semiquinone radical. Note that for simplification protonation equlibria of the reduced species were disregarded. (For details of protonation and deprotonation equilibria of reduced quinones see Jiang et al. (2009)).

molecules including biomolecules (e.g., fatty acids, carboxylic acids, alcohols, lignins, sugars), which, by the old macromolecular definition of HS, were not even part of HS (Stevenson 1994). However, the new model considers these molecules as part of HS (Sutton and Sposito 2005).

Different functional groups in HS (see below) are known to accept and donate electrons, i.e., to participate in redox reactions (Aiken et al. 1985; Visser 1964). Dunnivant et al. (1992) showed that natural OM can mediate the reduction of substituted nitrobenzenes by the bulk electron donor hydrogen sulfide. They proposed for the first time that this effect could be due to an electron shuttling activity of the OM, which was proposed to be reduced by the bulk electron donor and subsequently transfer its electrons to the organic pollutant. This model was further developed by Lovley et al. (1996), who were the first to show that HS can be reduced microbially and that the reduction of HS can support microbial growth. They also proposed that reduced HS can then transfer electrons abiotically to Fe(III) minerals and thus shuttle electrons between the microorganisms and Fe(III) minerals (Fig. 1) (Lovley et al. 1996). This process of electron shuttling by HS greatly enhances the rate of microbial Fe(III) reduction (see Sect. 4) (Jiang and Kappler 2008; Lovley et al. 1996).

When it was proposed for the first time that HS can function as electron shuttles, quinones were suggested to be the redox-active functional groups in HS (Dunnivant et al. 1992). Quinones are aromatic molecules that can accept electrons according to the formula shown in Fig. 2. As indicated, the reduction of quinones is a two-step process leading to the formation of a semiquinone radical and, upon transfer of a second electron, to the formation of a hydroquinone. Both electron transfer processes are reversible (Uchimiya and Stone 2009).

The hypothesis that quinones are the most important electron accepting functional groups also in microbial HS reduction (Lovley et al. 1996) was further supported by the work of Scott et al. (1998), who used electron spin resonance (ESR) measurements to quantify organic radicals in microbially reduced HS. They found that microbial reduction of HS results in an increase in radical content that is proportional to the number of electrons taken up by the respective HS samples (electron accepting capacity, see below). Furthermore, the ESR spectra obtained

for the HS samples were consistent with semiquinones being the main organic radicals (Scott et al. 1998). Furthermore, Chen et al. (2003) demonstrated that the electron accepting capacities of different OM samples was correlated to the aromaticity of the samples. These data indicate that quinones or quinone-like functional groups are the major functional groups responsible for the electron accepting capacity of HS samples.

Indications that quinones are the most important redox-active functional groups in HS at environmentally relevant conditions also come from the observation that electron transfer to HS is reversible (Aeschbacher et al. 2011; Bauer and Kappler 2009; Ratasuk and Nanny 2007) and from the comparison of electrochemical and spectroscopic properties of HS with those of selected model quinones (Fimmen et al. 2007; Nurmi and Tratnyek 2002; Ratasuk and Nanny 2007). Additionally, electrons and protons were consumed in a ratio close to 1:1 during electrochemical reduction of different HS samples, which also points toward the reduction of quinoid functional groups as the active electron acceptors (Aeschbacher et al. 2010, 2011; Maurer et al. 2010).

Besides quinoid functional groups, complexed metal ions such as iron were suggested to be responsible for part of the redox-active behavior of HS (Struyk and Sposito 2001). However, Chen et al. (2003) and Peretyazhko and Sposito (2006) found that the amount of electrons transferred by their HS samples (reducing capacities, see below) could not be explained by complexed iron alone. Furthermore, Lovley and Blunt-Harris (1999) showed that although the iron in HS was indeed being reduced during microbial HS reduction, the iron content of several commercially available HS [International humic substances society (IHSS)] was too small to contribute significantly to the reducing capacities measured.

These papers establish that functional groups other than complexed metal ions, e.g., quinone moieties, are the more important electron accepting functional groups. However, two important caveats must be mentioned. First, although they may represent only a small fraction of the electron accepting capacity, very reactive Fe species, could still be important for situations such as reduction of pollutants, e.g., chlorinated compounds or toxic metals such as Cr(VI), U(VI), or As(V). Second, the low Fe content in HS might be due to the harsh chemical extraction and purification treatment of the samples during HS isolation that will remove most of the Fe in the HS. It is therefore possible that HS in the environment contain much higher amounts of complexed metals than these purified IHSS samples and, in the environment, Fe could play a more important role than implied by these laboratory experiments with highly purified HS.

Besides quinones and complexed metal ions, non-quinone aromatic constituents (Chen et al. 2003) and sulfur-containing functional groups (Einsiedl et al. 2008) have also been suggested to be relevant as possible redox-active sites in humic substances. However, detailed investigations of the contributions of these functional groups to HS redox activity are missing until now.

Since HS consist of a variety of different redox-active functional groups with different redox potentials (see below), they can be present in a wide range of redox states. In order to describe the redox state of HS on a quantitative basis, the terms

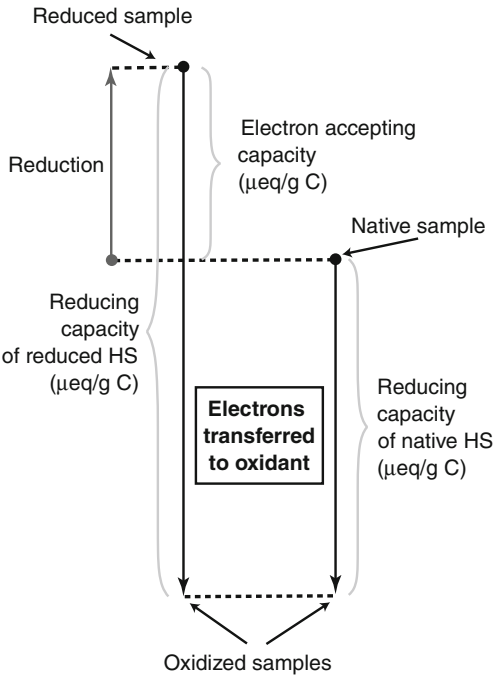

Fig. 3 Scheme illustrating the determination of the reducing capacity and electron accepting capacity of native and reduced HS samples

reducing capacity and electron accepting capacity are used (Sposito 2011). The redox state of HS is usually determined by oxidizing the HS with different oxidizing agents (e.g., ferric citrate, potassium ferricyanide, I_2 or electrochemically) and measuring the number of electrons that are transferred. This value is called the reducing capacity of the HS sample (Fig. 3) and is usually given in µeq (microequivalents, i.e., micromoles electrons transferred) per g HS or per g C.

The reducing capacity can be determined for native and for chemically as well as for microbially reduced HS. Upon reduction, the reducing capacity toward the same oxidizing agent increases (Fig. 3). The difference between the reducing capacity of the native and the reduced HS sample reflects the amount of electrons that are transferred to the HS during reduction, i.e., the electron accepting capacity of the HS (Fig. 3). As this approach is based on the assumption that all electrons that are transferred onto the HS during reduction can be recovered during oxidation, it is advisable also to determine the electron accepting capacity directly, e.g., by reduction with Zn (Blodau et al. 2009), in order to obtain a systematic characterization of the redox state of HS.

Reducing capacity values reported in the literature for non-reduced HS samples range from 50 to >10,000 µeq/g HS (Bauer and Kappler 2009; Benz et al. 1998; Jiang and Kappler 2008; Peretyazhko and Sposito 2006; Struyk and Sposito 2001; Wolf et al. 2009). These large variations are partly attributable to the different oxidizing agents used in determining these values, which differ in redox potential. Peretyazhko and Sposito (2006) and Bauer et al. (2007) compared reducing

capacities obtained with different oxidizing agents and found that reducing capacities measured with ferric citrate as oxidant are approximately one order of magnitude smaller than those measured with potassium ferricyanide and about two orders of magnitude smaller than those measured by oxidation with I_2. Peretyazhko and Sposito thus propose to standardize the protocol for determining reducing capacities to the use of ferric citrate, because its standard electrode potential of 309 mV and its chemical structure are most similar to naturally occurring soluble oxidants (Peretyazhko and Sposito 2006). However, although the absolute values of reducing capacities are not comparable if they were obtained with different oxidizing agents, the ratios between electron accepting capacities of different HS samples (e.g. HS of different origin) are still the same within the experimental error, regardless if they were measured toward ferric citrate, potassium ferricyanide, or electrochemically (Aeschbacher et al. 2010; Sposito 2011).

Even if the same oxidant is used, differences in reducing and electron accepting capacities are obtained for different HS samples. This is due to the fact that the composition of HS varies significantly depending on origin and genesis of the HS. These differences commonly affect the magnitude of the reducing and electron accepting capacities. The electron accepting capacity of humic acids is generally higher than that of fulvic acids extracted from the same source material (Aeschbacher et al. 2010; Ratasuk and Nanny 2007) and HS extracted from soils and sediments have higher electron accepting capacities than those from partially aquatic origin due to their higher aromatic content (Aeschbacher et al. 2010; Ratasuk and Nanny 2007; Scott et al. 1998).

As can be expected from the heterogeneity of HS composition and the range in reducing capacities measured, the redox potentials reported for HS at pH 7 span a wide range from −300 to +400 mV versus standard hydrogen electrode (Osterberg and Shirshova 1997; Straub et al. 2001; Visser 1964). Recent studies suggest that the redox potential of a HS sample cannot be expressed by a single value, but rather that HS contain a variety of redox-active functional moieties with a distribution of redox potentials. Thus, the overall redox potential of an HS sample was suggested to be expressed best as a continuum of redox potentials in the range of ca. –300 to +100 mV (Aeschbacher et al. 2011). The actual range of redox potentials and the frequency of redox-active moieties of a specific potential vary between different HS samples (Aeschbacher et al. 2011) depending not only on the origin of the HS but also on its history, i.e., the methods of extraction and pre-treatment of the HS.

3 Electron Shuttling by Humics: A Two-Step Process

Electron shuttling between microorganisms and Fe(III) minerals by HS consists of two steps: (i) The biotic reduction of the dissolved or non-dissolved HS, followed by (ii) an abiotic electron transfer from the reduced HS to the Fe(III) mineral (Fig. 1). The re-oxidation of the reduced HS by the Fe(III) leads once again to oxidized HS, which can again be re-reduced by the bacteria (Fig. 1). The same HS

molecule is thus recycled during this process and can transfer many electrons from the microorganisms to the Fe(III) without being consumed in the process (Ratasuk and Nanny 2007).

The first step of the electron shuttling process (Fig. 1) is the reduction of the HS. This process can be microbially mediated or can occur abiotically. Chemical reduction in laboratory experiments is commonly obtained by incubation of the HS samples under a H_2 atmosphere in the presence of a palladium catalyst (Kappler et al. 2004; Peretyazhko and Sposito 2006; Visser 1964). For microbial HS reduction, HS samples are incubated with HS-reducing bacteria, for example in cell suspensions with cell numbers of 10^8–10^{10} cells/mL or higher (Lovley et al. 1996; Nevin and Lovley 2000). Peretyazhko and Sposito (2006) and Jiang and Kappler (2008) showed for a number of different HS samples that the reducing capacities obtained after chemical reduction with H_2/Pd and after microbial reduction by a soil extract and by *Geobacter sulfurreducens*, respectively, were very similar. From this they conclude that chemical and microbial reduction both transfer electrons to essentially the same redox-active moieties in HS. These findings indicate that chemically reduced HS or HS-analogs can be used as a proxy for microbially reduced HS or HS-analogs, as it is commonly done in laboratory studies (e.g. Benz et al. 1998; Lovley et al. 1998, 1999). However, it should be noted that chemical reduction, especially in the presence of a palladium catalyst, can alter physicochemical properties and potentially even leave chemical traces (e.g. Pd-ions) in the HS which might affect the redox properties of the treated HS. A further possibility of abiotically reducing HS is by the use of an electrochemical cell, where the HS are reduced at an electrode surface (Kappler and Haderlein 2003). This method has the advantage that it allows precise control of the electrochemical endpoint of the oxidation and reduction. Further methodological developments by Aeschbacher et al. (2010) include the use of a glassy carbon working electrode, which prevents the reduction of H^+ leading to H_2 formation and, thus, enables the exact quantification of the number of electrons transferred to the HS. They also introduced the use of chemical mediators to facilitate electron transfer between organic matter and the electrode.

The first microorganisms with a demonstrated capacity for reducing HS were the dissimilatory Fe(III) reducers *G. metallireducens* and *Shewanella alga* (Lovley et al. 1996). In a subsequent study, Lovley et al. (1998) tested a number of different Fe(III)-reducing bacteria for their ability to reduce anthraquinone-2,6-disulfonate (AQDS), a model compound for quinone moieties in HS, and found that all Fe(III)-reducers studied were able to use AQDS as an electron acceptor. To our knowledge, this statement still holds true and there is still no report of any neutrophilic Fe(III)-reducer that is unable to reduce HS. However, Emmerich and Kappler (2012) recently demonstrated that the acidophilic Fe(III)-reducer *Acidiphilium* SJH was neither able to reduce HS nor AQDS. In addition to Fe(III)-reducers, fermenting (Benz et al. 1998), halorespiring, sulfate-reducing, and methanogenic microorganisms (Cervantes et al. 2002) have been shown to be able to reduce HS. Thus, the indirect reduction of Fe(III) via electron shuttling by HS is not restricted to microorganisms that are also able to directly reduce Fe(III).

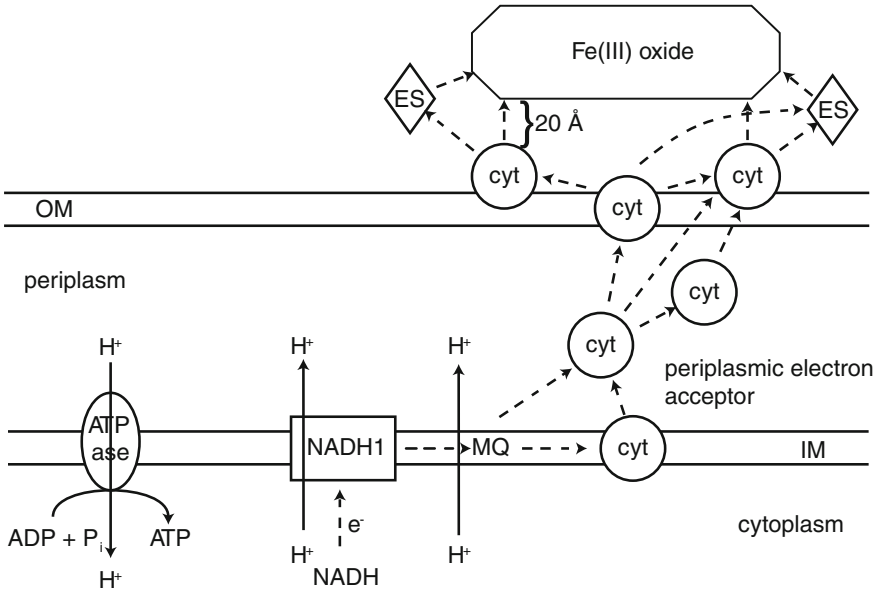

Fig. 4 Schematic illustration of the pathway of electron flow to poorly soluble Fe(III) oxides and dissolved electron shuttles in Fe(III)-reducing bacteria. Reduction of both electron acceptors [Fe(III) oxides and electron shuttles] proceeds largely via the same electron transfer system. *OM* outer membrane, *IM* inner membrane, *NADH1* NADH dehydrogenase, *MQ* menaquinone pool, *cyt* cytochrome of the electron transfer system, *ES* dissolved electron shuttle. *Dashed arrows* indicate electron flow

Electron shuttling thus increases the number of microorganisms that are able to indirectly reduce Fe(III) and, therefore also potentially increases the importance of Fe(III) reduction in the environment.

To date, the molecular mechanism of electron transfer from microbes to HS and other electron shuttles is not completely understood. There are several studies indicating that electrons are transferred to dissolved electron shuttles largely via the same protein complexes that are used for direct metal reduction (Gescher et al. 2008; Lies et al. 2005; Voordeckers et al. 2010). This electron transfer proceeds via a number of quinones and *c*-type cytochromes that transport the electrons from the oxidoreductase in the cytoplasm through the cytoplasmic membrane and the periplasm to the surface of the outer membrane (Fig. 4; chapter "The Biochemistry of Dissimilatory Ferric Iron and Manganese Reduction in *Shewanella oneidensis*"). These outer membrane cytochromes are particularly important in the reduction of poorly soluble electron acceptors such as iron and manganese oxides and oxyhydroxides, whereas some dissolved electron acceptors are expected to penetrate the outer membrane and take up electrons directly from the proteins located in the periplasm (Gescher et al. 2008). While there is some evidence for uptake of HS and AQDS into cells (Kulikova et al. 2010; Shyu et al. 2002), the main pathway of electron transfer to HS and AQDS seems to involve electron transfer at the surface of the outer membrane (Fig. 4).

Electron transfer through outer membrane proteins was demonstrated by the diminished ability of omc-mutants (defective in outer membrane proteins) to reduce AQDS or to indirectly reduce iron minerals by the use of electron shuttles (Gescher et al. 2008; Lies et al. 2005; Voordeckers et al. 2010). However, deletion of only one outer membrane cytochrome in *G. sulfurreducens* was not sufficient to completely inhibit HS and AQDS reduction, indicating that different cytochromes contribute to the reduction of electron shuttles (Voordeckers et al. 2010). The pool of proteins and reactive sites within proteins that are able to reduce dissolved electron shuttles also seems to include some sites which are either located in the periplasm or otherwise protected from access, making them inaccessible for poorly soluble minerals (Lies et al. 2005; Voordeckers et al. 2010). In the environment, electron shuttling to poorly soluble electron acceptors such as Fe(III) minerals via reduction of soluble electron shuttles such as HS might even take place simultaneously with the direct reduction of the Fe(III) minerals (Clarke et al. 2011; MacDonald et al. 2011).

The second step of the electron shuttling process is the transfer of electrons from reduced shuttles to the terminal electron acceptor. This means that after the HS are reduced microbially, they can be reoxidized by transferring their electrons for example to Fe(III) minerals (Fig. 1), leading to the indirect microbial reduction of Fe(III) minerals. While there are many studies on microbial HS reduction (see above), systematic studies of the kinetics and thermodynamics of abiotic electron transfer from reduced HS to different Fe(III) minerals are sparse. It is known from microbial iron mineral reduction experiments in the absence and presence of electron shuttles (HS, AQDS) that reduced shuttles are able to transfer electrons to a variety of different Fe(III) minerals and Fe(III) phases, including some phases such as goethite, hematite, and structural iron in clay minerals that are scarcely reducible by direct microbial reduction (Lovley et al. 1998).

However, the number of electrons that are transferred from reduced HS to different Fe(III) minerals and complexed Fe(III) depends on the redox potential of the respective Fe(III) phase (Bauer and Kappler 2009). Furthermore, Liu et al. showed that the electron transfer from reduced AQDS to hematite was limited, or at high concentrations even prevented, by sorption of Fe(II) and phosphate to the hematite surface (Liu et al. 2007). This indicates that the electron transfer between reduced shuttles and Fe(III) minerals strongly depends on geochemical conditions such as concentrations of different ions that might sorb to the Fe(III) minerals and the identity of the Fe(III) minerals themselves, such as biogenic versus abiogenic minerals. Therefore, it is unclear how fast and to what extent Fe(III) minerals in the environment are reduced by HS.

4 Advantages and Disadvantages of Electron Shuttling Versus Direct Contact Fe(III) Mineral Reduction

As discussed previously (chapter "Minerals and Aqueous Species of Iron and Manganese As Reactants and Products of Microbial Metal Respiration"), at near neutral pH values, Fe(III) minerals have very low solubilities and Fe(III) is

therefore present in the environment mostly in the form of solid (oxyhydr)oxides. This imposes a limitation on the rate and extent of direct microbial Fe(III) reduction, since microbes have to come within a distance of approximately 20 Å from the iron mineral surface in order to directly transfer electrons (Gray and Winkler 2005). Therefore, the addition of electron shuttling compounds such as HS is expected to stimulate microbial Fe(III) reduction by those cells that are not close enough for direct electron transfer to minerals. However, even when cells are attached to the mineral, the distance between the electron donating cytochromes and the electron accepting mineral surface might in some cases still be larger than 20 Å (Fig. 4). HS or other electron shuttles may facilitate electron transfer in this situation as well. Indeed, several studies have found increased rates of microbial Fe(III) reduction in the presence of HS and AQDS (e.g. Lovley et al. 1996; MacDonald et al. 2011; Wolf et al. 2009).

Jiang and Kappler (2008) compared the rates of direct ferrihydrite reduction by *Geobacter sulfurreducens* to the rate of HS reduction by *G. sulfurreducens* and to the rate of abiotic electron transfer from reduced HS to ferrihydrite. They found that HS are reduced 27 times faster than ferrihydrite and that the electron transfer from reduced HS to ferrihydrite proceeds at least seven times faster than the electron transfer from *G. sulfurreducens* to ferrihydrite. Thus, they showed that the overall electron shuttling process from *G. sulfurreducens* via HS to ferrihydrite is limited by the second, abiotic electron transfer step, but that it still proceeds at least seven times faster than the direct microbial ferrihydrite reduction. However, HS do not in all cases increase the Fe(III) reduction rate (see also Sect. 7). Studies with low concentrations of HS showed that at these concentrations HS can even lead to decreased microbial Fe(III) reduction rates compared to setups without HS (Amstaetter et al. 2012; Piepenbrock et al. 2011). These observations were attributed to the sorption of the HS to the mineral surface, thus reducing the bioavailable mineral surface area either by directly blocking surface sites or by increasing aggregation of the ferrihydrite particles.

Wolf et al. (2009) studied the effects of different model quinones on microbial Fe(III) reduction and found that the kinetics were mainly controlled by the redox potential of the shuttling compound. They hypothesized that there is an ideal redox potential for the electron shuttle as the most efficient shuttles all had a redox potential between –137 and –225 mV. This is high enough to provide sufficient redox potential difference to the electron donor (lactate or acetate in case of *Shewanella* and *Geobacter* sp., respectively) to allow the necessary amount of ATP synthesis for microbes but at the same time the redox potential of the shuttle is low enough to make the rate-limiting second electron transfer step to the terminal electron acceptor favorable. (Wolf et al. 2009).

When electron shuttling occurs, Fe(III) can be reduced at a higher rate than in the absence of electron shuttles. However, the energy that microbes can gain from a redox reaction depends on the redox potential difference between the electron donor and the microbial electron acceptor. As shown by Wolf et al. (2009), the redox potential of the electron shuttle must be between the standard redox potential of the electron donor and the electron acceptor in order to efficiently

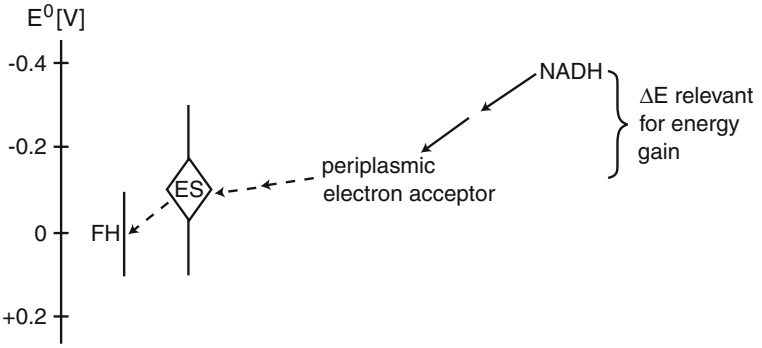

Fig. 5 Redox potential of the most important components involved in electron flow from NADH to ferrihydrite in the model for Fe(III) reducers presented in Fig. 4. *Solid arrows*, electron flow associated with ATP production; *dashed arrows*, electron flow without ATP production; *ES*, dissolved electron shuttle; *FH*, ferrihydrite

stimulate Fe(III) reduction. Hence, if bacteria reduce the shuttle instead of directly reducing the Fe(III) mineral, the redox potential difference is smaller and they can be expected to gain less energy from the reaction. However, this only holds true if the electron is transported through a long electron transport chain in the membrane, in the course of which protons are translocated out of the cytoplasm and a proton motive force is built up for ATP synthesis.

In the case of the most commonly studied Fe(III)-reducing bacteria, *Geobacter* and *Shewanella*, however, the electron transport chain involved in Fe(III) reduction seems to be rather short and proton translocation takes place only until the electron reaches the periplasm (Fig. 4) (chapter "The Biochemistry of Dissimilatory Ferric Iron and Manganese Reduction in *Shewanella oneidensis*"). Thus, the redox potential difference relevant for the energy gain is not the one between the electron donor and the terminal electron acceptor, but the one between the electron donor and the periplasmic electron acceptor (Fig. 5) (Bird et al. 2011). As the electron transport chain seems to be the same for the reduction of HS and Fe(III) (see Sect. 3, Fig. 4), the redox potential difference between electron donor and periplasmic electron acceptor and hence the overall energy gain should be the same for the direct reduction of Fe(III) minerals and the reduction of Fe(III) minerals via electron shuttles. Further evidence for this comes from studies with electrochemical cells which show that *G. sulfurreducens* yielded the same energy gain when grown at higher versus lower electrode potentials (Marsili et al. 2010).

In summary, the presence of electron shuttles (above a certain minimum concentration, see below) can increase the rate of microbial Fe(III) reduction. If the rate of microbial metabolism thus increases, the growth rate (increase in cell number per time) of bacteria that perform the electron shuttling can be expected to increase likewise. Hence, the use of electron shuttles provides an ecological advantage for bacteria as it enables them to outgrow other species. This is especially the case if there is indeed no loss in energy gain for the bacteria when

reducing the shuttle instead of reducing the mineral directly as discussed above. Thus, they can increase the rate of electron turnover and still generate the same amount of energy per electron transferred.

5 Reduction of Solid-Phase Humics

Until now, most research on HS electron shuttling has focused on dissolved HS. However, the highest fraction of HS in natural soils and sediments is in the solid form (Stevenson 1994). Kappler et al. (2004) presented the first evidence that solid-phase HS are also redox-active. They determined the reducing and electron accepting capacities of HS extracted from sediments with 0.1 M NaOH, and thus, they also extracted a fraction of HS that is particulate at circumneutral pH. They found that the HS were in a more reduced state in the deeper layers of the sediment. Although these authors did not determine to what extent the solid HS fraction contributed to the measured reducing capacities, this study showed that at least a fraction of the solid-phase redox-active humics was reduced by microorganisms.

Roden et al. (2010) were the first to systematically study the microbial reduction of solid-phase HS. They found that the two Fe(III)-reducers *G. sulfurreducens* and *S. oneidensis* were able to transfer electrons to Fe-stripped wetland sediments containing solid-phase HS. With a series of control experiments, they ruled out the possibility that the electron accepting capacity stemmed from inorganic constituents in the sediment. Although the electron accepting capacities determined per mg sediment were a lot lower than those for dissolved HS, the addition of the Fe-stripped sediments that contained solid-phase humic substances to microbial Fe(III) reduction experiments significantly increased the microbial Fe(III) reduction rates (Roden et al. 2010). Based on this evidence the authors suggested that solid-phase HS can also function as electron shuttles between microorganisms and poorly soluble terminal electron acceptors such as Fe(III) minerals. However, the relevance of microbial solid-phase HS reduction and electron shuttling in environmental systems is unknown and must be determined through future studies.

Evidence for the existence of long distance electron transfer via redox-active constituents comes from a recent study that showed electrons being transferred from sulfide produced within a marine sediment to oxygen present at the sediment surface over distances of more than a centimeter (Nielsen et al. 2010). The sulfide profile with depth in the sediment measured with microelectrodes showed an immediate response to the presence and absence of O_2 at the sediment surface, suggesting a direct redox coupling of sulfide oxidation to the overlying O_2. The very fast electron transfer rules out diffusion of dissolved redox-active molecules as the underlying mechanism, but requires electron transfer via a conductive network, as could be formed for example by solid-phase HS (Fig. 6) or (as suggested by the authors) by conductive bacterial nanowires or redox-active pyrite particles. However, if and to what extent solid-phase (and also dissolved) HS contribute to this electron transfer over cm-long distances remains currently unknown.

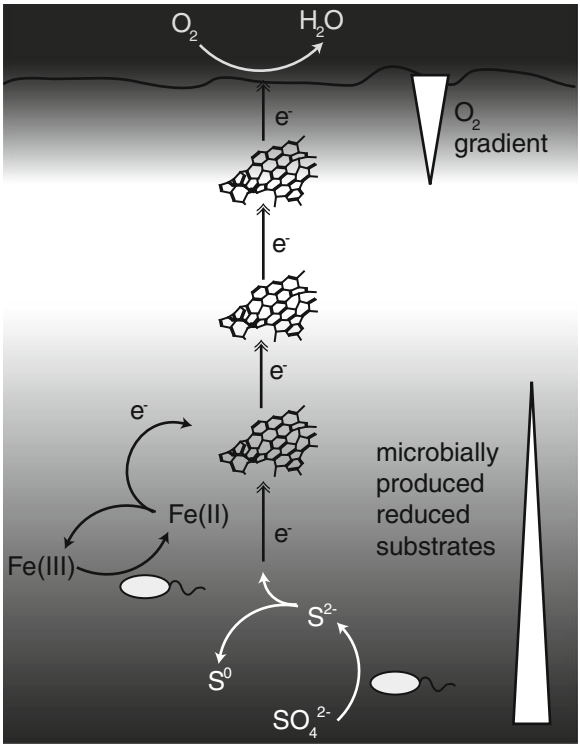

Fig. 6 Electron transfer from microbially produced reduced metabolites (Fe(II), S^{2-}, etc.) to electron acceptors with a more positive redox potential, e.g., O_2 over mm- or even cm-long distances via a conductive network including redox-active dissolved and solid-phase humic substances in sediments or soils

6 Other Extracellular Electron Shuttles and Humic Model Compounds

Besides HS, other organic and inorganic redox-active compounds such as sulfur species have been suggested to function as electron shuttles and to stimulate microbial Fe(III) reduction. In a comparative study, Nevin and Lovley (2000) analyzed the potential of U(IV) and several different sulfur species to function as electron shuttles for microbial Fe(III) reduction. They showed that the addition of U(IV) could stimulate Fe(III) reduction in cell suspension experiments with *G. metallireducens* and synthetic Fe(III) hydroxides as the electron acceptor. However, unlike HS or AQDS, uranium did not stimulate the reduction of Fe(III) present in aquifer sediments under environmentally relevant conditions. The same was true for S^0 species. These authors also observed that sulfur-containing amino acids at environmentally relevant concentrations did not stimulate Fe(III) reduction in cell suspension experiments (Nevin and Lovley 2000). In contrast, Straub and Schink (2004) proposed a model of electron shuttling by an unidentified sulfur species. They found that at low thiosulfate concentrations (50 μM) microbial reduction of the thiosulfate by *S. deleyianum* lead to the reoxidation of the produced sulfides by ferrihydrite leading to the formation of ferrous iron and oxidized

sulfur species, possibly polysulfides (Straub and Schink 2004). The identification of the oxidized sulfur species has not been accomplished and is an open question to be answered in future studies.

Among organic molecules, on the other hand, there are a variety of compounds that have been shown to stimulate microbial Fe(III) reduction, most of them quinones (Wolf et al. 2009). One of the most interesting of these quinones is AQDS (9,10-anthraquinone-2,6-disulfonic acid), since it is one of the most efficient electron shuttles (Wolf et al. 2009) and has often been used as a proxy for quinoid moieties in HS (e.g. Coates et al. 1998; Lovley et al. 1998). However, the replacement of HS with model quinones such as AQDS should be handled with care since AQDS differs from HS in some very important respects: first, while HS show strong sorption to Fe(III) (oxyhydr)oxides, AQDS sorption to Fe(III) minerals is more than one order of magnitude lower (Wolf et al. 2009). This difference in sorption behavior influences electron shuttling by HS and AQDS. While low concentrations of AQDS led to a significant stimulation of Fe(III) reduction, low HS concentrations, as mentioned above, even decreased the Fe(III) reduction rate (Piepenbrock et al. 2011). This was probably due to the fact that the concentration of dissolved shuttles was not high enough in the experiments with low HS concentrations (see below) and that the accessibility of the ferrihydrite surface was lowered by sorbed HS and potentially by consequential aggregation of the ferrihydrite particles (Amstaetter et al. 2012). The second important difference between AQDS and HS is that AQDS at high concentrations can have a toxic effect for some microorganisms (Shyu et al. 2002), which can lead to reduced Fe(III) reduction rates (Nevin and Lovley 2000). Furthermore, the redox potential of AQDS is close to the ideal redox potential for electron shuttling (Wolf et al. 2009), while most of the redox-active moieties in HS have a higher redox potential (Aeschbacher et al. 2011).

Other examples of extracellular organic electron shuttles are phenazines, which are produced by a variety of soil bacteria, e.g., *Pseudomonas* species, and enable these bacteria to reduce poorly soluble Fe(III) oxides (Hernandez et al. 2004). These and other endogenous electron shuttles are discussed in detail in the chapter "On the Role of Endogenous Electron Shuttles in Extracellular Electron Transfer".

7 Environmental Relevance

HS-reducing bacteria have been enriched and isolated from different environments such as aquifer and lake sediments, wetland soils, and marine sediments (Coates et al. 1998; Kappler et al. 2004; Snoeyenbos-West et al. 2000). Detailed protocols for the enrichment and isolation of these microorganisms can be found in (Straub et al. 2005). In these investigated environments, cell numbers of HS reducers were in the range of 10^4–10^6 cells/g or mL sediment (Coates et al. 1998; Kappler et al. 2004) and were as numerous as fermenting microorganisms, indicating that microbial HS reduction has the potential to contribute significantly to electron

fluxes in the environment (Kappler et al. 2004). The high cell numbers of HS reducers are probably due to the fact that HS are reduced not only by Fe(III) reducers but by a wide variety of different physiological groups of bacteria including fermenting microorganisms, sulfate reducers, methanogens and halorespirers (Benz et al. 1998; Cervantes et al. 2002). Thus, Fe(III) reduction is not restricted to microorganisms that directly reduce Fe(III), but also involves microorganisms that use HS as electron shuttles to indirectly reduce Fe(III). Thus, the number of microorganisms that contribute to Fe(III) reduction increases and also potentially the importance of Fe(III) reduction in the environment.

In anoxic systems where microbial Fe(III) reduction takes place, Fe(III) (oxyhydr)oxides are expected to be the most important oxidants for the re-oxidation of microbially reduced HS. However, reduced HS can also be oxidized by O_2 (Aeschbacher et al. 2010; Bauer and Kappler 2009; Ratasuk and Nanny 2007), for instance at oxic-anoxic interfaces. Bauer and Kappler (2009) quantified the amount of electrons transferred from reduced HS to O_2 and found that fewer electrons were transferred than would be expected based on the redox potential of O_2. This corresponds well to the finding that chemically or microbially reduced HS that are re-oxidized by O_2 do not return to the same redox state as before reduction, but that some redox-active sites, which can transfer electrons to Fe(III), are protected from rapid re-oxidation by O_2 (Bauer and Kappler 2009; Macalady and Ranville 1998). These findings indicate that electron transfer from HS to Fe(III) and, thus, electron shuttling between microorganisms and Fe(III) is not necessarily restricted to anoxic environments but has the potential to even take place under microoxic conditions, e.g., at oxic-anoxic interfaces (Bauer and Kappler 2009). However, if and to what extent HS electron shuttling really takes place in oxic environments is a question that remains to be answered in future studies.

In order to analyze the potential of HS electron shuttling in the environment, HS were added to microcosm experiments with soils or aquifer sediments (Nevin and Lovley 2000; Rakshit et al. 2009) and Fe(III) reduction rates were quantified with and without addition of electron shuttles. In both studies, microbial Fe(III) reduction rates were significantly higher in the presence of added AQDS and HS, indicating that, in the absence of added shuttles, microbial Fe(III) reduction was limited by the availability of electron accepting Fe(III) minerals (not by their abundance) and that HS also have the potential to increase reduction rates in complex environmental systems. However, these findings also show that the HS content originally present in the soil and sediment samples was not sufficient to exert the maximum stimulation possible on the Fe(III) reduction, since addition of electron shuttles further increased the Fe(III) reduction rates. This indicates that if and to what extent electron flow to Fe(III) in environmental systems proceeds via electron shuttling strongly depends on the HS concentration and on the ratio of HS to iron minerals.

Several studies demonstrated a linear correlation between the concentration of dissolved HS and the Fe(III) reduction rate (Amstaetter et al. 2012; Jiang and Kappler 2008). However, this is only true for a range of HS concentrations between a lower limit, below which no stimulation of Fe(III) reduction occurs (and in some cases even lower reduction rates were observed than in the absence of HS

(see Sect. 4), and an upper limit, above which no further increase of reduction rates with increasing HS concentrations takes place. Jiang and Kappler (2008) and Amstaetter et al. (2012) determined a lower limit for electron shuttling of 10–20 mg dissolved HS/L while Wolf et al. (2009) found a stimulation of the Fe(III) reduction even at 1 mg/L total HS (0.0025 mg/L dissolved HS). This indicates that the HS concentration necessary for stimulating microbial Fe(III) reduction strongly depends on the system, i.e., the iron mineral identity, mineral concentration, the number and type of microbial cells present, the type of HS, etc. The same is true for the upper limit of electron shuttling, as reported values vary between 50 and 240 mg HS/L (Amstaetter et al. 2012; Jiang and Kappler 2008). The HS concentrations necessary for electron shuttling also depend on the mechanism of electron transfer between the bacterial cell and the Fe(III) mineral. There are two models as to how electron shuttling over spatial distances could work (Fig 7): (1) the electron is transferred from the cell to a HS molecule that is located at a certain distance from the mineral surface. The reduced electron shuttle then diffuses to the Fe(III) mineral surface, where it transfers the electron to the mineral. The re-oxidized shuttle then returns (diffuses back) to the cell and can be re-reduced, thus functioning as electron shuttle between the cell and the mineral. In this case the electron transfer would be controlled by the diffusion of the shuttle to the mineral surface and back, and therefore by the distance between the cell and the mineral. (2) Alternatively, an electron is transferred from the cell to a first electron shuttle that is located at a certain distance from the mineral surface. But, instead of diffusion of the shuttle to the mineral surface, the electron is passed from the first HS molecule to the next one and the distance between the cell and the Fe(III) mineral is thus bridged by electron hopping. Since the maximum distance for each of these electron transfer steps is approximately 20 Å (Gray and Winkler 2005), a minimum concentration of HS is required to provide the necessary density of electron accepting sites (Fig 7).

Besides the concentration of HS and the ratio of HS to iron minerals, the ratio of microbial cells to Fe(III) minerals is also expected to be important for electron shuttling. If the Fe(III) minerals are present in excess and the mineral surface area is not limiting for microbial electron transfer (i.e., all cells are attached to the mineral surface), the addition of electron shuttles potentially leads only to a minor stimulation of microbial Fe(III) reduction. Such a stimulation by electron shuttles in a scenario where all cells are associated with the mineral surface could occur by dissolved shuttles functioning where the electron accepting Fe(III) mineral surface and outer membrane cytochromes are not close enough (see Fig 4). On the other hand, if the cells are in excess and the mineral surface area is not sufficient for all cells to attach, electron shuttling has the potential to significantly increase the reduction rate by enabling Fe(III) reduction by those cells that cannot directly transfer electrons to the mineral surface. Indeed, it was recently shown in our laboratory that increases in the rate of ferrihydrite reduction by *S. oneidensis* MR-1 in cell suspension experiments with varying cell densities in the presence of HS were present at both high and low cell densities but the increase was more prominent at high cell densities than at lower cell densities (Rohrbach,

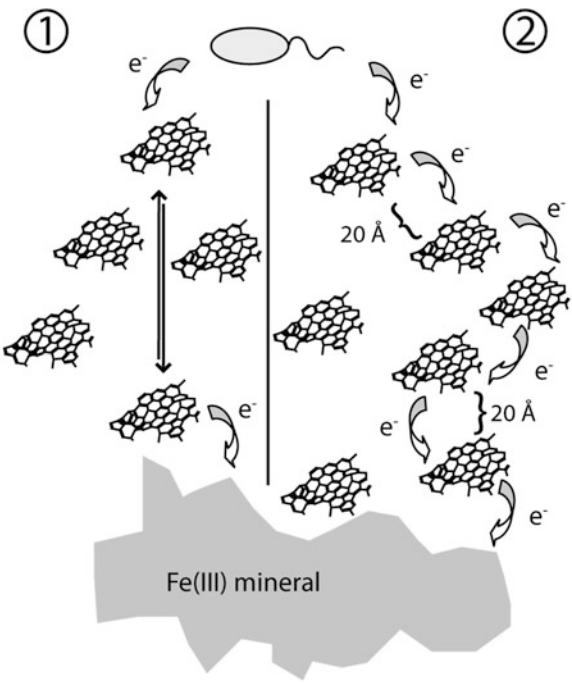

Fig. 7 Models for electron shuttling by humic substances between a microbial cell and a Fe(III) mineral by (1) diffusion of the electron shuttle (*left*) and (2) electron hopping (*right*). Electron hopping requires a maximum distance of approximately 20 Å between the redox-active sites of the involved shuttling molecules which can only be provided at a certain HS concentration

unpublished data). This suggests that at high cell densities, the shuttles enable electron transfer to the mineral surface from cells that are at a distance from the minerals, while at low cell densities the shuttles increase electron transfer to the mineral from cells that are attached to the mineral.

8 Open Questions and Future Research

Humic substances and other extracellular electron shuttles can contribute significantly to the electron fluxes during microbial respiration, in batch systems with pure cultures of microorganisms as well as in complex environmental systems including soil and sediment microcosms. Although a lot of recent research has focused on the role of HS as electron shuttles, there are still several key questions that remain unanswered. Most of them are related to the importance and relevance of electron shuttling in environmental systems, where electron shuttling is often very difficult to assess and quantify. This is particularly due to the absence of a specific enzymatic system that is involved in HS reduction. Therefore, it is not possible to quantify HS reduction in environmental systems via analysis of the expression and activity of functional genes. This is one of the reasons why we cannot easily evaluate which microorganisms are reducing HS in the environment and to what extent the different physiological groups contribute to HS reduction.

Furthermore, it is still unclear whether or not HS concentrations in the environment are really sufficient to function as electron shuttles and what are the contributions of HS reduction to the overall electron flow in the systems. This is especially the case since most of the studies on HS electron shuttling are conducted in batch cultures with single microbial strains and synthetic Fe(III) minerals, while in environmental systems consortia of different microbial strains are present, Fe(III) minerals are also of biogenic origin and the largest fraction of HS present is particulate. It is still unclear to what extent solid-phase HS contribute to electron transfer, especially over long distances (several cm).

These are two of the main topics on which future research should concentrate and which could help us to better understand the importance of HS electron shuttling in environmental systems.

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Metal Reducers and Reduction Targets. A Short Survey About the Distribution of Dissimilatory Metal Reducers and the Multitude of Terminal Electron Acceptors

Gunnar Sturm, Kerstin Dolch, Katrin Richter, Micha Rautenberg and Johannes Gescher

Abstract The longer we study the processes of microbial metal reduction the more we see the diversity within the processes. There are model organisms that are widely used to study the biochemistry of microbial metal respiration, but these strains are only the tip of the iceberg in terms of phylogenetic diversity. Dissimilatory metal reducers are highly abundant and widespread within the tree of life. Interestingly, the phylogenetic diversity seems to be mirrored by a biochemical diversity, which we are just beginning to assess. Diversity can also be seen in the terminal electron acceptors that can be used. Certainly, iron and manganese are the most influential environmental metallic electron acceptors and were therefore covered in the first chapter of this book. Nevertheless, other metals can serve as respiratory electron acceptors as well. These metals are mostly toxic and their reduction might not only be of respiratory purpose but also a detoxification process. Interestingly, in the past years, reduction of these alternative metallic electron acceptors became more and more an applied process for bioremediation, metal enrichment, and catalyst production. Therefore, this chapter deals with diversity and highlights phylogenetic diversity as well as the diversity within the usable metallic electron acceptors.

G. Sturm · K. Dolch · K. Richter · MichaRautenberg · J. Gescher (✉)
Institute for Applied Bioscience, Karlsruhe Institute of Technology,
Fritz-Haber Weg 2, 76131 Karlsruhe, Germany
e-mail: johannes.gescher@kit.edu

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1 Phylogenetic Diversity of Metal Respiring Bacteria and Archaea

New discoveries are always seen as rare or even bizarre exemptions from the rule in the beginning. Hence, bacterial isolates other than organisms belonging to the genera *Shewanella* or *Geobacter* were initially not screened for their potential ability to respire on metals as terminal electron acceptors. It was the continuous effort of leading groups in this field that gave this respiratory capability the attention that it deserves. Now, ferric iron is one of the routinely tested electron acceptors when novel strains are characterized, and consequently, the number of organisms that were discovered having this ability rises steadily. In the following paragraphs we will not be able to give a complete list of the known metal respiring organisms but will provide an extensive survey of our knowledge of the phylogenetic diversity of these microbes with a focus on ferric iron reducers.

1.1 Neutrophilic Bacteria

Proteobacteria seem to represent the majority of neutrophilic dissimilatory metal reducing bacteria. Here, the δ-subdivision contains the largest number of isolated and characterized organisms that are capable of extracellular metal respiration. Members of the genera *Geobacter*, *Desulfuromonas*, *Pelobacter*, and *Desulfuromusa* form a monophyletic group called Geobacteraceae and share the ability to reduce different metals and minerals.

G. sulfurreducens and *G. metallireducens* are the best characterized strains within the Geobacteraceae (see "Energetic and Molecular Constraints on the Mechanism of Environmental Fe(III) Reduction by *Geobacter*"). Although being closely related, the two *Geobacter* species differ quite substantially in some physiological aspects: *G. sulfurreducens* utilizes only two carbon sources under ferric iron reducing conditions (acetate and—after a prolonged lag-phase—lactate) (Summers et al. 2012), while *G. metallireducens* oxidizes almost 20 different carbon compounds with iron as terminal electron acceptor. Besides iron, *G. metallireducens* can use additional metallic electron acceptors like uranium and manganese (Lovley et al. 1993a). *G. sulfurreducens* can also reduce U(VI) (Lloyd et al. 2002), but is unable to respire on Mn(IV) (Caccavo et al. 1994). The genome of both species encodes an astonishingly high number of genes putatively encoding *c*-type cytochromes (see below), but only the genes for the periplasmic cytochromes MacA and PpcA seem to be conserved in both organisms (Aklujkar et al. 2009).

The genus *Geobacter* is closely related to the genus *Desulfuromonas*. *Desulfuromonas* species are well known for their ability to use elemental sulfur as electron acceptor (Biebl and Pfennig 1977). *D. palmitatis* and *D. acetooxidans* can also respire on different metallic electron acceptors. *D. palmitatis* couples the

oxidation of different short and long chain fatty acids to the reduction of various forms of soluble and insoluble iron and manganese. The bacterium contains *c*-type cytochromes that can be oxidized with iron in vitro, which was seen as an indication for their putative involvement in the electron transport chain to extracellular electron acceptors (Coates et al. 1995). *D. acetooxidans* is a marine microorganism and can conserve energy by coupling the oxidation of organic matter to the reduction of iron and manganese. The bacterium also contains cytochromes that were already in early experiments shown to be oxidizable with Fe(III)-citrate and Mn(IV)-oxalate (Pfennig and Biebl 1976; Roden and Lovley 1993). It should be noted though, that the observation of ferric iron being a suitable oxidant for isolated *c*-type cytochromes is not a direct indication that these proteins are involved in ferric iron reduction or that these organisms even use *Shewanella*- or *Geobacter*-like biochemical strategies to enable electron transfer to a mineral surface. A strong argument instead would be a localization of these cytochromes to the cell surface.

Although there is a close phylogenetic relationship between members of the genera *Geobacter* and *Desulfuromonas* and species of *Pelobacter*, the latter genus is physiologically outstanding within the Geobacteraceae. *Pelobacter* species have been considered to be completely fermentative and members are specialized to use a small range of rather rare substrates like butanediol, (poly)ethylene glycol, or gallic acid (Lovley et al. 1995). The close phylogenetic relationship to metal reducing genera leads to an investigation of metal reduction in *P. carbinolicus*, the closest relative to *D. acetoxidans*. Indeed, it could be demonstrated that *P. carbinolicus* can use ferric iron as terminal electron acceptor (Lovley et al. 1995). Interestingly, the first experimental attempts revealed no evidence of the presence of *c*-type cytochromes in ferric iron grown cells of this organism. Later genome analysis showed 14 genes putatively encoding for these proteins (Haveman et al. 2006). Three of these genes seem to be expressed under iron reducing conditions (Haveman et al. 2006). Nevertheless, there is no indication for outer membrane cytochromes and, compared to *Geobacter* and *Shewanella* species, the number of heme groups within the cytochromes is with five or less rather low (Haveman et al. 2006). These differences could be one reason for the surprising finding that *P. carbinolicus* does not produce current in a microbial fuel cell, which was thought to be a general feature of neutrophilic dissimilatory iron reducers (Richter et al. 2007).

Anaeromyxobacter dehalogenans belongs to the order Myxococcales (δ-proteobacteria) and strain 2CP-C has a high respiratory versatility (Wu et al. 2006). It is able to reduce soluble and amorphous forms of ferric iron and other metal species such as manganese or uranium as well as *ortho*-substituted halophenols, AQDS (anthraquinone 2,6 disulfonate, a humic acid analogue), oxygen, nitrate, nitrite, or fumarate (He and Sanford 2003). Interestingly, the genome of *A. dehalogenans* contains a high number of genes predicted to encode for *c*-type cytochromes (68 genes), including several putative multiheme cytochromes. The deduced amino acid sequence of one gene includes sequence information for 40 heme-binding motifs (Thomas et al. 2008). A high number of

cytochromes could be a reason for the respiratory versatility of *A. dehalogenans* in analogy to *Shewanella* species. Nevertheless, the mechanism of metal reduction is not known for *A. dehalogenans*. The mentioned versatility could lead to an application though, since reduction of water-soluble hexavalent uranium to insoluble uraninite makes *A. dehalogenans* an interesting candidate for remediation of subsurface uranium contaminations (Sanford et al. 2007).

Among the δ-proteobacteria are also several sulfate-reducing bacteria such as *Desulfovibrio desulfuricans*, *Desulfovibrio profundus*, and *Desulfobulbus propionicus*, which can also use metals as electron acceptors (Colemann et al. 1993; Bale et al. 1997; Holmes et al. 2004). The ability of reducing both sulfate and iron could be an advantage for these microorganisms at the interface of sulfate and Fe(III) reduction zones in aquatic sediments. For *D. propionicus* reduction of AQDS and current production at an anode surface was shown as well, but biochemical experiments that would point toward a potential mechanism for extracellular respiration are still due (Holmes et al. 2004).

The phylum of the γ-proteobacteria is also rich in metal respiring organisms. Besides the well-known genus *Shewanella* (see "The Biochemistry of Dissimilatory Ferric Iron and Manganese Reduction in *Shewanella Oneidensis*"), members of the genera *Aeromonas*, *Pantoea*, and *Ferrimonas* can respire on extracellular electron acceptors as well. *Ferrimonas balearica* is a marine microorganism and can utilize soluble and insoluble forms of iron and manganese (Nolan et al. 2010). *Pantoea agglomerans* is a member of the *Enterobacteriaceae* and can couple the oxidation of acetate and hydrogen to the reduction of iron, manganese, or AQDS. *P. agglomerans* is also able to transfer electrons to chromium. In contrast to iron and manganese, Cr(VI) is a soluble and highly toxic metal species that becomes insoluble and less toxic in the reduced Cr(III) state (see below). Only a couple of organisms are known that show growth supporting respiratory chromate reducing activity. *P. agglomerans*-catalyzed chromate respiration was shown to operate with hydrogen, acetate, and lactate as electron donors (Francis et al. 2000).

Aeromonas hydrophila was isolated from a microbial fuel cell and consequently also reduces ferric iron. Interestingly, anode reduction catalyzed by *A. hydrophila* is detectable only under anaerobic conditions and with Fe(III) in the medium (Pham et al. 2003). This is in contrast to *S. oneidensis*, which respires on anodes independent of the presence of Fe(III) (Kim et al. 1999), indicating that these two organisms, although being closely related, might have developed different regulatory mechanisms that enable or disable expression of proteins necessary for iron reduction (Pham et al. 2003).

Ferribacterium limneticum and *Rhodoferax ferrireducens* are two metal reducing β-proteobacteria. *F. limneticus* represents the first characterized iron reducer from the β-subclass and was isolated from mine wastewater. It reduces ferric iron but not manganese(IV) or arsenate (Cummings et al. 1999). *R. ferrireducens* is a psychrotolerant organism and can use iron and manganese as electron acceptors. *R. ferrireducens* is the first described dissimilatory metal reducer in the genus *Rhodoferax* and is, unlike other *Rhodoferax* species, an obligate chemotroph. Of note, *R. ferrireducens* can couple the complete oxidation

of sugars such as glucose or fructose to ferric iron or anode reduction (Chaudhuri and Lovley 2003). This is remarkable, since most dissimilatory metal reducers are typically limited to organic acids such as acetate or lactate as carbon and electron sources.

Rhodobacter capsulatus has to be mentioned as a member of the α-subclass of the proteobacteria. This photosynthetic bacterium can use various terminal electron acceptors including different iron species. Iron reductase activity seems to be localized to the membrane fraction. Cytochromes play a role in the respiratory process, since a mutant lacking cytochrome *c*2 and cytochrome *bc*1 complex reduces Fe(III) with lower rates (Dobbin et al. 1996).

Apart from the multitude of metal reducing bacteria among the proteobacteria, there are a number of other, phylogenetically widespread, organisms that also have the ability to respire on metals. *Geothrix fermentans* belongs to the Acidobacteria. It is a Gram-negative strictly anaerobic organism and can reduce iron, manganese, and AQDS (Coates et al. 1999). *G. fermentans* is able to reduce iron species even when they are incorporated into microporous alginate beads, which precludes direct contact to the electron acceptor. This indicates that *G. fermentans* releases an electron shuttling compound (see "On the Role of Endogenous Electron Shuttles in Extracellular Electron Transfer"). The electron shuttle is also produced when an anode is introduced to the medium as sole electron acceptor. The compound is similar to water-soluble quinones and does not stimulate the reduction rates of *G. metallireducens*. As an alternative strategy to reach inaccessible iron, *G. fermentans* can also solubilize Fe(III) during exponential growth. Both the shuttling and the chelating compounds have not been identified yet (Coates et al. 1999; Nevin and Lovley 2002; Bond and Lovley 2005).

Geovibrio ferrireducens was isolated from the sediment of a hydrocarbon-contaminated ditch and can reduce ferric iron among other electron donors with proline as carbon and electron source. *G. ferrireducens* is phylogenetically distinct from all other iron reducers and belongs to the Deferribacteraceae. The spectrum from 400 to 600 nm reveals typical maxima for *c*-type cytochromes (Caccavo et al. 1996).

All organisms described so far are Gram-negative bacteria. Nevertheless, dissimilatory metal reducers were also detected in the group of Gram-positives. Until now, these organisms were investigated only to a minor extent. *Thermincola potens*, a member of the Peptococcaceae family in the phylum Firmicutes, was isolated from a thermophilic microbial fuel cell and can transfer electrons to an electrode as well as to insoluble ferric iron species or AQDS. *T. potens* does not seem to produce a shuttling component. Instead, electron transfer via direct contact was suggested (Wrighton et al. 2011). Thirty-two genes putatively encoding mulitheme *c*-type cytochromes were identified in the genome of the organism. The deduced amino acid sequences suggest an average number of 11.9 hemes per *c*-type cytochrome protein (Wrighton et al. 2008, 2011; Byrne-Bailey et al. 2010). Several of these multiheme cytochromes are expressed under iron- or AQDS-reducing conditions and some of them are located in the cell wall or toward the cell surface in a manner that provides evidence for an electron transfer mechanism

similar to Gram-negative metal reducers (Carlson et al. 2012). *Thermoanaerobacter acetoethylicus* is another thermophilic member of the Firmicutes. This organism shows growth around 60 °C and reduces amorphous ferric iron with hydrogen or peptone as electron donor (Slobodkin et al. 1999).

Bacillus strain SG1, certainly also belonging to the Firmicutes, was isolated from a manganese-rich environment and is capable of direct manganese reduction. The manganese reductase seems to be located in the membrane, since spheroblasts reduced manganese oxide at the same rate as untreated cells. *C*-type cytochromes were detected spectrophotometrically. Manganese reduction is inhibited by the addition of antimycin or HQNO indicating that the reduction indeed is a respiratory process and not based on facilitated fermentation (de Vrind et al. 1986). Another *Bacillus* strain, *B. infernus* is also capable of metal respiration. This strictly anaerobic, thermophilic organism was isolated from the deep subsurface and couples the reduction of manganese to the oxidation of lactate or formate (Boone et al. 1995a). In a comparative experiment, reduction of crystalline magnetite was measured in *G. metallireducens* and *B. infernus* cultures and no difference in reduction rate between both organisms could be detected. The reduction was very fast at the beginning of the experiment but stopped rapidly, probably due to the precipitation of vivianite on the magnetite surface and a resulting inaccessibility to the bacteria (Crespo et al. 2007).

Another Gram-positive species known for metal respiration within the Firmicutes belongs to the spore-forming Gram-positive sulfate-reducing bacteria. *Desulfotomaculum reducens* was isolated from heavy metal polluted soil and can reduce Fe(III), Mn(IV), U(VI), and Cr(VI). Butyrate, lactate, or valerate oxidation to acetate either with these metals or sulfate leads to almost identical growth rates. Nothing is known about the mechanism of metal reduction in *D. reducens* yet, but again cytochromes are present in the cells (Tebo and Obraztsova 1998). Spores of *D. reducens* can also reduce U(VI) with H_2 as electron donor and here, a shuttling component seems to be involved in the reduction process, since cell-free, spent medium from a fermentative culture is required and no immediate U(VI) reduction can be detected if spores are transferred into fresh medium (Junier et al. 2009).

The next group of ferric iron reducers was used to provide evidence for the hypothesis that the development of a respiratory chain to ferric iron could be a very early microbial invention on our planet. Respiratory energy generation is believed to start at a point in the Earth‘s history at which due to reducing conditions the availability of potential electron acceptors was limiting. Ferric iron was most probably available at that point because of the photochemical oxidation of ferrous iron released from hydrothermal vents. Biological evidence for catabolic iron reduction as an early form of respiration was derived from the distribution of dissimilatory metal reducers within the phylogenetic tree of life. Many archaeal and bacterial species that are most closely related to the putative last common ancestor were observed to reduce Fe(III) to Fe(II) (Vargas et al. 1998). Hence, if the phylogenetic tree that leads to this last common ancestor that is located between the bacteria and archaea is correct, then ferric iron could indeed be considered as competitor to sulfur for the title of being the first terminal electron

acceptor used. Organisms belonging to the Thermotogales are such deeply branching thermophilic microorganisms. It was shown that *Thermotoga subterranea* as well as *Thermotoga maritima* are capable of reducing amorphous ferric oxide with hydrogen or peptone as electron acceptor and that this reduction process is growth supporting (Slobodkin et al. 1999). Interestingly, *Petrotoga* sp. SL27, also a member of the Thermotogales, cannot respire on ferric iron under identical conditions. This could indicate that this organism might have lost its ferric iron reducing capabilities as a secondary effect. It could also mean that the assumption of ferric iron respiration, being a general capability of organisms phylogenetically located close to the branching point between archaea and bacteria, should be treated carefully.

The last phylum that will be mentioned in this section are the Thermodesulfobacteria. Members of this phylum were thought to be thermophilic sulfate reducers. However, using samples from the Obsidian Pool area in Yellowstone National Park, Kashefi and colleagues were able to isolate *Geothermobacterium ferridreducens* (Kashefi et al. 2002a). This organism grows chemolithoautotrophically using hydrogen as electron donor and ferric iron as electron acceptor. Interestingly, it is not able to use sulfur species as electron acceptors. Thereafter, other members of the Thermodesulfobacteria were also screened for their ability to respire on ferric iron and *T. commune* could also reduce ferric iron with hydrogen but not with lactate as electron donor, which it could use with sulfate as electron acceptor (Kashefi et al. 2002a). What can be learned from this study is that ferric iron reduction might be far more abundant than believed and that environmental sequence data has to be treated carefully, since phylogenetic similarities do not necessarily mean physiological resemblance.

1.2 Acidophilic Bacteria

Acidic environments demand adaptations in terms of pH homoeostasis and they also influence biochemical strategies for dissimilatory metal reduction. This is foremost due to the altered solubility and redox potential of certain metals. For instance, a shift from pH 7 to 2.5 alters the redox potential of the Fe(III)/Fe(II) pair from values of around −100 to +100 mV for insoluble ferric iron at neutral pH to +770 mV for soluble Fe(III)/Fe(II) (see "Minerals and Aqueous Species of Iron and Manganese as Reactants and Products of Microbial Metal Respiration", Straub et al. 2001). Solubility and redox potential convert ferric iron into an attractive terminal electron acceptor that is frequently produced in large quantities by the pyrite oxidizing activity of *Leptospirillum* and *Acidithiobacillus* strains. Of note, reduction of soluble ferric iron is an acid generating process (Eq. 1) and organisms that respire on soluble iron therefore have to cope with a further decrease in the surrounding pH.

$$24\,Fe^{3+} + C_6H_{12}O_6 + 6H_2O \;->\; 24\,Fe^{2+} + 6\,CO_2 + 24H^+ \qquad (1)$$

In contrast, reduction of minerals such as schwertmannite could lead to a net alkalinization of the microbial microenvironment (2) (Coupland and Johnson 2008)

$$3Fe_8O_8(OH)_6(SO_4) + C_6H_{12}O_6 + 6H_2O \rightarrow 24Fe^{2+} + 6CO_2 + 3SO_4^{2-} + 42OH^- \quad (2)$$

Neutrophilic dissimilatory metal respiring microbes reduce ferric iron only under anaerobic conditions. Acidophiles are able to reduce ferric iron and oxygen often simultaneously, which might be due to very similar redox potentials of the O_2/H_2O and Fe(III)/Fe(II) redox couples and the oxygen-based autoxidation of a number of *c*-type cytochromes. Another difference is the substrate spectrum of acidophilic versus neutrophilic iron reducers. Neutrophiles often use fermentation end products such as lactate, acetate, or propionate, whereas acidophiles face the problem that these compounds have pK_a values between 3 and 5. Consequently, these organic acids enter the cell fully protonated and deprotonate under the neutrophilic intracellular conditions, which leads to a severe acidification of the cytoplasm. As a consequence of the so-called *acid trap* described above, fermentative acidophiles have not been identified yet (Slonczewski et al. 2009).

C-type cytochromes seem to be the key components of electron transport chains to ferric iron in many of the known neutrophiles. Several *c*-type cytochromes have been found in acidophilic dissimilatory metal reducers as well. Interestingly, a comparison with neutrophilic dissimilatory metal reducers such as *Shewanella* or *Geobacter* strains reveals that a difference can be found within the number of heme cofactors per protein. While neutrophiles often express multiheme proteins, acidophiles seem to have a strong bias toward mono- and dihemes. The advantage of dihemes and monohemes under acidic conditions might be the compact structure and therefore the enhanced stability (Giudici-Orticoni et al. 2000).

In comparison with neutrophils, acidophilic metal reducers are usually grown in media containing up to 0.02 % yeast extract, but it is not yet clear why this undefined component is added or whether it has to be added at all to each of the isolates. Since yeast extract could certainly contain potential electron shuttles (e.g. riboflavin) further work is required to define what purpose this media supplement fulfills for these organisms. So far, bacterial acidophilic dissimilatory metal reducers have been spotted in the Proteobacteria, Firmicutes, and Actinobacteria.

Acidiphilium cryptum is the best studied acidophilic dissimilatory metal reducer and belongs to the α-proteobacteria. It was first isolated from a supposedly pure culture of *Acidithiobacillus ferrooxidans* (Harrison 1981) and thereafter several others members of *Acidiphilium* spp. were found and isolated from mine rivers and acidic lakes (Johnson and Hallberg 2009). *A. cryptum* couples the oxidation of glucose to CO_2 to the reduction of ferric iron and can grow from pH 1.9 to 5.9 (Harrison 1981). This is possible under oxic as well as under anoxic conditions (Küsel et al. 1999). While the proteins for iron reduction are inducible in *A. cryptum*, *A. acidophilum*, and *A. rubrum*, they seem to be constitutively expressed in *A. organovorum*, *A. multivorum* and *A.* SJH (Küsel et al. 2002;

Johnson and Bridge 2002). All *Acidiphilium* species have in common that they can reduce ferric iron; however, they might have developed different respiratory pathways. A number of *c*-type cytochromes were spotted in *A. cryptum*, but only a few of them were characterized and the role of these proteins is mostly not understood. The periplasmatic *c*-type cytochrome (ApcA) from *A. cryptum* was isolated and studied in more detail. ApcA is a 10.1 kDa monoheme closely related to the mitochondrial cytochrome *c* Mcc (Khare et al. 2006). ApcA was found to be an in vitro chromate reductase when reduced with menadiol (Magnuson et al. 2010).

The α-proteobacterium *Acidocella facilis* (formerly called *Acidiphilium facilis*) is dependent on pH-values higher than 2.3 to be able to reduce iron in the form of schwertmannite (Coupland and Johnson 2008). *A. facilis* is not able to reduce soluble iron, probably due to the pH shift accompanied with this process (see Eq. 1) (Johnson and McGinness 1991; Coupland and Johnson 2008). *Acidicaldus organivorans* belongs to the α-proteobacteria as well. It is a heterotrophic bacterium, which can either grow aerobically with oxygen or anaerobically with ferric iron as terminal electron acceptor. The organism grows between 40 and 65 °C and pH 1.75–3.0 (Johnson et al. 2006).

A. ferrooxidans belongs to the γ-proteobacteria and was formerly called *Thiobacillus ferrooxidans*. *At. ferrooxidans* is well known for ferrous iron oxidation and autotrophic growth. Under oxygen limited condition *At. ferrooxidans* is able to oxidize sulfur and reduce ferric iron (Pronk et al. 1992).

Sulfobacillus spp. are members of the firmicutes and known iron oxidizers. Under oxygen limited condition *S. thermosulfidooxidans* and *S. acidophilus* are able to reduce ferric to ferrous iron (Bridge and Johnson 1998). Strain *S. benefaciens* is furthermore capable of growing autotrophically (Johnson et al. 2008). Actinobacteria are also able to oxidize and reduce iron. *Acidimicrobium ferrooxidans* and *Ferrimicrobium acidiphilium* are mesophilic examples while *Ferrithrix thermotolerans* is a thermophile (Bridge and Johnson 1998; Johnson et al. 2009).

1.3 Alkaliphilic Bacteria

Recently, there have been a couple of studies on alkaliphilic dissimilatory metal reducers as they are part of the natural community in soda lakes and soda deserts of naturally occurring alkaline environments and artificial environments like borate leachate ponds (Ye et al. 2004). From these environments a few microorganisms were isolated which were able to reduce metals such as ferric iron (Boone et al. 1995b), chromium, and cobalt (Ye et al. 2004) and metalloids such as arsenic and selenium (Switzer Blum et al. 1998; Gorlenko et al. 2004). However, all of them were grown with up to 0.25 % LB or 0.02 % yeast extract, respectively. Therefore, it is not clear whether ferric iron is reduced directly or by a potential redox shuttle in the LB or yeast extract supplement. Metal reduction is also not

necessarily a respiratory mechanism but a way to facilitate fermentation. *Bacillus* sp. SFB uses 12.5 % of the reducing equivalents for the reduction of ferric iron and thereby stimulates fermentative growth (Pollock et al. 2007).

Bacillus subterraneus can grow fermentatively or with iron and manganese as electron acceptors at pH values ranging from 7.0 to 9.0 (Kanso et al. 2002). *Anaerobranca californiensis*, *A. horikoshi*, and *A. gottschalkii* also belong to the firmicutes and reduce Fe(III) and Se(IV) under anaerobic and thermophilic conditions with an optimal pH > 8.5 (Gorlenko et al. 2004).

Alkaliphilus metalliredigens was isolated from leachate ponds at the U.S. Borax Company in Boron, Calif. and can grow anaerobically at a pH range from 7.5 to 11.0. This is the highest pH value under which iron reduction is reported to be possible. Fe(III)-citrate, Fe(III)-EDTA, Co(III), Cr(VI), and AQDS support growth as electron acceptors and yeast extract or lactate as electron donors. No aerobic or fermentative growth was observed with glucose, starch, or sucrose (Ye et al. 2004).

1.4 Archaea

The ability to reduce iron is a common characteristic especially among hyperthermophilic archaea (Vargas et al. 1998). However, the biochemical mechanisms of archaeal ferric iron reduction are studied only to a minor extent and this will certainly be a fascinating research field in the following years.

The first archaeal iron reducer reported was *Sulfolobus acidocaldarius*, which reduces (soluble) ferric iron with elemental sulfur (Brock and Gustafson 1976). Since then, several other iron reducing archaea were identified.

Pyrobaculum islandicum is the archaeal model organism for Fe(III) reduction. It is a neutrophilic hyperthermophilic organism belonging to the crenarchaeota, which was isolated from hot sulfataric and geothermal waters in Iceland (Huber et al. 1987). The organism couples the oxidation of hydrogen to the reduction of insoluble Fe(III)oxides as well as to soluble ferric iron forms (Vargas et al. 1998). Besides iron, *P. islandicum* can also reduce metals such as Mn(IV), U(VI), Cr(VI), and Co(III) (Kashefi and Lovley 2000). Similar to many mesophilic bacteria, extracellular quinone shuttles can stimulate reduction of solid iron forms in *P. islandicum*. Even low concentrations of 50 μM AQDS have a great impact on the reduction rates of poorly crystalline Fe(III), hematite, and goethite (Lovley et al. 2000). However, Holden et al. demonstrated that *P. islandicum* does not produce an endogenous electron shuttle and is dependent on direct contact to the electron acceptor iron. *C*-type cytochromes do not seem to play a role in iron reduction (Feinberg and Holden 2006; Feinberg et al. 2008). Although genome analysis revealed evidence for three putative *c*-type cytochromes, none of these was detectable under iron reducing conditions (Feinberg and Holden 2006). *Pyrobaculum aerophilum* is closely related to *P. islandicum* but differs considerably in the mechanism of iron reduction. Unlike *P. islandicum, P. aerophilum* can

grow when separated from the electron acceptor by a barrier and no Fe(III) or Fe(II) could be detected outside the dialysis tubing (Feinberg et al. 2008). This indicates the production of an extracellular shuttle and not a chelator for iron reduction in *P. aerophilum*. Moreover, *P. aerophilum* contains 13 putative monoheme *c*-type cytochromes and two putative diheme cytochromes. However, under iron reducing conditions, only one cytochrome was detectable in the membrane fraction (Feinberg et al. 2008; Childers and Lovley 2001).

Members of the crenarchaeota were also described to reduce ferric iron under acidic condition. *Acidianus manzaensis* grows autotrophically either with H_2 or S^0 as electron donor and iron or oxygen as terminal electron acceptor. The optimal temperature is 80 °C and the optimal pH is between 1.2 and 1.5 (Yoshida et al. 2006).

Archaeoglobus fulgidus is an anaerobic, sulfate-reducing organism belonging to the euryarchaeota that was first isolated from marine thermal vents. Vadas et al. (1999) could purify a ferric reductase from the soluble protein fraction that is strictly dependent on FMN or FAD and can reduce complexed iron species such as ferric-EDTA, sodium ferricyanide, ferric-citrate, or ferric-NTA. The authors speculated that this enzyme could potentially act as a dissimilatory ferric iron reductase (Fig. 1). Nevertheless, it was not possible so far to grow this organism with ferric iron as electron acceptor, although cell suspension assays revealed a hydrogen-dependent ferric iron reduction (Vargas et al. 1998; Vadas et al. 1999).

Ferric iron reduction was also shown for methanogens such as *Methanosarcina barkeri*, and *Methanococcus voltaei*. *M. barkeri* is able to reduce structural Fe(III) in goethite minerals. This capability is enhanced in the presence of AQDS (Bond and Lovley, 2002). The organism can also reduce iron in clay minerals like nontronite with H_2/CO_2 or methanol as electron donor but not with acetate (Liu et al. 2011). *M. voltaei* is able to use Fe(III) oxides as electron acceptor in the presence of hydrogen as electron donor. Addition of iron generally inhibits methanogenesis from H_2/CO_2, which is understandable since electrons needed for CO_2 reduction are transferred onto ferric iron (Bond and Lovley 2002). It is not yet clear what kind of mechanism is used for ferric iron reduction by methanogens and whether it really is a respiratory mechanism or rather an unavoidable loss of electrons in the presence of ferric iron or humic acids.

Obligate ferric iron reducers were spotted in the Geoglobales belonging to the euryarchaeota. These organisms are thermophiles and known species were isolated from deep-sea hydrothermal vents. *Geoglobus ahangari* and *Geobglobus acetivorans* are very versatile (Kashefi et al. 2002b, 2008; Slobodkina et al. 2009). They can grow with a number of organic carbon sources organoheterotrophically, while they are also able to grow with hydrogen and CO_2 as lithoautotrophs. Interestingly, both organisms are unable to use AQDS as electron acceptor. Hence, the process of ferric iron reduction seems to be more specific than neutrophilic iron reduction by *Shewanella* or *Geobacter* strains.

Ferroplasma acidarmanus is mesophilic organism with a pH optimum between pH of 1.0–1.7 (Dopson et al. 2004, 2007) and belongs also to the euryarchaeota. It is able to couple the oxidation of organic carbon to the reduction of ferric iron.

Yeast extract, glucose, glycerol, or acetate is used as electron donor. Yeast extract is always necessary as a medium supplement. However, $Fe_2(SO_4)_3$ is also necessary as electron acceptor and therefore yeast extract is not just a substrate for fermentative growth.

Reysenbach and colleagues made a very interesting observation while following strain isolation. They recognized that a euryarchaeal lineage called DHEV2 (deep-sea hydrothermal ven euroarchaeotic 2) is widely distributed at hydrothermal vent sites (Reysenbach et al. 2006). Organisms belonging to this phylum *represented* up to 15 % of the archaeal population. The authors were able to isolate one strain named '*Aciduliprofundum boonei*' that grows heterotrophically with either sulfur or ferric iron as terminal electron acceptor. The strain is phylogenetically related to organisms belonging to the genus Thermoplasma and *Picrophilus*. Nevertheless, organisms belonging to these genera were so far not reported to be ferric iron reducers.

2 Metal Respiration and *c*-Type Cytochromes

Neutrophilic dissimilatory metal-reducing model organisms of the genera *Shewanella* and *Geobacter* have an astonishingly high number of genes putatively encoding for *c*-type cytochromes. Moreover, the electron transport chain from the cytoplasmic membrane to the cell surface in *Geobacter* and *Shewanella* strains consists mainly of *c*-type cytochromes. Nevertheless, in the previous paragraph a number of organisms were mentioned that do not seem to be dependent on a high *c*-type cytochrome content, while others were not evaluated in terms of cytochrome diversity or concentration. Therefore, we developed a simple bioinformatic method to screen microbial genomes for *c*-type cytochrome encoding genes. In this assay, deduced protein sequences were first screened for sec-transport-dependent export signals using the program signal P. Positive candidates were thereafter searched for CXXCH motifs that characterize heme-binding sites in these proteins. To avoid false positives, the signal P screening was performed rather strictly and only proteins that undoubtedly were predicted to contain an export signal were chosen for further analysis. Hence, the number of hereby detected *c*-type cytochrome encoding genes might be lower than it actually is, since some export signal might just not be as pronounced as others. For instance *S. oneidensis* MR-1 has in our high-throughput screen a predicted number of 38 c-type cytochromes, while a more detailed analysis revealed a number of 41 genes (Romine et al. 2008). Nevertheless, for a simple comparative screen this accuracy is sufficient.

What conclusion can be drawn from this screening of proteobacterial genomes? The average number of *c*-type cytochrome encoding genes per genome determined from all 483 proteobacterial genomes available in July 2011 is 13. This is far below the c-type cytochrome encoding gene content in metal-reducing *Shewanella* and *Geobacter* strains. Interestingly, *S. denitrificans* is a member of the genus

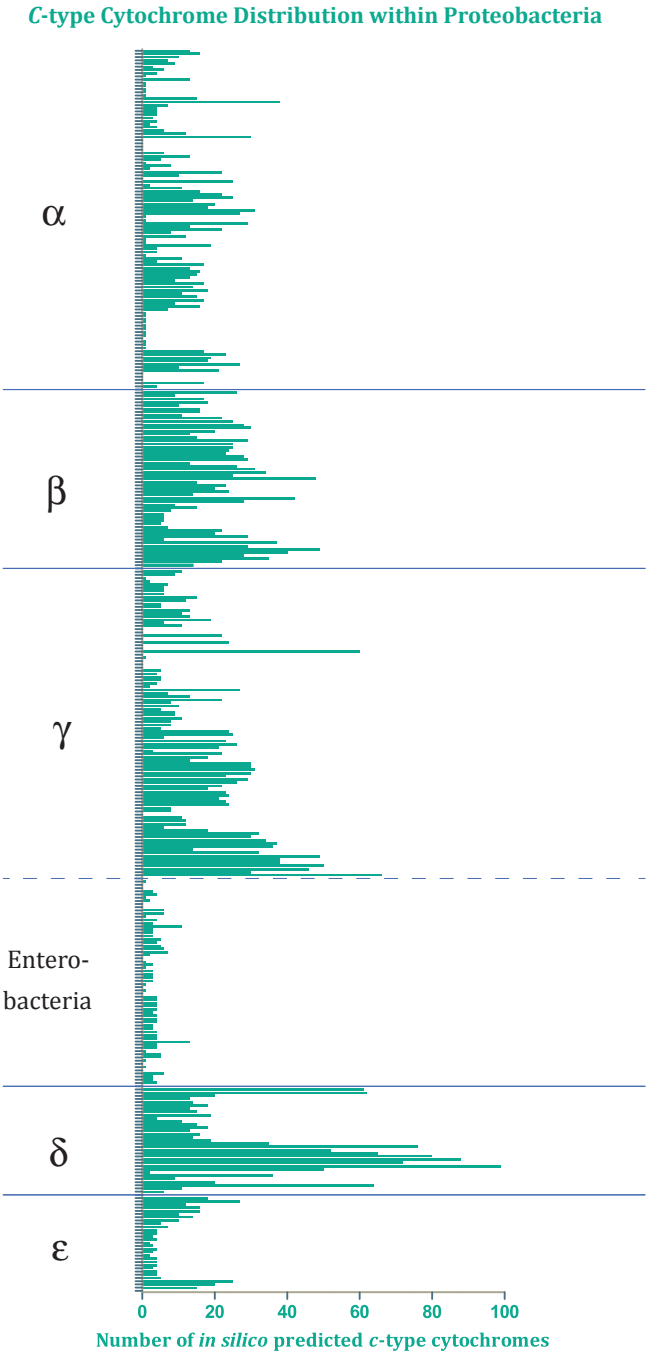


Fig. 1 Bioinformatic analysis of the number of cytochrome encoding genes in available proteobacterial genomes. Organisms that are mentioned in the text as well as known dissimilatory ferric iron reducers are marked in *red*

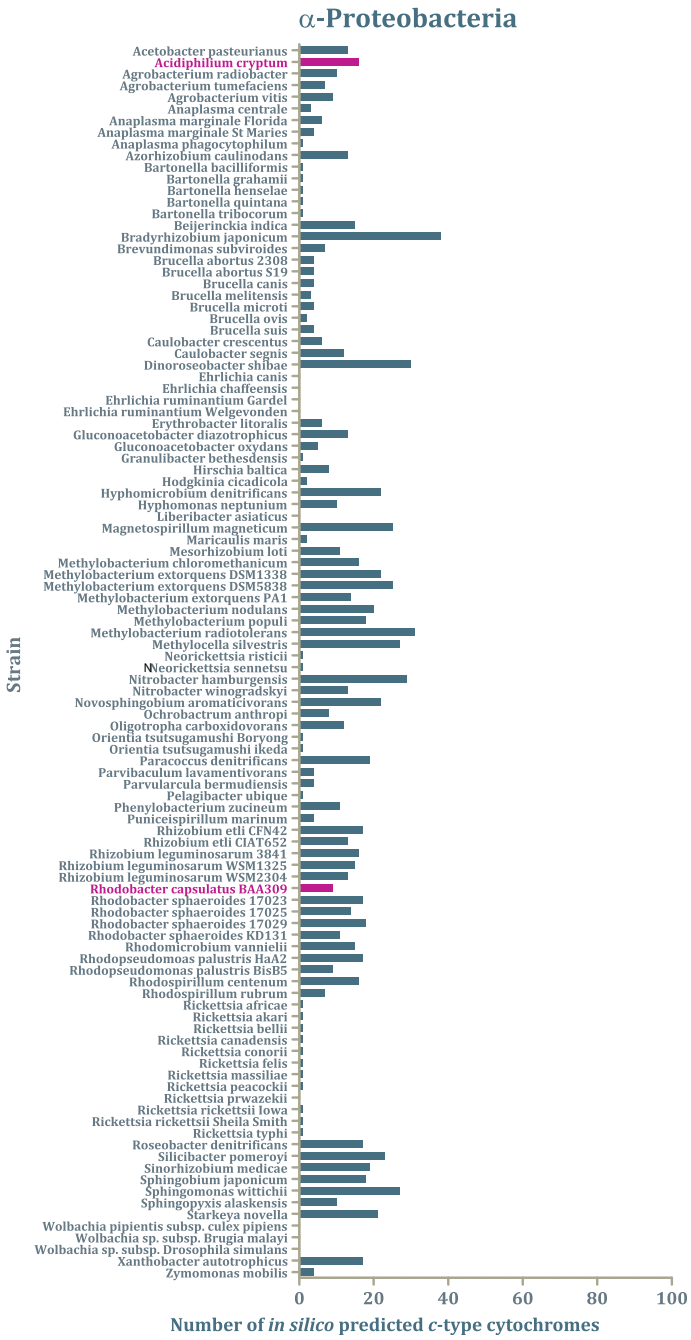
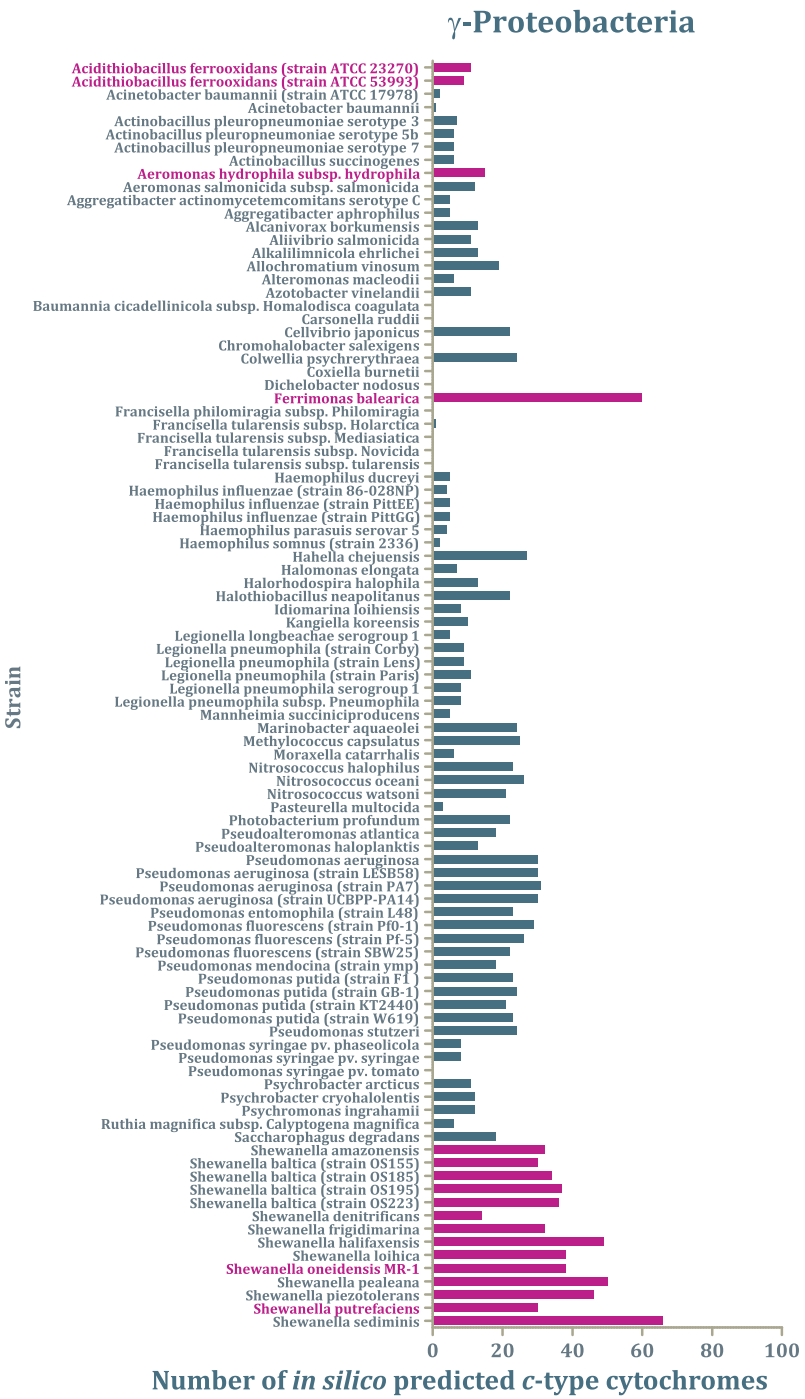


Fig. 1 continued

Fig. 1 continued



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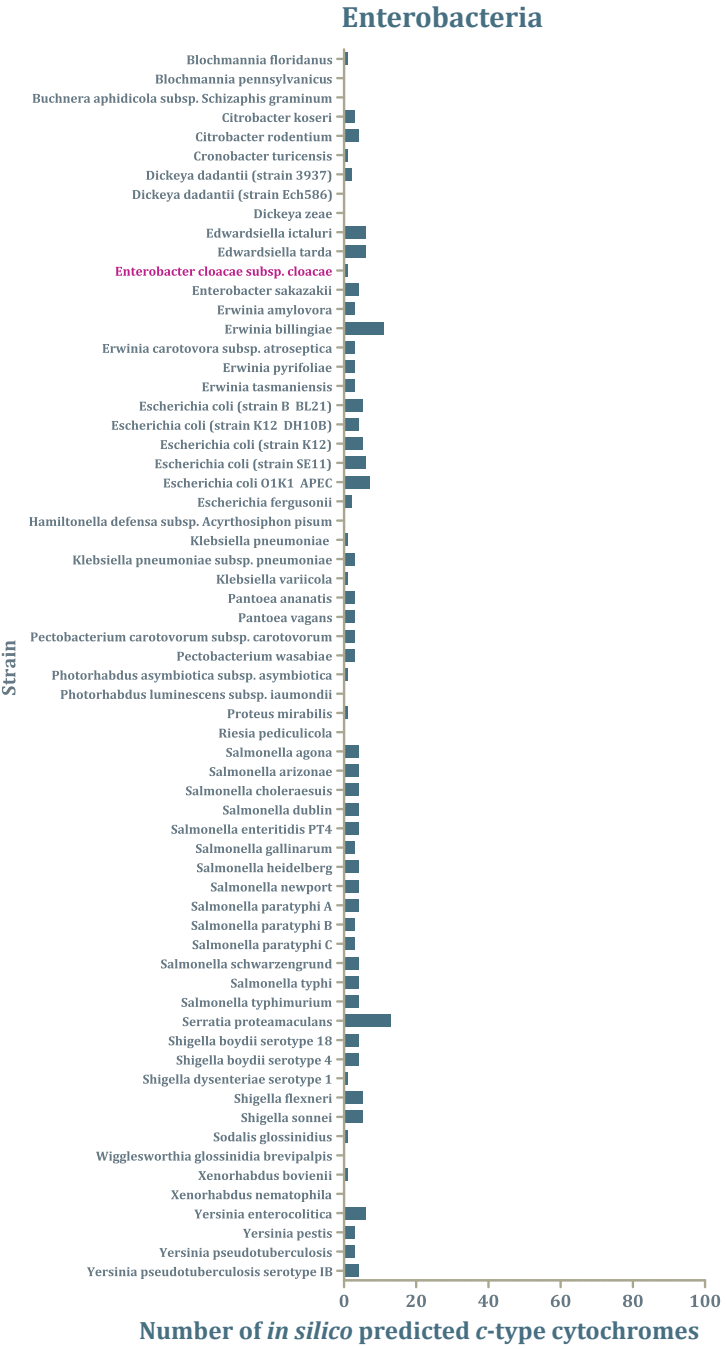

Fig. 1 continued

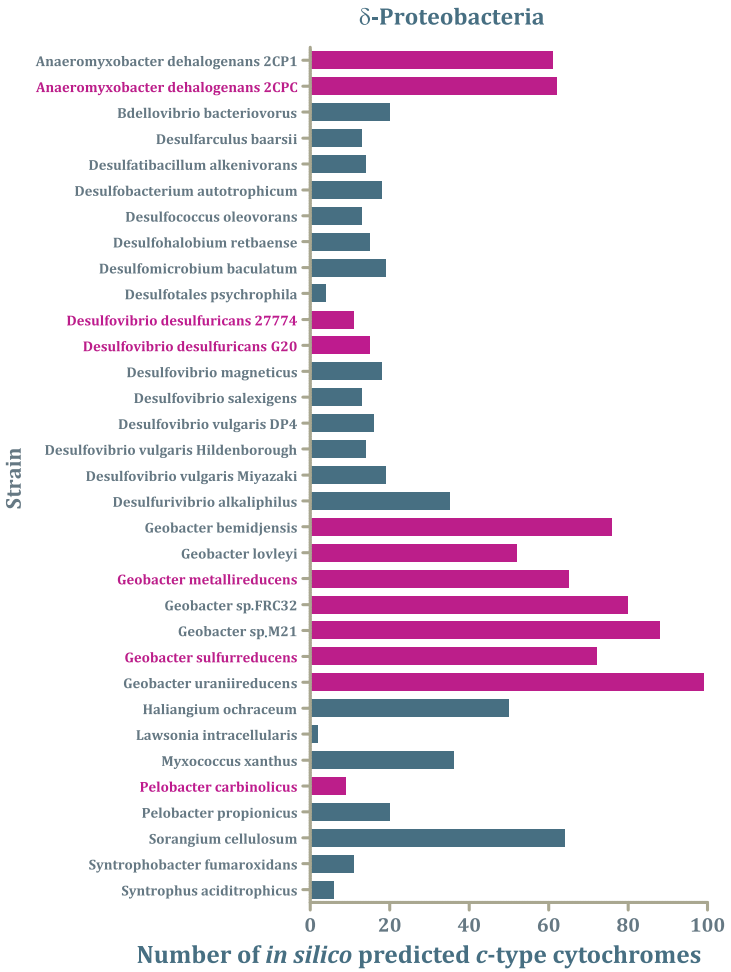

Fig. 1 continued

Shewanella that is unable to reduce extracellular electron acceptors. This organisms shows, maybe consequently, only 14 putative *c*-type cytochrome encoding genes.

So is there a strong correlation between the number of *c*-type cytochromes and metal respiration? It does not seem to be that easy. Yes, metal reducing *Geobacter*, *Shewanella*, and *Anaeromyxobacter* strains contain way more than the average number of *c*-type cytochrome encoding genes in proteobacteria. Nevertheless, this is also true for a number of other organisms (e.g. *Bradyrhizobium japonicum*) that at least are not known to have the ability of extracellular metal respiration. Therefore, the sheer number is a good indicator to investigate further whether a certain strain might have the ability to respire on insoluble metals but is far from

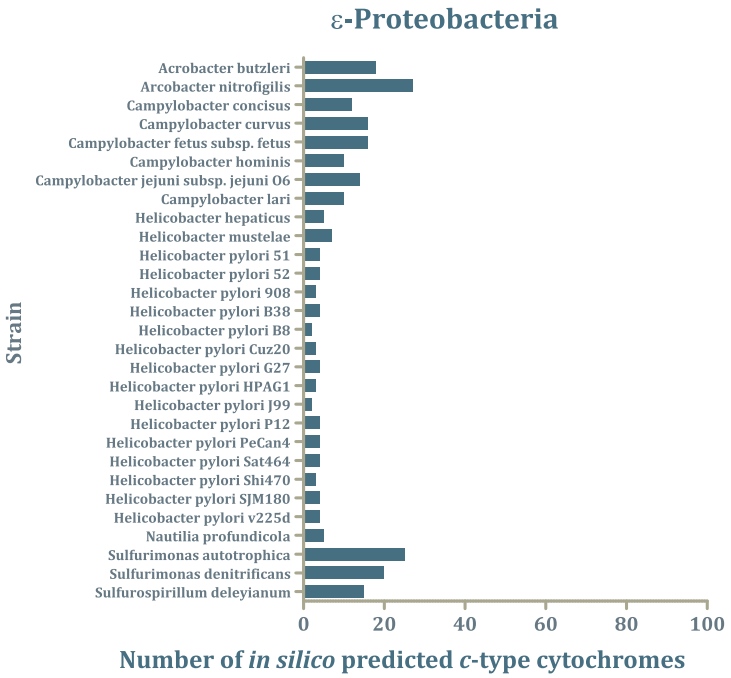

Fig. 1 continued

being sufficient evidence to claim with certainty that this has to be the case. It is furthermore interesting to note that some neutrophilic metal reducers do not have an above-average number of *c*-type cytochrome encoding genes (e.g. *Pelobacter carbinolicus, D. desulfuricans*). It will be interesting to study how these organisms developed a way to reach the insoluble electron acceptor. Last but not least, acidophilic ferric iron reducers such as *Acidithiobacillus* and *Acidiphilium* strains do not seem to be dependent on a multitude of *c*-type cytochromes. Yet, these strains need a different mechanism for iron reduction since their substrate will be mostly soluble and the major challenge rather is to avoid a precipitation process in the periplasm than to develop a respiratory chain to the cell surface.

3 Diversity of Metallic Electron Acceptors

Iron and manganese species were presented in detail in "Minerals and Aqueous Species of Iron and Manganese as Reactants and Products of Microbial Metal Respiration" as targets for metal respiration. Nevertheless, the number of metallic reduction targets is by far larger. A number of these reduction targets are toxic and it has to be distinguished between respiratory reduction, reduction for the purpose of detoxification, or a mixture of both. Although the metals that are presented in

the following section are not as abundant as iron or manganese species, their reduction has greater implications at least in applied sciences. The production of metal nanoparticles as catalysts or the detoxifications of subsurface plumes of toxic oxidized metals like uranium are examples for these applied processes. A more extensive coverage of the concomittant applied processes can be found in Chap. 7 of this book.

3.1 Vanadium

Vanadium is known as a compound used not only in the steel industry but also as a trace element with a potential function as a stimulant for growth and glycolysis. In certain prokaryotes, vanadium has been found in nitrogenase (replacing molybdenum). Interestingly, it is also part of the oxygen-carrying blood pigment in ascidian worms (Lyalkova and Yurkova 1992). At neutral pH it typically occurs either in the form of vanadyl V(IV) or vanadate V(V) ions. Vanadate is structurally similar to phosphate and phosphate transporters are therefore the entry gates for this metal into the cell. In higher concentration V(V) is known to cause oxidative cell damage. Vanadate is highly water soluble, while its reduced form is less soluble and due to its decreased bioavailability and less toxic. Therefore, reduction of vanadium can be a resistance mechanism and also an applied process to reduce the concentration of bioavailable toxic V(V) in the environment. A number of publications also provide evidence that this reduction process can be coupled to respiration. Judging from the redox potential of +0.127 V at pH 7.4 for the V(V)/V(IV) couple, this process could easily be growth supporting. Not surprisingly, known iron reducers like *Geobacter* or *Shewanella* species are capable of respiratory vanadium reduction. In the case of *S. oneidensis* there had been a controversy whether vanadium reduction could be growth supporting. Earlier experiments carried out by Myers et al. (2004) suggested that this is not the case, while later studies by Carpentier et al. (2005) showed that a few growth medium adjustments are necessary to enable respiration. As expected, key players in ferric iron reduction, such as MtrB, MtrC, CymA, and members of the cytochrome maturation machinery, are necessary for the organism to conduct this form of respiration (see "The Biochemistry of Dissimilatory Ferric Iron and Manganese Reduction in *Shewanella Oneidensis*"). *G. metallireducens* was also shown to grow with V(V) as terminal electron acceptor. Ortiz-Bernard et al. (2004) could furthermore demonstrate that *G. metallireducens* catalyzed vanadium reduction diminishes the level of soluble vanadium to values below the human health risk-based concentration of 6 μM. Interestingly, several strains that are not able to respire on Fe(III) are capable of coupling growth to vanadium reduction. Lyalikova reported about two *Pseudomonas* strains (*P. vanadiumreductans*, *P. issachenkovii*) that could grow lithotrophically but also with a number of organic electron sources with vanadium as electron acceptor (Lyalkova and Yurkova 1992; Yurkova and Lyalikova 1990). Recently, an *Enterobacter cloacae*

strain isolated from a gold mine was also demonstrated to have a membrane anchored vanadium reductase (van Marwijk et al. 2009). Of note, the biochemistry of vanadium reduction in these and other strains is not thoroughly studied yet. For *P. issachenkovii* it was suggested that the nitrate reductase of this organism might be the terminal vanadium reductase as well.

3.2 Chromium

Chromium is frequently used in the leather and steel industry. Unfortunately, this frequent use leads to a number of contaminated field sites. The most common oxidation states of chromium are Cr(VI) and Cr(III). Structurally, chromate ions resemble sulfate and are therefore transported into the cell via sulfate uptake mechanisms. Similar to vanadium, Cr(VI) is highly water soluble and toxic, while the reduced form precipitates rapidly and is therefore not or only slightly toxic. Consequently, chromium reduction represents a widespread resistance mechanism. While some strains are able to use Cr(VI) as a terminal electron acceptor in anaerobic respiration (Liu et al. 2002) and hence combine respiration and resistance, (Guha et al. 2001), the majority of the so far known chromium reducing strains do not seem to gain energy from the reduction process which frequently is catalyzed by soluble cytoplasmic, NAD(P)H-dependent proteins. Another reducing mechanism is known from sulfate reducers that produce Cr(III) indirectly via the abiotic reaction with hydrogen sulfite (the end product of sulfate reduction). Nevertheless, Lovley and Philips gained evidence that at least *Desulvovibrio vulgaris* can reduce chromate also enzymatically. P. agglomerans was already mentioned as another example for a chromate respiring organism. For *Shewanella alga* BrY-MT, *S. putrefaciens* CN32, and *S. oneidensis* MR-1, it was shown that these strains are able to reduce Cr(VI) in an anaerobic defined environment where only Cr(VI) was given as electron acceptor (Guha et al. 2001; Liu et al. 2002). A recent study revealed that resting cells of *Shewanella oneidensis* reduce Cr(VI) for the most part by using the same outer membrane cytochromes that also catabolically reduce ferric iron (Belchik et al. 2011). However, TEM/EELS analysis showed that Cr(III) precipitates can be found outside the cell as well as in the cytoplasm of the cells suggesting that the extracellular reduction is not fast enough to prevent all Cr(VI) from entering the cell (Middleton et al. 2003).

3.3 Uranium

Common oxidation states of uranium are +4 and +6. U(VI) usually is a mobile form, while U(IV) is insoluble. A number of microbes were shown to be able to reduce U(VI) to U(IV) and this process was applied to immobilize subsurface uranium contaminations (Lovley et al. 1991; Lovley and Phillips 1992; Cardenas

et al. 2008). Interestingly, there seems to be a microbially controlled formation of U(IV) minerals as Boyanov et al. (2011) could show that some strains seem to form uraninite (UO_2) as reduction product while others transform U(VI) into carbonate-complexed U(IV) species. Lovley et al. suggested that microorganisms capable of Fe(III) reduction are also able to reduce uranium U(VI) (Lovley and Phillips 1992). Moreover, it was proven that these organisms, namely *Geobacter metallireducens* and *Shewanella putrefaciens*, can thrive using respiratory U(VI) reduction (Lovley et al. 1991). *D. desulfuricans* was also shown to reduce U(VI), but in contrast with *G. metallireducens* and *S. putrefaciens* it is not able to grow with U(VI) as the sole electron acceptor. In growth experiments *D. desulfuricans* grew only if sulfate was added to the medium. However, Lovley et al. demonstrated that the U(VI) reduction is conducted enzymatically, due to the temperature-dependent reduction activity of *D. desulfuricans* cultures (Lovley and Phillips 1992). Bencheikh-Latmani et al. (2005) reported that *S. oneidensis* strain MR-1 also has the ability to reduce U(VI) and furthermore is able to grow with U(VI) as the sole electron acceptor. In gene expression studies, they could also show that proteins which have originally been identified to be involved in Fe(III) reduction were upregulated under U(VI) reducing conditions. Wade et al. found furthermore, that a knock-out in SO3980 of *S. putrefaciens* 200 which encodes for the nitrite reductase NrfA leads to an inability to reduce U(VI), implicating that NrfA represents the or a major terminal U(VI) reductase (Wade and DiChristina 2000). In *Desulfovibrio vulgaris* cytochrome c3 was identified in vitro to function as an anaerobic U(VI) reductase. Nevertheless, so far it was not possible to grow *D. vulgaris* with U(VI) as sole electron acceptor (Lovley et al. 1991, 1993b; Lovley and Phillips 1992).

3.4 Plutonium

Plutonium redox chemistry is far more elaborate than that of the other redox systems discussed so far. The oxidation states III, IV, V, and VI can exist under environmental conditions. Pu(V) and (VI) are more soluble than the more reduced forms. Studies show that cell suspensions of *S. oneidensis* and *G. metallireducens* are able to reduce Pu(V/VI) (Icopini et al. 2009). However, in negative controls in which the electron donor was omitted, plutonium reduction was also detectable although on a lower level. In these cell suspension experiments a green precipitate was formed which likely contained Pu(IV). There is also evidence that plutonium reduction could support growth of *G. metallireducens* and *S. oneidensis* MR-1.

3.5 Neptunium

Neptunium occurs under environmental conditions mostly in the oxidation states +5 and +4. Similar to the other metal species described before, the oxidized state is more soluble and hence more toxic than the reduced form, which is due to its

sparsely soluble state in the absence of chelators not bioavailable. Experiments by Icopini et al. point out that *S. oneidensis* and *G. metallireducens* are not able to use Np(V) as the sole terminal electron acceptor in anaerobic respiration. However, they showed that in cell suspension experiments, *S. oneidensis* cells can reduce Np(V) to Np(IV) if a growth medium with ferric citrate as electron acceptor is supplemented with Np(V). Moreover, sodium citrate added to the medium led to a stimulation of Np(V) reduction by *S. oneidensis* as well as by *G. metallireducens*, probably caused by the thermodynamically driven preference of Np(IV)-citrate complexes (Icopini et al. 2007). Of note, *G. metallireducens* cultures showed nearly the same reduction rate in media in which addition of the electron donor was omitted. This might suggest a non-respiratory redox activity. Neptunium reduction was also observed in cultures of sulfate reducing bacteria but again the reduction might be indirectly via the production of hydrogen sulfide.

3.6 Technetium

Microbial technetium reduction has been a topic of several studies. Similar to the elements mentioned before the technetium high valence form Tc(VII) can be reduced by different bacteria. *G. sulfurreducens*, *S. putrefaciens*, *D. desuluricans*, and *E. coli* were described to couple the oxidation of H_2 or organic carbon sources to Tc(VII) reduction. As described before, there is a difference between "simple" reduction and an energy generating respiratory process. Llyod et al. showed that *G. metallireducens* is not able to conserve energy by Tc(VII) reduction. *D. desulfuricans*, *D. fructosovorans*, and *S. putrefaciens* CN32 were also able to reduce Tc(VII) but this reduction was recorded in resting cultures (Lloyd et al. 1998, 2000; Wildung et al. 2000; De Luca et al. 2001). Even though bacterial growth is not stimulated by Tc(VII) reduction, it can be used for bioremediation purposes. Tc(IV), the main reduction product, forms insoluble precipitates which are in many cases directly associated to the bacterial periplasm or outer membrane, thereby removing the soluble technetium species from the medium.

3.7 Molybdenum

Molybdenum is an important trace element that occurs typically in the +4 and +6 oxidation states. It is cofactor of a number of enzymes including nitrogenase and nitrate reductase. Only a few studies described the use of molybdenum as terminal electron acceptor for microorganisms. Pregrown sulfate reducing *D. desulfuricans* cells showed an Mo(VI) reduction activity that was not growth supporting. These cells and other sulfate reducers are also able to reduce molybdenum indirectly via H_2S formation. The acidophile *Acidithiobacillus ferrooxidans* was shown to reduce Mo(VI) using sulfur as electron donor. Sugio et al. (1992) suggested that the molybdenum reducing enzyme is in fact the sulfur:ferric iron oxidoreductase of the organism.

3.8 Cobalt

A number of publications describe microbial redox transformations on cobalt. This is most probably due to colbalt-60 being a known contaminant at a number of field sites that were used for radioactive waste storage. Cobalt is furthermore used for radiotherapy and radiation-based sterilization. Co(III) is mobile, especially in an EDTA-complexed form. Co(II) is less mobile and EDTA complexes not as stable compared with the Co(III) complexes. Earlier work by Yuri Gorby established that some ferric iron reducers are able to reduce Co(III) as well (Gorby et al. 1998). Later work by Hau et al. (2008) showed that at least for *S. oneidensis* the electron transfer route to Co(III) goes via the same enzymes and electron transferring proteins as the respiratory chain to ferric iron. Gorby et al. (1998) demonstrated that reduced cobalt can effectively transfer electrons onto Mn(IV) thereby acting as an inorganic electron shuttle.

3.9 Palladium

Palladium is widely used as a catalyst in a multitude of applications. Hence, biological reduction of soluble Pd(II) to insoluble Pd(0) received and still receives attention. Early work described palladium reduction by *D. desulfuricans* on electrodes that provided the bacterium with hydrogen, which supposedly is the reductant of microbial palladium reduction. Hydrogenase is a periplasmic enzyme and consequently, palladium precipitates were detected in this bacterial compartment. A role of hydrogenase was furthermore confirmed in studies with *D. fructovorans* and *Escherichia coli*. Nevertheless, this suggests that palladium reduction is not part of a respiratory process but that hydrogenase catalyzed electron transfer is a futile reaction. *S. oneidensis* was also used as a catalyst for Pd(II) reduction but this reduction seems to be nothing but a detoxification mechanism, which does not matter for its potential industrial application. It should be noted that the enzymatic activity might not be crucial for the reduction process, since Rotaru et al. (2012) described that bacterial surfaces might be sufficient as nucleation points for palladium recovery.

3.10 Mercury

The geomicrobiology of mercury is complex since not only redox transformations are involved but also methylation that transforms Hg^{2+} into $(CH_3)Hg^+$ or $(CH_3)_2Hg$. Since living tissues have a high affinity for methylmercury, this compound can be concentrated in the food chain several thousand-fold which is part of the risk that companions mercury contaminations. Nevertheless, Hg^{2+}

reduction to volatile metallic mercury is a common detoxification mechanism found in a number of bacterial isolates. The necessary proteins for this process are encoded by the *mer*-genes (*merA*, *merP*, and *merT*). Interestingly, Wiatrowski et al. (2006) could demonstrate that dissimilatory iron reducers like *S. oneidensis* or *G. sulfurreducens* have also another mechanism for Hg reduction, which most probably relies on the proteins necessary for ferric iron reduction. Nevertheless, this other mercury reduction mechanism is not present in all dissimilatory iron reducers since the authors could show that *A. dehalogenans* was not able to produce metallic mercury from Hg^{2+}.

3.11 Gold

Reduction of Au(III) to insoluble Au(0) particles might be a way to recover trace amounts of gold from solutions and to produce nanoparticles with certain activities. Although the actual mechanism of gold reduction is not clear yet, Kashefi et al. (2001) could show that a number of ferric iron reducing bacteria and archaea can reduce Au(III). Nevertheless, this process was not growth supporting, which might be due to Au(III) toxicity. Hydrogen as electron donor was necessary for this process, which might again (similar to palladium particle formation) suggest that hydrogenase acts as unspecific reducing enzyme and nucleation point for gold particle formation. It was furthermore speculated that dissimilatory metal reducing organisms could contribute to gold ore formation, although this process might not be growth supporting and moreover substracts electrons that would otherwise be used for the formation of cell energy.

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Bioremediation via Microbial Metal Reduction

Mathew P. Watts and Jonathan R. Lloyd

Abstract The ability of microbes to reductively transform a variety of metals has wide-reaching implications for controlling the mobility of contaminants in the subsurface, resulting in the degradation of toxic organics or the reductive immobilization of metals. For example, soluble toxic metal contaminants, including Cr(VI), Hg(II), V(V), Co(III), U(VI), Tc(VII) and Np(V) can be reduced directly and removed from solution by enzymatic processes, often being used as terminal electron acceptors during anoxic respiration. In many cases these transformations can also be mediated indirectly via reactive end products of metal reduction, including biogenic Fe(II) or Pd(0). Similar indirect mechanisms for the reductive transformations of organics, such as chlorinated solvents, are also possible, as is the enzymatic oxidation of several organic "xenobiotics", coupled directly to the reduction of metals such as Fe(III). Many of these processes occur naturally within contaminant systems, and the ability to accelerate them during bioremediation applications has attracted much recent interest. Over the past two decades, studies have sought to understand these processes from the molecular level while applying them at field or industrial scale. This review seeks to summarise these findings and address areas of current and prospective progress in the bioremediation field.

M. P. Watts · J. R. Lloyd (✉)
The Williamson Research Centre for Molecular Environmental Science and the School of Earth Atmospheric and Environmental Sciences, The University of Manchester, Manchester, M13 9PL, UK
e-mail: Jon.Lloyd@manchester.ac.uk

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1 Introduction

Anthropogenic activities have resulted in the widespread contamination of the environment with both inorganic and organic pollutants. In many examples, pollutant behaviour is regulated by complex biogeochemical reactions mediated by extant microbial communities. The harnessing of the inherent ability of microorganisms, to detoxify or precipitate inorganic and degrade organic contaminants, can be key to their bioremediation. Due to the near ubiquitous presence of such microbes in contaminated environments, and their enormous gene pool, the potential for their use in bioremediation applications is significant (Lovley et al. 2004).

Many inorganic contaminants are redox active and can be reduced to less harmful or less mobile forms. Mounting research has highlighted the role of microbes in the reduction of inorganics, including transition metals and radionuclides, and often used as the terminal electron acceptor in anaerobic metabolism (Lloyd 2003). Organic contaminants can also play a direct role in microbial metal reduction as an electron donor for Fe(III) reduction (Lovley et al. 1989a) or can be reduced by resulting biogenic Fe(II) (McCormick et al. 2002).

In order to better understand contaminant processes coupled to microbial metal reduction, a variety of approaches from the molecular to the field scale have been addressed. The subsequent application of microbes with metal-reducing properties for bioremediation has led to many proposed application technologies. These typically encompass "biostimulation" strategies to enhance the activities of indigenous microbes and "bioaugmentation", via the addition of model metal-reducing microbes. Many bioremediation approaches have been proven at laboratory scale, while others have reached pilot (Wagner-Döbler 2003) and field-scale in situ application (Li et al. 2009).

This review aims to give an overview of a variety of bioremediation processes underpinned by microbial metal reduction. This will include studies concerning the diversity of the microorganisms involved, the mechanisms of contaminant transformation, the environmental fate of the contaminant, strategies for optimising these processes and finally the impact of the latest post-genomic approaches on increasing our knowledge of these complex biogeochemical systems.

2 Mechanisms of Microbial Fe(III) Reduction

The biogeochemical cycling of Fe is an important global process and has influence over the behaviour of many other bulk and trace elements. The cycling between the oxidised (Fe(III)), and reduced (Fe(II)), states is often microbially mediated, where Fe(III) is used as a terminal electron acceptor in anaerobic metabolism and Fe(II) as an electron donor in both aerobic and anaerobic processes (Weber et al. 2006).

The microbial reduction of Fe(III) plays a key role in the oxidation of organics within the anaerobic subsurface, where Fe(III) is often the most abundant electron acceptor (Nealson and Myers 1992). The ability of microbes to reduce Fe(III) as an electron acceptor during metabolism has long been established (see Lovley and Phillips, 1986). The isolation of organisms capable of Fe(III) reduction coupled to growth came later with the isolation of *Geobacter metallireducens* (formerly GS-15) (Lovley and Phillips 1988) and the realisation that *Shewanella oneidensis* (formerly *Alteromonas putrefaciens*) could also respire Fe(III) (Lovley et al. 1989b). Following these findings, many of the strains isolated, capable of Fe(III) reduction, have belonged to the family *Geobacteraceae* of the δ-Proteobacteria (Lloyd 2003). This is significant as members of the family *Geobacteraceae* are commonly identified within natural environments, and are capable of coupling Fe(III) reduction to the oxidation of acetate and many other organic substrates (Lovley et al. 2004). Many Fe(III)-reducing bacteria can also utilise other redox active metals and reducible organics as electron acceptors (Lovley 1993). For an extensive review of the diversity of organisms capable of Fe(III) reduction, please Refer to Chap. 6 of this book and to (Lovley et al. 2004). Further studies have extended the range of environments in which Fe(III) reduction can occur, to encompass bacteria adapted to extreme environments including thermophiles (Tor and Lovley 2001) acidophiles (Coupland and Johnson 2008) and alkaliphiles (Roh et al. 2007).

Under near-neutral environments, Fe(III) is typically poorly soluble, occurring as Fe(III) oxides or adsorbed onto mineral surfaces (Weber et al. 2006). The rate of microbial reductive dissolution of the Fe(III) phase is thought to be controlled by the surface area (Roden and Zachara 1996) and the crystallinity of the Fe(III) phase (Hansel et al. 2003b; Cutting et al. 2009). The accumulation of Fe(II) on the Fe(III) oxide surface has also been found to influence the rate and extent of reduction by preventing further reduction of the bulk mineral (Roden 2006).

To deal with the poor solubility of Fe(III) in the environment, microbes have evolved a variety of mechanisms which enable electron transfer to the microbe-mineral interface, resulting in reductive dissolution of extracellular Fe(III) minerals (Lovley et al. 2004). Early studies suggested that outer membrane cytochromes were a key component for electron transfer of *Shewanella* spp. (Myers and Myers 1992). The cellular electron transfer apparatus, in the model Fe(III) reducers, *Geobacter* spp. and *Shewanella* spp., have now been characterised in some detail (Lovley et al. 2004; Hartshorne et al. 2009). This is reflected in the corresponding bacterial genomes, where genes encoding *c*-type cyctochromes are present in abundance (Richter et al. 2012).

Some Fe(III)-reducing bacteria were found to have the ability to produce soluble compounds, termed "electron shuttles", which can accelerate electron transfer between a remote cell and Fe(III) mineral (Lloyd et al. 2003). The ability to produce such compounds was first demonstrated in *Shewanella* (von Canstein et al. 2008; Marsili et al. 2008). The secretion of such "endogenous" electron shuttles appears to be lacking in *Geobacter* spp. (Nevin and Lovley 2000). However, other naturally occurring organic substances, for example humics

(Lovley et al. 1996), can be opportunistically used as electron shuttles by a wide variety of Fe(III)-reducing bacteria. In addition, other organisms such as *Geothrix fermentans*, are also capable of producing iron chelating compounds in order to promote Fe(III) dissolution and thereby increasing accessibility for reduction (Nevin and Lovley 2002).

The production of pili has also been observed in *Geobacter* spp., and these structures were hypothesised to aid contact between cells and Fe(III) minerals (Childers et al. 2002). However, more recent studies have noted these pili to be conductive, therefore implicating a role of electron transfer in *Geobacter* spp. (Reguera et al. 2005; Reguera et al. 2007) and also *Shewanella* spp. (Gorby et al. 2006).

The resulting biogenic Fe(II) not only adsorbs to the original Fe(III) phase, but can form discrete secondary biominerals (Lloyd et al. 2008). The mineralogy of the resulting Fe(II) phase is determined by a complex interaction of microbial and geochemical controls (Hansel et al. 2003a). Although a variety of biominerals can result from Fe(III) reduction; siderite, green rust and vivianite for example, the common end member biomineral formed is often biogenic magnetite, especially in laboratory experiments utilising ferrihydrite as the Fe(III) mineral phase (Lloyd et al. 2008; Coker et al. 2006).

2.1 Oxidation of Organic Contaminants Coupled to Fe(III) Reduction

Many organic molecules occur as subsurface contaminants due to their release during a variety of anthropogenic activities. These organic molecules often consist of aromatic compounds which, due to the thermodynamic stability of the benzene ring, are persistent in the environment (Carmona and Díaz 2005). Anaerobic conditions are established rapidly within organic contaminant plumes, where oxygen is quickly utilised as the favourable electron acceptor in catabolism of the abundant organic electron donors (Carmona et al. 2009). During the resulting redox sequence, an abundance of Fe(III) oxides within the subsurface can result in much of the plume being located within the Fe(III) reduction zone (Lloyd et al. 2003). In situ monitoring of such environments has confirmed degradation of organic contaminants under Fe(III)-reducing conditions (Lyngkilde and Christensen 1992; Nielsen et al. 1995; Christensen et al. 2001), although the persistence of many aromatics within Fe(III) reducing zones suggests this process in the subsurface can be kinetically slow (Lovley, 1995).

The ability of a single organism to couple Fe(III) reduction to the oxidation of aromatic hydrocarbons was first reported for the bacterium *G. metallireducens* (Lovley et al. 1989a). This bacterium, isolated from a hydrocarbon-contaminated aquifer, was able to obtain energy from oxidation of benzoate, toluene, phenol or *p*-cresol using Fe(III) as the sole electron acceptor (Lovley and Lonergan 1990).

The bacterium in this study was able to mineralise the aromatic compounds to CO_2, with little accumulation of intermediate compounds. For an in-depth genomic view of the aromatic catabolism of *G. metallireducens* refer to (Wischgoll et al. 2005) and (Carmona et al. 2009). However, few further isolated *Geobacter* sp. have exhibited the ability to couple Fe(III) reduction to the oxidation of aromatic compounds (Butler et al. 2007). Exceptions include *Geobacter hydrogenophilus* and *Geobacter grbiciae,* both able to oxidise benzoate and the latter bacterium toluene (Coates et al. 2001). Outside the *Geobacter* genus, few axenic cultures of bacteria have demonstrated Fe(III) reduction coupled to aromatic metabolism. Notable examples are the thermophile, *Ferroglobus placidus*, which could indicate aromatic oxidation coupled to Fe(III) reduction is an important metabolic process in hot subsurface environments (Tor and Lovley 2001). Another recent study described the isolation of two bacteria capable of this metabolic trait, *Geobacter toluenoxydans* and *Desulfitobacterium aromaticivoran*, the latter being a member of the Gram-positive Clostridia group (Kunapuli et al. 2010).

Although the ability of bacteria to couple Fe(III) reduction to aromatic oxidation seems to be not expressed widely across known Fe(III)-reducing bacteria outside the family *Geobacteraceae*, the abundance of *Geobacter* species in many sedimentary environments where Fe(III)-reducing conditions prevail suggests that these processes are of environmental significance (Coates et al. 1996). Using microbial ecology techniques, such as 16S rDNA profiling, these organisms have been found to contribute a significant proportion of the microbial community in subsurface environments contaminated with aromatics (Rooney-Varga et al. 1999; Roling et al. 2001; Staats et al. 2011; Snoeyenbos-West et al. 2000). Thus, it would seem reasonable that biostimulation of these organisms could offer a viable bioremediation strategy, to enhance biodegradation. Here, a key limiting step in the process could be the poor accessibility of insoluble Fe(III) oxides to microbial Fe(III) reduction (Lovley et al. 1994; Lovley et al. 1996). A study using anoxic aquifer sediments was able to increase aromatic oxidation rates, coupled to Fe(III) reduction, to values approaching those seen in oxic systems, by the addition of ligands which increased Fe(III) oxide solubility (Lovley et al. 1994). The promotion of Fe(III) reduction using electron shuttling compounds was also linked to an increased rate of aromatic oxidation within the subsurface (Borch et al. 2010). Multiple studies have demonstrated this through the use of the humic analogue AQDS (anthraquinone-2,6-disulphonic acid) (Snoeyenbos-West et al. 2000; Jahn et al. 2005). For a more detailed review on the addition of electron shuttles to promote the degradation of contaminants see (Van der Zee and Cervantes 2009). Fe(III) reduction coupled to these processes also leads to the production of reactive Fe(II) species. A recent study demonstrated Fe(II)-mediated reduction of an enzymatically recalcitrant contaminant, a nitroaromatic compound, using Fe(II) generated from the oxidation of BTEX (benzene, toluene, ethylbenzene and xylene) compounds by *G. metallireducens* (Tobler et al. 2007). Thus, this study demonstrated that microbial Fe(III) reduction coupled to aromatic oxidation, can result in abiotic degradation of co-contaminants that cannot be utilised directly (Tobler et al. 2007).

2.2 Fe(II)-Mediated Contaminant Reduction

As noted above, the microbial reduction of Fe(III) can mediate reductive transformation of contaminants via abiotic electron exchange reactions with biogenic Fe(II), see Fig. 1 (Lloyd 2003). Indeed, ionic Fe(II) is a well-known reductant commonly used in industrial processes and during chemical remediation of contaminants (Charlet et al. 1998).

Biogenic Fe(II) is often associated with Fe(II)-bearing biominerals, either within the structure of the mineral itself or sorbed to the surface (Cutting et al. 2009). The reactivity of various synthetic Fe(II)-bearing minerals towards dehalogenation of hexachloroethane and nitroaryl reduction of 4-chloronitrobenzene has been demonstrated (Elsner et al. 2004). This study found variability within surface area normalised reaction rates, dependent upon the Fe mineral composition, and where Fe sulphides exhibited the highest reaction rates. As magnetite is ubiquitous in the subsurface environment (and also a common product of microbial Fe(III) reduction) it has received much attention regarding reactivity towards contaminants (Gorski and Scherer 2009; Gorski et al. 2010). The latter of these studies demonstrated an increasing rate of reactivity towards nitrobenzene, of synthetic magnetites, with increasing Fe(II) content up to those of stoichiometric magnetite.

Several studies have carried out contaminant interaction experiments using biogenic magnetite (McCormick et al. 2002; McCormick and Adriaens 2004; Williams et al. 2005; Telling et al. 2009; Cutting et al. 2010). These biogenic magnetites were synthesised via reduction of an amorphous Fe(III)-starting mineral (e.g., ferrihydrite) using Fe(III)-reducing bacteria of the genus *Geobacter*. In one study, the resulting biogenic magnetite was used to transform recalcitrant carbon tetrachloride (McCormick and Adriaens 2004). A variety of daughter compounds formed during exposure, including chloroform and the intermediate dichloromethane. A proportion of this was subsequently hydrolysed to carbon monoxide or was further reduced to methane, although the mechanism of methane formation was not elucidated. The formation of chloroform and dichloromethane is of concern as these products are also toxic; however, a large proportion ($\sim$47 %) of carbon tetrachloride was fully dechlorinated to the relatively benign CO and CH_4. In a separate study, biogenic magnetite, with minor components of siderite and an unknown Fe(II) phase, was demonstrated to be active for transformation of the toxic groundwater contaminant, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), a component of military explosives (Williams et al. 2005). Sequential reaction of a series of nitroso products occurred, eventually leading to the accumulation of 1,3,5-trinitroso-1,3,5-triazacyclohexane, although the products only accounted for a maximum of 30 % of the original RDX. Interestingly, this paper also noted a decrease in reactivity of the biogenic minerals after increased incubation time with the bacterium *G. metallireducens* during mineral biosynthesis. This effect was hypothesised to be possibly the result of the release of cell lysis products.

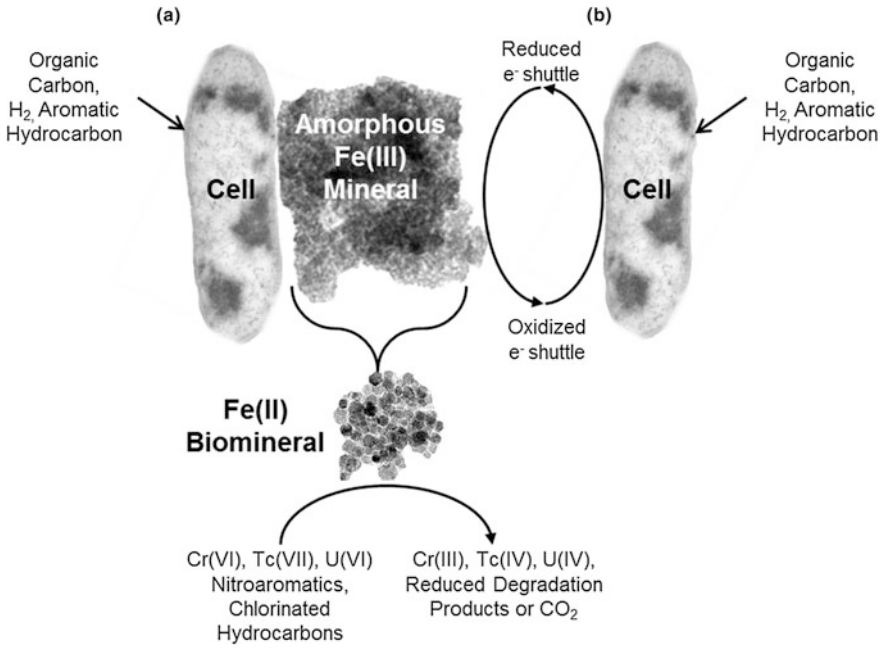

Fig. 1 A schematic representation of biogenic Fe(II)-mediated reduction of contaminants linked to microbial Fe(III) reduction. The two mechanisms correspond to **a** direct contact between the cell and Fe(III) mineral surfaces and **b** the utilisation of an extracellular electron shuttle

Biogenic magnetite was also demonstrated to be effective for the reduction of the common soluble inorganic contaminant Cr(VI) to the insoluble and non-toxic Cr(III), and the reduction of the fission product Tc(VII) to insoluble Tc(IV) (Cutting et al. 2010), see Sects. 3.1 and 4.2 for more details of Cr and Tc contamination, respectively. This study utilised biogenic magnetites produced from different starting Fe(III) phases, which exhibited contrasting Fe(II) yields, and these corresponded to differing activities against Cr(VI). The Cr was shown, using X-ray magnetic circular dichroism (XMCD) analysis, to be converted into Cr(III) and incorporated into the octahedral site of a $CrFe_2O_4$ spinel, at the surface of the magnetite. Retention of ^{99m}Tc(VII) was investigated using flow through columns of quartz sand with a reactive layer of the biogenic magnetite, and the quantities of ^{99m}Tc retained in the column and in the effluent were monitored using a gamma camera imaging system. Efficient retention, 77.9 and 97.8 %, for ferrihydrite- and schwertmannite-derived magnetites, respectively, was observed compared to only 16.2 % accumulation in control columns with no magnetite.

Other studies have focussed on biogenic Fe(II)-mediated contaminant transformations using direct culturing of Fe(III)-reducing bacteria grown with reducible Fe(III) phases (Wielinga et al. 2001; Hansel et al. 2003b; Borch et al. 2005). The degradation of 2,4,6-trinitrotoluene (TNT), a groundwater contaminant from the

explosives industries, was investigated using a *Cellulomonas* sp. incubated with and without ferrihydrite and the electron shuttle AQDS (Borch et al. 2005). The TNT reduction products, which are inferred to be less mobile than the parent molecule, were formed more rapidly and to a greater extent in the presence of the Fe(III)-bearing substrate ferrihydrite and AQDS. Studies using direct culturing of *Shewanella alga* in contact with ferrihydrite and a Cr(VI) solution also showed significant Fe(II)-mediated reduction and removal of Cr(VI) (Wielinga et al. 2001; Hansel et al. 2003b). The first of these studies also noted that, under Fe-limiting conditions, cycling of Fe between the two valence states occurred with microbial Fe(III) reduction, and then subsequent abiotic Fe(II) oxidation coupled to Cr(VI) reduction. The latter study characterised the resulting Cr(III) phase by X-ray absorption near-edge structure (XANES) and extended X-ray absorption fine structure (EXAFS), identifying an insoluble mixed Fe(III)-Cr(III) hydroxide.

The capacity for indigenous Fe(III) microbial communities to mediate organic degradation was investigated for TNT degradation by charging a column containing ferrogenic aquifer sediments with acetate (Hofstetter et al. 1999). Rapid and complete reductive degradation, via biogenic Fe(II), to 2,4,6-triaminotoluene was observed in amended columns, while unamended columns exhibited a slower rate of reduction.

A series of studies on alkaline Cr(VI) containing leachates, from chromite processing ore residue waste sites, indicated in situ biogenic immobilisation of Cr(VI) by Fe(II) in adjacent soils (Stewart et al. 2007; Stewart et al. 2010; Whittleston et al. 2011a). When the soils, with alkaline Cr(VI) leachates, were amended with the electron donor acetate, the utilisation of the cascade of electron acceptors occurred, passing from nitrate reduction to sulphate reduction via Fe(III) reduction (Whittleston et al. 2011a). Accumulation of Fe(II) coincided with the removal of Cr(VI) from solution (Whittleston et al. 2011a). Enrichments of a bacterial consortium from the Fe(III)-reducing soils were dominated by Fe(III)-reducing *Fermicutes* species (Whittleston et al. 2011b). These studies appear to show the natural occurrence of a microbially-mediated Fe(III)-reducing reactive zone which had the potential for reducing and sequestering alkaline Cr(VI) leachates.

Reduction and immobilisation of Tc(VII) is well documented within anoxic sediments (Lloyd et al. 2000a; Wildung et al. 2004; Burke et al. 2005, 2006; McBeth et al. 2007). Reduction typically coincides with microbial Fe(III) reduction, and is thought to be mediated indirectly by the microbial production of the reductant Fe(II) (Lear et al. 2009). This is in part due to low concentrations of Tc(VII) present in most Tc-contaminated environments, and poor recognition of the substrate by the hydrogenases believed to be responsible for enzymatic Tc(VII) reduction in microbes (Lloyd et al. 1999). The microbial reduction of Fe(III) within sediments, from the US Department of Energy's Hanford and Oak Ridge sites, inoculated with the Fe(III)-reducing bacterium *Shewanella oneidensis*, was shown to increase the capacity for Tc(VII) reduction and immobilisation (Fredrickson et al. 2004). Alternative studies have also shown the effectiveness of stimulation of indigenous Fe(III)-reducing bacterial consortia for indirect

reduction of Tc(VII) (Burke et al. 2005; McBeth et al. 2007; Lear et al. 2009). Upon amendment with an electron donor, the reduction and removal of Tc(VII) from estuarine or aquifer sediment microcosms, coincided with microbial Fe(III) reduction (Burke et al. 2005; McBeth et al. 2007). In the sediments of the latter study, the known Fe(III)-reducing bacteria *Geothrix* and *Geobacter* species were confirmed to be present by PCR-based analyses. X-ray absorption spectroscopy (XAS) analysis confirmed reduction to Tc(IV)(as TcO_2) within the sediments (Burke et al. 2005). It should be noted that upon exposure to air, reoxidation of immobilised reduced Tc(IV), resulted in the release of Tc(VII) back into solution, of ~50 % (Burke et al. 2006) and ~80 % (McBeth et al. 2007) of total Tc, although much lower release values were noted when nitrate (a common nuclear contaminant) was used to reoxidise the reduced Tc(IV). A recent study used a γ-camera to image retention of the radiotracer ^{99m}Tc, in a biostimulated flow-through sediment column, alongside geochemical and molecular ecology techniques (Lear et al. 2009). This was able to spatially correlate areas of Fe(II) accumulation, Tc(VII) reduction and immobilisation and an increase in the proportion of Fe(III)-reducing bacteria.

3 Enzymatic Transition Metal Contaminant Reduction

A variety of transition metals occur as contaminants, and many of these are redox active and amenable to enzymatic reductive remediation, see Fig. 2. Other transition elements do not typically occur as contaminants, for example Pd(II), but may present opportunities for remediation processes by their enzymatic reduction, forming highly active precious metal catalysts, useful for contaminant destruction.

3.1 Reduction of Cr(VI)

The pervasive pollutant, Cr, is associated with a significant proportion of contaminated sites worldwide. This is as a consequence of its widespread industrial use, from its initial mining and processing as a chromite ore through to its use in metallurgy, leather tanning, pigment production and wood preservation (Kamaludeen et al. 2003).

The metal is redox active, forming two valence states which dominate in environmental systems; Cr(VI) and Cr(III). Cr(III) species are dominant under acidic and moderate to reducing conditions while Cr(VI) is dominant in more oxidising and alkaline conditions (Kimbrough et al. 1999). Cr(III), being relatively insoluble in all but highly acidic environments, typically forms chromium (oxyhydr)-oxides, often in association with iron or strongly adsorbed to mineral surfaces (Fendorf 1995). The Cr(VI) species; H_2CrO_4, $HCrO^{4-}$, CrO_4^{2-} and

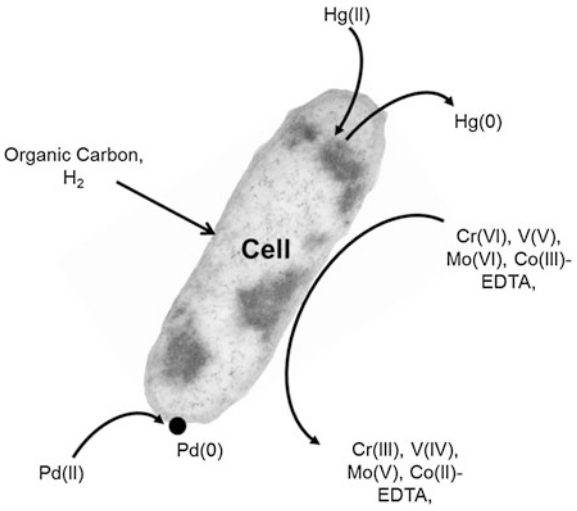

Fig. 2 A schematic representation of enzymatic transition metal reduction related to contaminant processes

$C_2O_4^{2-}$, are by contrast readily soluble and weakly adsorb to mineral surfaces (Kimbrough et al. 1999).

Cr(VI) has a variety of toxic properties behaving as an irritant, carcinogen and allergen (Chen and Hao 1998). In humans, this manifests itself as dermal irritation upon skin contact, lung and respiratory tract damage upon inhalation and liver and kidney damage upon ingestion (Gad 1989). This toxic effect is in part due to the mobile nature of the Cr(VI) which enables diffusion across cell membranes, where intracellular reduction causes oxidative damage to the cell (Dayan and Paine 2001). Cr(III), however, is by contrast regarded as non-toxic and an essential trace nutrient necessary for glucose and lipid metabolism (Wang 2000). The risks involved with exposure to Cr(VI) have led to it being regarded as a priority pollutant by the US Environmental Protection Agency (USEPA 2009), and its use controlled by the European Restriction of Hazardous Substances Directive (RoHS 2003). The World Health Organisation currently sets an upper limit of 0.05 mg L^{-1} for Cr(VI) in drinking water (WHO 2008).

Conventional treatments for Cr(VI)-containing wastes target chemical reduction and precipitation as poorly soluble Cr(III) (oxyhydr)-oxides. Due to the high Cr(VI)/Cr(III) redox couple, re-oxidation by contact with dissolved oxygen is kinetically very slow (Rai et al. 1989). While more oxidising species found in waters and soils such as Mn oxides are capable of re-oxidation of Cr(III), this process is dissolution controlled and kinetically slow (Rai et al. 1989).

Although reduction can be a consequence of indirect microbial reduction via the formation of intermediary reducing species, such as Fe(II) and sulphides, (see Sect. 2.2) direct enzymatic Cr(VI) reduction can occur (Lovley and Phillips 1994). Since the first recorded isolations of organisms capable of enzymatic microbial Cr(VI) reduction in the late 1970s, *Pseudomonas dechromaticen* (Romanenko and Koren'kov 1977) and *Pseudomonas chromatophila* (Lebedeva and Lialikova 1979),

a diverse group of Cr(VI)-reducing microbes have been identified (Cervantes et al. 2007). The majority are facultative anaerobes which can be found in natural unpolluted systems (Wang and Shen 1995). Early studies primarily identified bacteria that did not conserve energy through Cr(VI) reduction and was likely due to the secondary activity of enzymes with different physiological functions (Ishibashi et al. 1990). Later studies, however, also identified bacteria capable of coupling Cr(VI) reduction to growth (Tebo and Obraztsova 1998; Francis et al. 2000). Enzymatic Cr(VI) reduction has been demonstrated under aerobic and anaerobic conditions, with some bacteria exhibiting reduction under both conditions; for example *Escherichia coli* ATCC 33456 (Shen and Wang 1993).

Multiple enzymatic reduction mechanisms are reported within the literature, and appear to be associated with specific locations within the cell (Cervantes et al. 2007). Typically, membrane-bound Cr(VI) reduction is thought to be mediated by the electron transfer chain and related cytochromes, while cytoplasmic Cr(VI) reduction occurs by flavin-dependent reductases (Magnuson et al. 2010). Early studies of *Pseudomonas ambigua* G-1 and *Pseudomonas putida* exhibited Cr(VI) reduction via soluble Cr(VI) reductases under aerobic conditions, dependent upon NAD(P)H as the electron donor; (Suzuki et al. 1992) and (Ishibashi et al. 1990; Suzuki et al. 1992) respectively. Since these studies, other soluble Cr(VI)-reducing enzymes have been identified. Some of the more extensively studied include; ChrR (formerly referred to as YieF when expressed in *E. coli*), ChrR6 and the nitroreductase NfsA (Ackerley et al. 2004, Barak et al. 2006). The reduction mechanism is enzyme specific, for example the NfsA dimer (of *E. coli*) reduces Cr(VI) via a combination of one and two electron transfer reactions (Ackerley et al. 2004). This mechanism proceeds via the reactive intermediary Cr(V), generating reactive oxygen species (ROS) which are implicated in Cr(VI) toxicity within the cell (Barak et al. 2006). Indeed, a proteomics study correlated Cr(VI) stress in *Pseudomonas aeruginosa* to overexpressed production of the ROS detoxification protein glutathione (Kilic et al. 2009). Other soluble reductases, such as ChrR of *E. coli*, which normally transfers four electrons in redox reactions, reduces Cr(VI) to Cr(III) in a one-step three electron transfer, with one electron going to molecular oxygen, thus limiting ROS generation (Ackerley et al. 2004).

Anaerobic Cr(VI) reduction, in which Cr(VI) acts as the terminal electron acceptor, has been demonstrated in several obligate anaerobes. Sulphate-reducing bacteria have received much attention as they are able to couple lactate oxidation to Cr(VI) reduction, due to similarities between the sulphate and chromate anions (Lloyd et al. 2001). In sulphate-reducing bacteria (*Desulfovibrio* spp.), reduction has been shown to occur via *c*-type cytochromes operating with hydrogenases, facilitating the use of hydrogen as an electron donor for Cr(VI) (Lovley and Phillips 1994). A recent study of *S. oneidensis* was able to identify the MtrC and OmcA as the cytochromes acting as the terminal reductases of the electron transfer chain responsible for extracellular Cr(VI) reduction (Belchik et al. 2011). This was confirmed via deletion of the respective genes, *mtrC* and *omcA*, resulting in a loss of reduction efficiency and lack of extracellular Cr(III) precipitation with a concurrent increase in intracellular Cr(III) precipitation (Belchik et al. 2011).

The development of microbial Cr(VI) reduction to a feasible bioremediation strategy has been the aim of numerous studies. For example, *ex situ* and wastewater treatment bioreactors have demonstrated efficient Cr(VI) reduction. These have been composed of simple batch cultures of planktonic cells (Tripathi 2002) or immobilised cultures, forming a biofilm upon a support substrate (Konovalova et al. 2003). A noteworthy study compared Cr(VI) removal within a variety of bioreactors, using the model microbe *P. aeruginosa* A2Chr (Tripathi 2002). The results indicate that cells entrapped in biofilms are less susceptible to Cr(VI) toxicity than planktonic forms, and thus more applicable to higher Cr(VI) concentrations. Immobilised biofilm beds have also been demonstrated using *Bacillus* spp. (Chirwa and Wang 1997), mixed cultures of sulphate reducers (Smith 2001) and mixed cultures obtained from Cr(VI) contaminated environments (Nancharaiah et al. 2010). The latter study was effective under both aerobic and anaerobic conditions. Biomass-dependent Cr(VI) removal was observed to be slightly greater in the absence of oxygen, at 0.17 mM $day^{-1}g^{-1}$ compared to 0.15 mM $day^{-1}g^{-1}$ for aerobic conditions, inferred to be due to oxygen acting as a competing electron acceptor. A study, using a mixed culture, to investigate optimum parameters for microbial Cr(VI) reduction found that biomass exerted the overall control over the reaction (Jeyasingh and Philip 2005). However, the pH of the medium also exerted influence with loss in reduction efficiency away from circum-neutral values.

The potential in situ treatment of Cr(VI)-contaminated sites has also attracted significant interest. The diversity of microbes capable of Cr(VI) reduction means most soils and sediments have an indigenous population of Cr(VI) reducers (Bader et al. 1999). Stimulation of this metabolic capability is achievable by a variety of amendments. For example, addition of tryptic soy broth and a mix of glucose and mineral salts stimulated CO_2 evolution, as a product of respiration, coupled to significant Cr(VI) reduction over 128 days from a contaminated soil from an electroplating facility (Turick et al. 1998). In situ conditions are not easily controlled and thus not often optimum for metabolic activity, for example, most subsurface aquifer conditions are typically relatively low temperature, and limited in electron donor concentrations. However, a study of Cr(VI)-contaminated sediments from the Hanford facility led to culturing of an indigenous psychrophilic bacterium, *Arthrobacter aurescens*, capable of complete reduction of 60 mg/L Cr(VI) within 120 h at 10 °C (Horton et al. 2006). Extremes of pH are also a key factor for in situ Cr(VI) remediation, and Cr(VI) is particularly associated with highly alkaline environments. Several recent publications have however isolated bacteria from alkaline environments capable of high pH (typically pH 9–10) Cr(VI) reduction; *Alkaliphilus metalliredigens* (QYMF) (Roh et al. 2007), *Burkholderia cepacia* MCMB-821 (Wani et al. 2007) and *Halomonas* sp. (VanEngelen et al. 2008). Bioaugmentation with known Cr(VI) reducers has also been effective in laboratory-based studies where addition of mixed cultures, obtained from contaminated samples, has been undertaken (Jeyasingh and Philip 2005; Jeyasingh et al. 2010). The former study of a contaminated soil found that the addition of a mixed culture of Cr(VI)-reducing bacteria was capable of complete Cr(VI) reduction. The latter study trialled targeted inoculation in a “bio-barrier”, which

effectively creates a reactive zone of Cr(VI)-reducing bacteria perpendicular to groundwater flow within a laboratory-based system. Under highly controlled conditions of moderate and uniform groundwater flow, with high biomass, effective removal of Cr(VI) from an artificial groundwater plume was observed.

3.2 Reduction of Hg(II)

The bioremediation of mercury (Hg), another important pollutant, has also received significant attention. Although significant natural emissions occur, anthropogenic emissions (2479 Mg/yr^{-1} in 2006) (Streets et al. 2009), represent a considerable input to the Hg biogeochemical cycle. These primarily result from fossil fuel combustion, mining, gold and nonferrous metal production and the chloralkali processes (von Canstein et al. 2002).

The toxic properties of Hg are dependent upon its valence state, with ionic Hg(II) believed to be the most toxic species in comparison to the less toxic Hg(0) (Clarkson 1997). The toxicity of Hg(II) is the result of its ability to bind to, and thus inactivate, key metabolic enzymes (Barkay et al. 2003). In humans, this is manifest by acute toxic shock at high exposures (Clarkson 1997) and predominantly neuronal disorders at lower doses (Wagner-Döbler 2003). Methylated Hg, MeHg, is considered of greater concern due to its more mobile nature, although its toxicity is a result of the intracellular de-methylation to Hg(II) (Morel et al. 1998).

The acute toxicity and natural occurrence of Hg has led to the evolution of a highly conserved bacterial Hg(II) detoxification mechanism (Wagner-Dobler et al. 2000). This consists of uptake, followed by the intracellular reduction of toxic Hg(II) to the far less toxic Hg(0), which is subsequently expelled due to its low solubility and high vapour pressure (Barkay et al. 2005). This phenotype is expressed by a sequence of genes, the *mer* operon, encoding proteins used in Hg transport and transformation (Barkay et al. 2005). The *mer* operon is located on transposable elements and plasmids found within a diverse variety of both Gram-positive and Gram-negative bacteria (Wagner-Döbler 2003). Expression is induced by the presence of Hg(II) which binds to the regulatory MerR protein uncoupling it from, and thus activating, the promoter region of the *mer* operon (Wagner-Döbler 2003).

Unusually, the *mer* detoxification mechanism (see Fig. 3) actively transports Hg(II) into the cell. This occurs via a succession of specific uptake proteins including periplasmic MerP (in Gram-negative bacteria), and the cytoplasmic membrane-bound proteins MerT, MerC, MerF and Mer E (Barkay et al. 2003). Once inside the cell, the Hg(II) is transferred to the MerA enzyme via redox buffers, such as glutathione or cysteine, or directly from the MerT membrane protein (Barkay et al. 2003). The MerA enzyme is an NAD(P)H dependent mercuric reductase responsible for the reduction of the Hg(II) ion to Hg(0), which passively diffuses out of the cell membrane (Barkay et al. 2003).

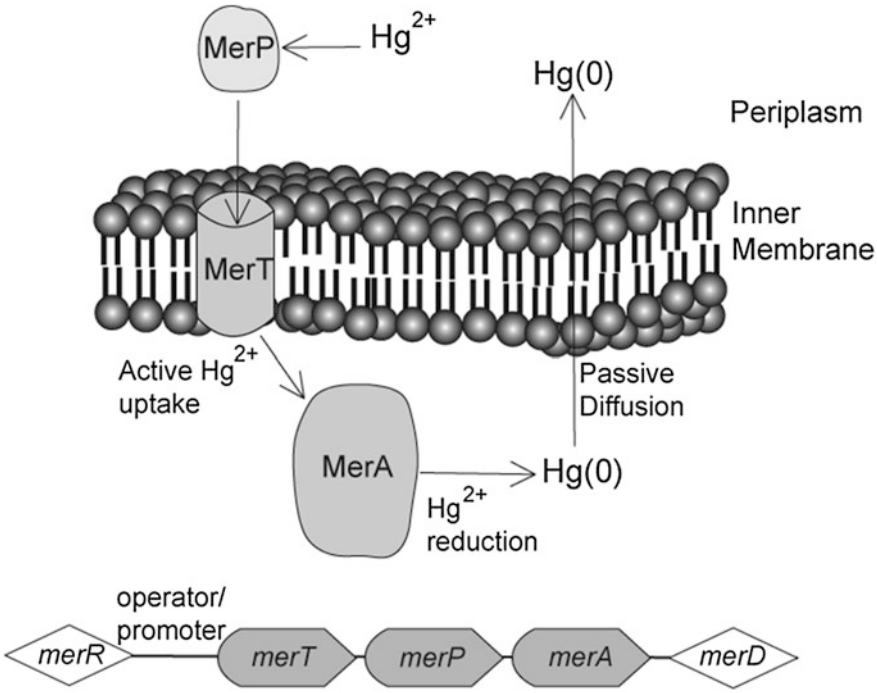

Fig. 3 A model of the "core" Hg resistance mechanism and associated operon in Gram-negative cells. The alternative transport proteins, MerC, MerE and MerF, may be present in the Gram-negative cell's inner membrane. For the *mer* operon diagram below; *white* boxes indicate regulatory function genes, while *grey* boxes are transport and transformation genes. The operator/promoter region for the operon is located after the *merR* gene. The *merD* gene is present in Gram-negative bacteria and antagonises *merR* transcription. Diagram adapted from (Barkay et al. 2003, 2005)

The bioremediation of Hg via the microbial *mer* detoxification mechanism has undergone trials as both a wastewater treatment (von Canstein et al. 1999; Wagner-Döbler 2003) and as an in situ treatment for waters, soils and sediments (Saouter et al. 1995; Nakamura et al. 1999). As a wastewater treatment, a fixed bed bioreactor was employed at technical scale for treating chloralkali electrolysis wastewater (Wagner-Dobler et al. 2000; von Canstein et al. 2002). The aim of the bioreactor was to reduce aqueous Hg(II) from the water and retain the Hg(0) precipitates within the reactor. The bioreactor consisted of an inert carrier matrix, such as pumice, within the main chamber followed by a carbon filter which adsorbed the remaining Hg from the outflow (Wagner-Döbler 2003). The carrier matrix was inoculated with natural isolates of Hg-resistant microbes, predominantly of the *Pseudomonas* genus, which formed a thick biofilm of cells and exopolysaccharides (EPS) (Wagner-Dobler et al. 2000). The technology was trialled using the wastes of multiple chloralkali plants in Europe alongside synthetic waste analogues (von Canstein et al. 1999). The chloralkali wastewater contained 1.6–7.6 mg/L total Hg, high chloride content of 25 g/L and either acidic

or alkaline pH (von Canstein et al. 1999). The wastewater was pre-treated by nutrient amendment, neutralisation of the pH and where necessary dilution, reactor retention times varied between 15 and 60 min. During pilot studies the bioreactor maintained effluent Hg concentrations approaching the theoretical minimum, due to a water solubility of Hg(0) of 50 μg/L, independent of the Hg(II) influent concentrations, which were up to 7 mg/L (von Canstein et al. 1999). The technical-scale bioreactor was able to maintain an Hg removal average of 90 %, including technical failures, retaining 45 kg Hg(0) per 1 m^3 bioreactor volume from 10,080 m^3 wastewater over 8 months (Wagner-Dobler et al. 2000). Interestingly, microbial community analysis showed a decrease in the abundance of inoculated strains within the biofilm in favour of invading bacteria (von Canstein et al. 2002). The same study also showed that overall diversity was dictated by the selective pressure of mercury contamination, where diversity was lowest at the influent end of the bioreactor and overall diversity lowered during times of higher mercury influent concentrations.

The treatment of Hg-contaminated environments is also a potentially useful in situ bioremediation application. However, due to the complexities of the Hg biogeochemical cycle, fewer studies have used environmental samples. A study of note used an Hg-resistant isolate, *Aeromonas hydrophila* KT20 obtained from an Hg-contaminated lake, to inoculate microcosms containing contaminated lake water (Saouter et al. 1995). This caused an increase in the flux of Hg(0) gas produced, which was taken as a proxy for microbial Hg(II) reduction. However, this only accounted for the removal of 5 % of the Hg input. The genetic engineering of strains to express or overexpress *mer* detoxification proteins has also been cited as a possible strategy to improve removal efficiency of microbes (Brim et al. 2000), although environmental applications are limited due to tight regulations on release of genetically engineered microbes (Lloyd et al. 2003). One study in this area focused on genetically engineering the radiation-resistant bacterium, *Deinococcus radiodurans,* for use in remediation of sites co-contaminated with radionuclides and Hg (Brim et al. 2000). This strain contained the cloned Hg resistance gene *merA* and was able to reduce Hg(II) to Hg(0) at high concentrations, even when within a highly irradiated environment.

It should also be noted that some metal-reducing bacteria (Kerin et al. 2006) and a wide variety of sulphate-reducing bacteria (King et al. 2000) can also be responsible for production of highly toxic MeHg. Indeed, this is believed to be a key source of MeHg within anoxic sediments (Compeau and Bartha 1985).

3.3 Reduction of V(V)

Vanadium is a relatively abundant transition metal used primarily in the metallurgy industry. Its accumulation to problematic concentrations is commonly associated with mining of titaniferous magnetite deposits (Teng et al. 2006), and its release from the combustion of oil fuels (Costigan and Cary 2001).

The environmental chemistry of vanadium is comparatively complex, existing in three valence states; V(III), V(IV) and V(V), with a variety of complexes, ion pairs and polymers associated with each (Wanty and Goldhaber 1992). Generally, in environmental systems oxic dissolution of vanadium results in the oxidation of V(III) and V(IV) to V(V), which exists primarily as soluble vanadate (Wanty and Goldhaber 1992). Vanadium is an essential trace nutrient in many organisms (French and Jones 1993) and the human health risks of exposure primarily focus upon respiratory tract problems as a result of inhalation (Costigan and Cary 2001). Acute toxicology studies in rats and mice have shown both V(IV) and mobile V(V) compounds to be toxic to mammals when administered intravenously and orally (Llobet and Domingo 1984).

Remediation of mobile, toxic V(V) focuses upon stabilisation via reductive precipitation to the less mobile V(IV). Enzymatic microbial reduction was noted as early as 1962 where the vanadate (V) anion was reduced, with H_2 as the electron donor, to the vanadyl (IV) cation by *Micrococcus lactilyticus* (Woolfolk and Whiteley 1962). More recent studies have identified vanadium-resistant microbes able to reduce vanadate(V), even when exposed to high concentrations of up to 16 mM (Bisconti et al. 1997). Reduction coupled to growth, with V(V) as the sole electron acceptor, has been reported in the anaerobic metal reducers *G. metallireducens* (Ortiz-Bernad et al. 2004b) and *S. oneidensis* MR-1 (Carpentier et al. 2005; Carpentier et al. 2003). For the MR-1 strain, reduction was mediated by an electron transfer chain including the outer membrane cytochromes OmcA and OmcB, the periplasmic CymA and the cytoplasmic menaquinone (Myers et al. 2004). It should be noted however that these are also required for the reduction of other transition metals, and are not necessarily specific to V(V) reduction (Carpentier et al. 2005). The study of V(V) reduction by *G. metallireducens* presented evidence of in situ V(V) reduction at the Rifle, Colorado field site (Ortiz-Bernad et al. 2004b). The electron donor acetate was injected into groundwater containing up to 50 μM soluble V(V). Within 39 days of injection, some downstream monitoring wells recorded V(V) concentrations below detection limits, while others fell to below 6 μM V(V). The injection of acetate caused a dramatic increase in the proportion of microbes of the *Geobacteraceae* family, inferred to be responsible for either direct enzymatic V(V) reduction or indirect reduction via biogenic Fe(II). Recently, the V(V)-reducing bacterium *Enterobacter cloacae* EV-SA01 was isolated from a South African deep gold mine, suggesting a widespread occurrence of bacteria capable of V(V) reduction (van Marwijk et al. 2009).

3.4 Reduction of Co(III)

The radioactive isotope ^{60}Co occurs as a contaminant at several US Department of Energy sites, typically, with organic chelates such as EDTA (Means et al. 1978). This can be in the form of the Co(II) or Co(III) valence states, although the Co(III)-EDTA complex is more stable in the environment (Blessing et al. 2001).

Despite the short half-life of ^{60}Co (5.27 years), it still causes concern as Co(III)-EDTA is highly mobile and can quickly migrate in the subsurface, outside site boundaries (Hau et al. 2008). Reduction to Co(II)-EDTA can be a favourable remediation reaction as it dissociates, releasing ionic Co(II) which readily sorbs to iron oxides (Gorby et al. 1998).

Dissimilatory reduction of the Co(III)-EDTA complex coupled to growth by the Fe(III)-reducing bacterium *G. sulfurreducens* was first observed in (Caccavo et al. 1994). Since this study, other bacteria exhibiting Co(III)-EDTA reduction have been studied; *S. alga* (Gorby et al. 1998), *S. oneidensis* (Hau et al. 2008) and *Desulfovibrio vulgaris* (Blessing et al. 2001). Reduction by *D. vulgaris* was noted not to be related to growth, and was inhibited in the presence of sulphate, although abiotic reduction can take place via the formation of sulphides (Blessing et al. 2001). The presence of Mn(IV) oxides can also prevent effective Co immobilisation, as it was shown to re-oxidise Co(II)-EDTA to Co(III)-EDTA (Gorby et al. 1998). The common occurrence of Mn(IV) oxides within the subsurface could have implications for any in situ bioremediation attempts, although it should be noted that they are readily reduced by many metal-reducing bacteria.

The mechanism of Co(III)-EDTA reduction was investigated for *S. oneidensis* (Hau et al. 2008). This study demonstrated, using mutants, that the Mtr extracellular respiratory pathway was important for Co(III) reduction. Specific gene deletions showed the greatest loss in reduction when the periplasmic c-type cytochrome MtrA and the outer membrane protein MtrB were not expressed. Interestingly this study also found that Co(II)-EDTA had a profound toxic effect upon *S. oneidensis*.

3.5 Reduction of Pd(II)

The enzymatic reduction of soluble Pd(II) to nanoparticulate Pd(0) can be used to remove Pd from solution, while making a powerful catalyst for contaminant remediation (Lloyd et al. 2003). Synthetic nano-Pd(0), typically supported on a substrate, has received much attention as a catalyst capable of reducing a variety of organic and inorganic contaminants (Zhang 2003). When supplied with an electron donor, typically H_2 gas or formate, nano-Pd(0) can maintain high levels of reductive reactivity (Kopinke et al. 2004).

Conventional Pd(0) synthesis techniques utilise toxic chemicals and can be prohibitively expensive (De Corte et al. 2011). Therefore, biogenic methods provide a more cost effective and environmentally benign alternative (Lloyd et al. 2010). Pd is a highly valuable commodity and its extensive industrial uses, as catalysts, means the market for recovering used Pd is large (Lloyd et al. 2003). Early work focused upon harnessing the metal-reducing potential of sulphate-reducing bacteria to reduce and remove Pd(II) from solution (Lloyd et al. 1998). In this study, enzymatic Pd(II) reduction to nanoparticulate cell surface bound Pd(0) was observed by cells of *Desulfovibrio desulfuricans* utilising pyruvate, formate or H_2 as the electron donor. The use of H_2 as an electron donor, the inhibition of

activity by Cu(II) and the periplasmic deposition implicated hydrogenase and possibly cytochrome c_3 activity in this reduction.

Later studies, also using sulphate-reducing bacteria, found that in the absence of an added electron donor, an initial Pd(II) biosorption step occurred (Yong et al. 2002b). Subsequent electron donor additions, as formate, initiated reduction and precipitation to Pd(0) at an optimal acidic pH. During these studies, it is believed that the cell acts as a convenient nucleation site for metal precipitation on to the cell (Yong et al. 2002a). Later work expanded upon these findings, using another sulphate reducer, *Desulfovibrio fructosivorans* (Mikheenko et al. 2008). This study compared nucleation sites of three hydrogenase-deficient mutant strains to a wild-type bacterium, which has both periplasmic hydrogenases and cytoplasmic membrane-bound hydrogenases. In the wild-type strain, Pd(0) nucleation occurred in both the cytoplasmic membrane and the periplasm, whereas in the periplasmic hydrogenase-deficient strain nucleation sites were confined to the cytoplasm. These data implicated both cytoplasmic and periplasmic hydrogenases in Pd(0) reduction and nucleation. The cell-supported nano-Pd(0) formed had varying morphological and catalytic properties, dependent upon the bioreduction/biosynthesis methodology. This was compared for bio-synthesised Pd(0) catalysts by two differing bacterial species; *D. desulfuricans* and *Rhodobacter sphaeroides* (Redwood et al. 2007). The *D. desulfuricans* exhibited a higher frequency of nucleation sites, thus precipitating larger quantities of smaller Pd(0). These smaller Pd(0) particles recorded higher catalytic activity, measuring H_2 release, upon reaction with hypophosphite. Both of the cell types supported bio-Pd(0) particles with higher catalytic activity than a synthetic analogue. A study, using the metal reducer *S. oneidensis*, showed that cell distribution and particle area of the Pd(0) was controlled by the Pd(II) concentration cell ratio (De Windt et al. 2006). Interestingly, this study found that the larger particles were more effective for polychlorinated biphenyls (PCB) degradation, while smaller dispersed particles were optimised for perchlorate degradation.

Alongside direct enzymatic Pd(0) synthesis, a novel two-step biologically mediated synthesis technique was outlined in (Coker et al. 2010), yielding a nanoscale magnetically recoverable catalyst. Initially, a biomagnetite carrier was synthesised via the reduction of ferrihydrite, using cell suspensions of *G. sulfurreducens*. The resulting reactive bio-magnetite was then added to a solution of $NaPd(II)Cl_4$, where rapid reductive precipitation of Pd(0) occurred on the surface of the biomagnetite nanoparticles. The magnetically recoverable Pd(0) catalyst has potential for industrial synthesis reactions (e.g. Heck coupling) and for contaminant remediation (Table 4.1).

The resulting biogenic Pd(0) catalysts have been used to demonstrate a variety of reductive, dehalogenation and hydrogenation reactions (Lloyd et al. 2010). These have targeted a variety of organic contaminants including; PCBs (Baxter-Plant et al. 2003; De Windt et al. 2006; Harrad et al. 2007), flame retardant polybrominated diphenyl ether (PBDE) (Harrad et al. 2007; Deplanche et al. 2009), lindane (Mertens et al. 2007), trichloroethylene (TCE) (Hennebel et al.

Table 1 A summary of Pd(II)-reducing bacteria and the application of Pd(0) catalysts to contaminant systems. Adapted from (De Corte et al. 2011)

Pd(II) reducing bacterium	Pd(0) support	Target contaminant	Catalytic electron donor	References
Desulfovibrio desulfuricans *Desulfovibrio vulgaris* *Desulfovibrio* sp. Oz-7	biomass	PCBs	formate, H_2	Baxter-Plant et al. (2003)
Serratia sp. NCIMB 40259	biofilm	Cr(VI)	H_2	Beauregard et al. (2010)
Cupriavidus necator *Pseudomonas putida* *Paracoccus denitrificans*	biomass	–	–	Bunge et al. (2010)
Clostridium pasteurianum	biomass, biofilm	Cr(VI)	formate, H_2	Chidambaram et al. (2010)
N/A	biogenic magnetite	–	–	Coker et al. (2010)
Desulfovibrio desulfuricans *Bacillus sphaericus*	biomass	Cr(VI), 3-nitrostyrene, 4-azidoaniline	formate, H_2	Creamer et al. (2008)
Shewanella oneidensis	biomass	PCBs	formate, H_2	de Windt et al. (2006)
Shewanella oneidensis	biomass	PCBs, perchlorate	formate, H_2	de Windt et al. (2006)
Desulfovibrio desulfuricans	biomass	PBDE #47	H_2	deplanche et al. (2009)
Desulfovibrio desulfuricans	biomass	PBDE #47, PCBs	formate	Harrad et al. (2007)
Shewanella oneidensis	biofilm	TCE	H_2	Hennebel et al. (2009a, b)
Shewanella oneidensis	plate membrane reactor	TCE	formate, H_2	Hennebel et al. (2009a)
Shewanella oneidensis	electrolysis cell	TCE	H_2	Hennebel et al. (2011)
Desulfovibrio desulfuricans	biomass	Cr(VI)	formate	Mabbett and Macaskie (2002)
Shewanella oneidensis	biomass	lindane	formate	Mertens et al. (2007)
Desulfovibrio fructosivorans	–	–	–	Mikheenko et al. (2008)
Desulfovibrio desulfuricans *Rhodobacter sphaeroides*	biomass	PCP	formate	Redwood et al. (2007)
Desulfovibrio desulfuricans	biomass	–	–	Yong et al. (2002a, b)

2009b; Hennebel et al. 2009a; Hennebel et al. 2011) and the inorganic contaminant; Cr(VI) (Beauregard et al. 2010; Chidambaram et al. 2010).

Recent studies have focussed upon the development of remediation applications for bio-Pd(0), and several novel technologies have been proposed. Two studies, (Mertens et al., 2007, Hennebel et al. 2009a), utilised *S. oneidensis*-derived, cell-supported Pd(0) in membrane reactors to, respectively, dechlorinate lindane and dehalogenate TCE. The bio-Pd(0) was encapsulated within dialysis membranes and the contaminant stream cycled through the reactor as a treatment for wastewaters. Both studies observed efficient degradation with both formate and H_2 gas as the hydrogen donor, with H_2 exhibiting stronger catalytic activity for TCE degradation.

An alternative TCE wastewater treatment process incorporated a fixed bed reactor which encapsulated bio-Pd(0), also from *S. oneidensis*, within a variety of polymer and silica beads or supported on zeolites (Hennebel et al. 2009b). Reaction rates varied dependent upon porosity of the support, with the zeolite recording highest degradation rates. All however were substantially lower than those recorded for bio-Pd(0) suspensions and approximately 50 % of the aforementioned TCE membrane reactor, although the fixed bed reactor is thought to be a more cost-effective method. The fixed bed reactor technique was also used, in a modified form, as a support for a biofilm supported Pd(0) (Beauregard et al. 2010). A matrix of polyurethane foam disks were colonised with *Serratia* sp. bacteria in an air-lift fermenter and subsequently fitted into glass columns along with un-colonised controls. The column was then charged with Pd(II) solution and supplied with H_2 gas as the electron donor to promote reductive precipitation to Pd(0). After charging the bio-Pd(0) films with formate, Cr(VI) reduction was monitored in batch and continuous flow experiments. This was then monitored by UV–visible spectroscopy to quantify Cr(VI) concentration in solution and magnetic resonance imaging (MRI) to visualise the reduced Cr(III) in the columns. Both flow methods observed efficient reduction of Cr(VI) to Cr(III), although significant spatial heterogeneity was observed due to uneven bio-Pd(0) covering.

Few in situ remediation methods using BioPd have been reported, this is likely as a result of the high cost of Pd, which would be difficult to retrieve, and the difficulty of effective hydrogen supply to the subsurface (although formate could be a viable alternative). A notable exception provided a novel solution to hydrogen supply problems in the subsurface for the treatment of Cr(VI) detailed in (Chidambaram et al. 2010). Using the anaerobic metal-reducing and fermenting bacterium, *Clostridium pasteurianum* BC1, quartz sand was inoculated allowing a biofilm to coat the grains surface and precipitate cell supported Pd(0). Fermentation of a glucose medium by the bacterium subsequently produced an in situ hydrogen supply system. Reduction of Cr(VI) and immobilisation of reduced Cr(III) was confirmed by XANES and μX-ray Fluorescence (μXRF) analysis and effluent conditions showed efficient Cr(VI) removal compared to controls.

3.6 Reduction of Se(VI), Se(IV) and Se(0)

The metalloid, Se, can occur naturally in soils and waters at varying concentrations, ranging from "selenium deficient" to "seleniferous" (Garbisu et al. 1996). Alongside naturally occurring problematic high concentrations of Se, in seleniferous soils, anthropogenic activities can concentrate Se through processes such as agricultural irrigation, combustion and processing of fossil fuels (Haygarth 1994). Well-known examples are in the agricultural regions of California, where irrigation waters mobilize Se and transfer elevated concentrations to surface waters (Presser and Ohlendorf 1987).

Under environmental conditions, Se occurs in the following valence states; Se(VI), Se (IV) and Se(0) (Dungan and Frankenberger 1999). The former two species predominantly form the soluble and toxic anions; $Se(VI)O_4^{2-}$ and $Se(IV)O_3^{2-}$, while Se(0) is insoluble (Masscheleyn et al. 1990), thus making reductive stabilization to Se(0) and further to Se(-II) (selenide) a desirable remediation reaction (Lenz and Lens 2009). It should be noted however that re-oxidation and re-solubilization of Se(0) has been recorded indicating reductive precipitation is not a long-term solution (Losi and Frankenberger 1998).

Se uptake concentrations in animals are important, due to its requirement as an essential element; deficiency can cause a variety of severe health problems (Lenz and Lens 2009). An excess of Se, however, can also cause severe health problems, including dermal or neurological effects. Like other toxic oxyanions this is believed to be related to oxidative stress upon the cell (Lenz and Lens 2009). Toxicity is particularly a problem in livestock where bioaccumulation in feedstock can cause severe Se poisoning symptoms (Spallholz 1994). The effect of elevated Se levels has also been widely reported at the ecosystem level, in particular the bioaccumulation and poisoning of wildlife in the Kesterson area of California, resulting from highly seleniferous geology (Presser 1994).

The reduction of Se(VI) and Se(IV) oxyanions to particulate Se(0) and Se(-II) by bacteria has long been established in both pure cultures and environmental samples (Oremland et al. 1989). Reduction can be due to a detoxification strategy or coupled to growth. The dissimilatory reduction of Se oxyanions is expressed in a variety of bacteria (Dungan and Frankenberger 1999). A study using a facultative isolate from seleniferous agricultural wastewater, identified as *E. cloacae*, was able to reduce SeO_4^{2-} via the intermediary SeO_3^{2-} to nanoparticulate Se(0) (Losi and Frankenberger 1997). This study indicates intracellular reduction, alongside NO_3^- reduction, by membrane bound reductases followed by expulsion of the Se(0) nanoparticle. The intensively studied organisms *S. oneidensis* and *G. sulfurreducens* have been shown to exhibit the capacity for Se(IV) reductions (Pearce et al. 2009). This study demonstrated different mechanisms for reduction between the two metal reducers, resulting in different end products of metabolism, whereby *G. sulfurreducens* was able to reduce Se(IV) to Se(0), and then further to Se(-II) while *S. oneidensis* could only reduce Se(IV) to nanoparticulate Se(0) phases. This study also implicated the involvement of *c*-type cytochromes and ferredoxin in the

formation of Se(0) nanoparticles. Further reduction of Se(0) to Se(-II) is noted in other bacterial strains such as; *Bacillus selenitireducens* (Herbel et al. 2003) and *Veillonella atypical* (Pearce et al. 2008). The former of these studies found when incubated with seleniferous sediments the end product of bacterial reduction was as solid precipitate of FeSe as opposed to soluble HSe^-, indicating a possibly in situ treatment application for the organism. The latter study showed that biogenic selenide, after reaction with transition metals, could be used to synthesize quantum dots for photonic applications. *Ex situ* bioreactor treatment using the Se-reducing organism *Bacillus* sp. SF-1 has also been explored (Fujita et al. 2002). This study demonstrated reduction of Se(VI), via Se(IV), to Se(0) in a synthetic waste-stream by the organism, using lactate as an electron donor.

4 Enzymatic Reduction of Actinides and Fission Products

Human exposure to radiation is principally from naturally occurring radionuclides. There is, however, public concern regarding exposure as a result of anthropogenic activities such as nuclear weapons testing and nuclear power generation (MacKenzie 2000). Currently, nuclear power activities are the principal anthropogenic release of radionuclides (Lloyd et al. 2002). Several processes in the nuclear fuel cycle result in localised radionuclide release alongside concerns over the resulting radioactive waste generated (Macaskie 1991). Much public concern regarding nuclear power generating processes is as a result of high profile and large-scale accidents such as the 1986 Chernobyl and 2011 Fukushima disasters.

The bioremediation of radioactive waste has also received much attention. In common with many transition metal, several key radionuclides including both actinides and fission products such a technetium are redox active, and become less soluble in their reduced state (Lloyd 2003); see Fig. 4 for a graphical representation of key oxidation states of key priority radionuclides. Microbial reduction has therefore been the focus of several bioremediation strategies (Lloyd 2003).

4.1 Reduction of U(VI)

Uranium is a common radionuclide contaminant occurring at sites associated with various aspects of the nuclear fuel cycle. Under environmental conditions it most commonly occurs as the U(IV) and U(VI) oxidation states. The former occurring primarily as poorly soluble U(IV) oxides and the latter as widely soluble U(VI) carbonate complexes, for example $UO_2((CO_3)_2^{2-}$ and $UO_2((CO_3)_3^{4-}$ (Clark et al. 1995). Reduction can therefore lead to immobilisation of uranium and could offer

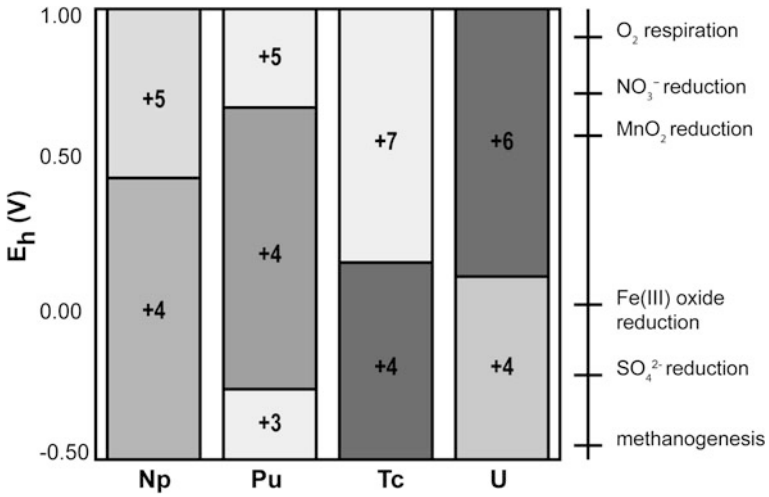

Fig. 4 Oxidation states of key contaminant actinides and fission products addressed within this chapter, presented as standard reduction potentials at pH 7. A redox tower of common metabolic processes is located on the right for comparison. Adapted from (Silva and Nitsche 1995; Lloyd et al. 2002; Stumm 1996; Banaszak et al. 1999)

a useful remediation mechanism, preventing migration of U(VI) to groundwater resources.

The accumulation of uranium within anaerobic environments had previously been explained by indirect reduction mechanisms, for example linked to the microbial production of reducing agents such as Fe(II) or sulphide (Lovley et al. 1991). Direct enzymatic microbial U(VI) reduction coupled to growth was first demonstrated for the dissimilatory metal reducers *G. metallireducens* and *S. oneidensis,* with acetate and H_2 as the respective electron donors (Lovley et al. 1991). This work was soon followed by the demonstration of U(VI) reduction using the sulphate-reducing bacteria, *D. desulfuricans*, (Lovley and Phillips 1992) and *D. vulgaris* (Lovley et al. 1993). Although these sulphate reducers were unable to conserve energy for growth in these experiments, U(VI) did rapidly transform to U(IV), forming poorly soluble uraninite (UO_2). Importantly enzymatic reduction occurred faster than abiological mechanisms, suggesting an environmentally relevant role for enzymatic U(VI) reduction (Lovley et al. 1991). The mechanism of enzymatic U(VI) reduction in *D. vulgaris* was shown to be mediated by cytochrome c_3 (which is also a Cr(VI) reductase), as reductive capability was lost when this electron transfer protein was removed from the soluble fraction of cells via a cationic exchange column (Lovley et al. 1993). The mechanism of U(VI) reduction by *G. sulfurreducens* seems to be more complicated, with several of the many cytochromes in this organism implicated, including those in the periplasm (Lloyd et al. 2002) and outer membrane (Shelobolina et al. 2007), while a recent report has suggested that extracellular nanowires could also be involved (Cologgi et al. 2011).

Whatever the precise mechanism of electron transfer to U(VI) in this important model organism, reduction most likely proceeds via a single electron transfer to form an unstable U(V) intermediate, which then disproportionates to give U(IV) (Renshaw et al. 2005).

Several studies have addressed the complex behaviour of U(VI) within environmentally relevant substrates. A study using a variety of synthetic and natural Fe(III) oxides and the U(VI) reducer *G. sulfurreducens* found that U(VI) reduction was slower in the presence of natural Fe(III) oxide phases (Jeon et al. 2004), suggesting that at circumneutral pH, aqueous U(VI) can be removed by reduction, while the adsorbed fraction can remain and potentially desorb over time if under non-reducing conditions (Jeon et al. 2004). These findings were supported by experiments with U(VI) contaminated sediments, where the rate of microbial reduction of aqueous U(VI) was far greater than that of adsorbed U(VI) (Ortiz-Bernad et al. 2004a). Studies using sediments and waters collected from near a uranium mill tailings pile, located in Shiprock, New Mexico, also found that under anaerobic conditions addition of acetate or glucose stimulated reduction from 10 μM soluble U(VI) to <1 μM U(VI) within 15 days (Finneran et al. 2002). This reduction was considered enzymatic, as addition of the abiotic reductants; Fe(II), sulphide and reduced AQDS, did not reduce U(VI) in the sediments. Using the same sediments, 16S rDNA analysis found that acetate amendment, and also Fe(III) and U(VI) reduction, corresponded to an increase in the proportion of sequences belonging to the family *Geobacteraceae*, to ca. 40 % (in comparison to 5 % in un-amended controls; (Holmes et al. 2002)). These studies support the hypothesis that bacteria of the family *Geobacteraceae* can, via electron donor addition, enzymatically reduce and remove aqueous U(VI) from contaminated soils and sediments.

A significant number of studies have looked at the possible bioremediation of U(VI) via in situ enzymatic reduction at U.S. Department of Energy (U.S. DOE) sites (Roh et al. 2000). One such site, at Oak Ridge, Tennessee, has extensive U(VI) contamination with levels up to 60 mg/L U(VI) in groundwater and 800 mg/kg in sediments (Cardenas et al. 2008). The groundwater conditions in some parts of the site present challenges to in situ bioremediation due to an acidic pH, ~3.4, high concentrations of clogging agents such as Al and Ca, and U(VI) reduction inhibitors (Wu et al. 2006a). Therefore, prior to bioremediation tests, a pre-treatment groundwater recirculation system was established consisting of a series of pumps and wells, detailed in (Wu et al. 2006a). This enabled pumping and *ex situ* treatment of groundwater to remove the problematic components and buffer the pH to a circumneutral value. It should be noted during groundwater pre-treatment, the U(VI) concentration fell from ~300 to ~5 μM linked to neutralisation and subsequent uranium precipitation. Ethanol was chosen as the electron donor for in situ biostimulation, as bench experiments indicated faster U(VI) reduction than with other electron donors (Wu et al. 2006b). The results of this trial, presented in (Wu et al. 2006b), show that periodic additions of ethanol and low level carbonate to desorb U(VI), over an approximately 400 day period, first resulted in a denitrification phase followed by U(VI) reduction. Groundwater

U(VI) concentrations fell from 5 to ~1 μM. The proportion of uranium as reduced U(IV), determined by XANES analysis of sediment samples, increased from below detection limit prior to treatment to 51 %, 35 % and 28 % after treatment for the injection, monitoring and extraction wells, respectively. Further studies of this treatment area note that after 2 years of operation the uranium levels fell below the U.S. EPA drinking water maximum of 0.126 μM (Cardenas et al. 2008, Xu et al. 2010). Treated wells showed an increase in denitrifiers, sulphate and iron-reducing bacteria compared to untreated wells from 16S rRNA analysis (Cardenas et al. 2008). Specific U(VI)-reducing bacteria were identified including *Geobacter* spp., *Anaeromyxobacter* spp., *Desulfovibrio* spp., *Desulfosporosinus* spp. and *Acidovorax* spp. A subsequent study carried out functional gene array (GeoChip) analysis of sediment from the electron donor treated and untreated wells (Xu et al. 2010). Treated wells recorded higher total signal intensities of functional gene categories, including those associated with U(VI) reduction; e.g. cytochrome *c* and metal resistance genes. The sum of these studies at the Oak Ridge site have given a deeper insight into the complexities of in situ U(VI) bioremediation projects.

A further extensive field study focuses on a U(VI)-contaminated aquifer at the former uranium ore processing facility in Rifle, Colorado (Anderson et al. 2003). In situ biostimulation treatment of the U(VI) groundwater plume, described in detail in (Anderson et al. 2003, Williams et al. 2011), was assessed by a series of 15 monitoring wells downgradient and untreated control wells upgradient of the injection wells. To stimulate microbial activity the electron donor acetate (1–3 mM) was injected into the groundwater plume. Within 9 days of injection, aqueous U(VI) concentrations had fallen from 0.6–1.2 μM to below the target limit of 0.18 μM in some of the wells, coinciding with an increase in Fe(II). However, it was noted that after 50 days U(VI) concentrations began to increase again, and Fe(II) levels decreased. This initial study also correlated U(VI) removal and Fe(II) production with an increase in bulk biomass and *Geobacter* species abundance. The authors inferred that *Geobacter* species were predominantly responsible for enzymatic U(VI) and Fe(III) reduction. It should be noted the later increase in U(VI) correlates with a period of sulphate reduction where sulphate reducers dominate the microbial ecology of the study site, suggesting these are not able to reduce U(VI) in these sediments. Interestingly lactate (but not acetate)-dependent sulphate reducers are known to reduce U(VI). A further study used a more detailed sampling arrangement, including depth profiles, of the same field sampling array (Vrionis et al. 2005). This study reported greater heterogeneity of the geochemistry and microbial ecology than previously demonstrated with areas of high U(VI) reduction occurring at disparate areas of the array. Again, increased representation of *Geobacter* 16S rRNA gene sequences correlated with areas of maximal U(VI) removal and Fe(II) production. A more recent study has applied proteomic monitoring of the bacterial species at this site (Wilkins et al. 2009). Peptide analyses of planktonic organisms in the areas, where *Geobacter* species dominated, were compared to those expected from those encoded by seven *Geobacter* genome sequences. Closest matches were to *Geobacter bemidjiensis*

proteins, while data suggested that other species closely related to *Geobacter lovleyi* became dominant during later stages of the field trial. The proteome of the samples, throughout acetate addition, were dominated by enzymes related to acetate metabolism and energy generation and thus inferred to represent microbial proliferation and growth linked with acetate-driven U(VI) and Fe(III) reduction. The series of studies at this site again depict the complexities encountered during in situ biostimulation remediation of U(VI), and have enabled greater knowledge of how to approach such projects over prolonged periods of U(IV) bioreduction.

4.2 Reduction of Tc(VII)

Technetium is a product of nuclear fission with appreciable amounts produced during nuclear weapon tests and as a by-product of the nuclear fuel cycle (Schulte and Scoppa 1987). It occurs predominantly as the β-emitting ^{99}Tc isotope which due to its long half-life, 2.15×10^5 years, is an important component of high and medium level wastes (Schulte and Scoppa 1987). Within most oxic environmental systems, technetium occurs in the Tc(VII) valence state as the pertechnetate anion (TcO_4^-). Pertechnetate is highly soluble in water and exhibits poor adsorption to mineral surfaces, making it highly mobile in the subsurface where its migration can contaminate groundwater resources (Icenhower et al. 2008). However, technetium under reducing conditions usually exists as relatively insoluble Tc(IV), as less soluble hydrated TcO_2 or mineral surface adsorbed Tc(IV) (Lear et al. 2009).

Early studies recorded a removal of Tc(VII) from solution under anaerobic conditions in the presence of mixed bacterial cultures (Henrot 1989) or sediments (Pignolet et al. 1989). These were, however, unable to identify the mechanism of removal. Direct enzymatic microbial reduction of Tc(VII) was later confirmed in cultures of *G. metallireducens* and *S. oneidensis* (Lloyd and Macaskie 1996). In this study *G. metallireducens* was able to remove 70 % Tc(VII) from a ~0.27 mM solution within 22 h. *S. oneidensis* was able to reduce 60 % of the Tc(VII), but the reduced form in cultures of this organism were largely soluble under the conditions tested. Both *S. oneidensis* (Wildung et al. 2000) and another *Geobacter* spp.; *G. sulfurreducens* (Lloyd et al. 2000a) respectively, exhibited optimal Tc(VII) reduction capacity when H_2, (rather than lactate or acetate), was used as the electron donor. The fate of the reduced Tc(IV) in the former study consisted of a dark Tc(IV) oxide associated with the periplasm and cell surface, although when in bicarbonate medium, extracellular particulates and possible carbonate complexes formed. The latter study used transmission electron microscopy (TEM) and XAS to confirm Tc(VII) reduction to Tc(IV) as TcO_2, see Fig. 5. In this latter study, very rapid and efficient reduction of Tc(VII) mediated by biogenic Fe(II) e.g. associated with magnetite, was also demonstrated. Given the very efficient scavenging of Tc(VII) by biogenic Fe(II), and the less efficient enzymatic (hydrogenase mediated) mechanism (Lloyd et al. 1997a, b, 1999, 2000a), many subsequent studies have focused on indirect mechanisms of Tc(VII) reduction. These include

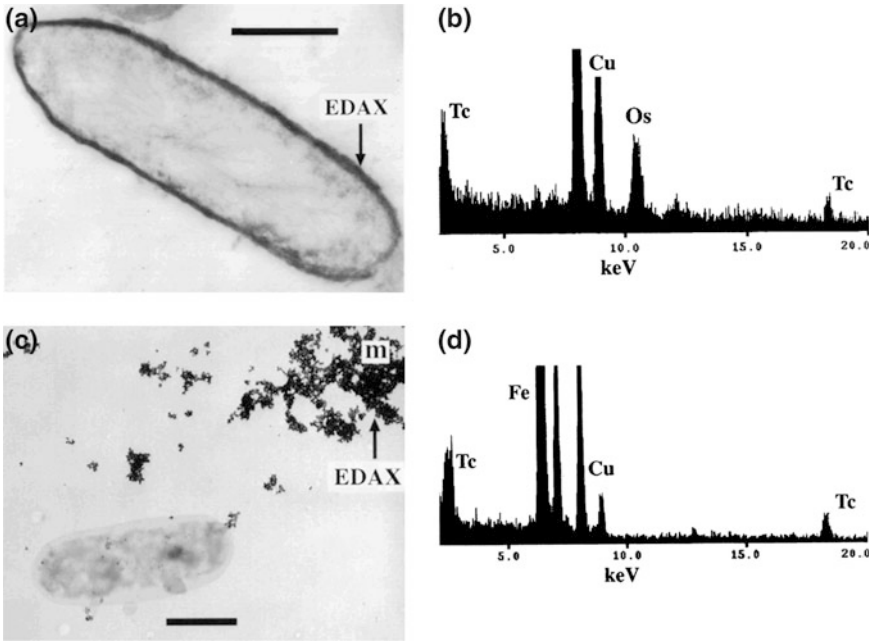

Fig. 5 TEM images of thin sections of *G. sulfurreducens* with Tc-containing precipitates. **a** An electron dense periplasmic Tc precipitate formed via direct enzymatic reduction. **b** EDX spectrum associated with the periplasmic precipitate in a. **c** TEM image of air dried whole-cell preparations with extracellular Tc-containing magnetite crystals via indirect Fe(II) mediated Tc(VII) reduction. **d** EDX spectrum of the annotated area in C, showing Tc presence on the magnetite. Bars 0.5 μm. Diagram adapted from (Lloyd et al. 2000a)

studies on aquifer sediments, collected from the Oak Ridge Field Research Centre (McBeth et al. 2007), estuarine sediments (Burke et al. 2005) and model Fe(II) biomineral phases (McBeth et al. 2011). The clear involvement of Fe(III)-reducing bacteria in Oak Ridge sediments has also been confirmed using novel nuclear imaging techniques, in combination with molecular ecology and mineralogical approaches (Lear et al. 2009).

4.3 Reduction of Np(V)

Neptunium (Np), like Tc, has a long half-life (2.14×10^6 years) and is considered an important radioactive component of high and intermediate level wastes over geological timespans (Sasaki et al. 2001). This is primarily due to the isotope ^{237}Np which increases in proportion within wastes due to the radioactive decay of the short lived isotope ^{241}Am (half-life = 423.7 years) (Kaszuba and Runde 1999). ^{237}Np is a key α-emitting isotope and is regarded as chemically and

radiologically toxic to life (Ruggiero et al. 2005). Under most environmental conditions Np is stable as the Np(IV) and Np(V) valence states (Kaszuba and Runde 1999). Np(V) occurs primarily in oxic conditions, as the soluble NpO_2^+ species which is highly mobile in the subsurface as it sorbs poorly to mineral phases (Kaszuba and Runde 1999). By contrast Np(IV) species, which dominate in reducing conditions, are less soluble and can form complexes which are easily adsorbed to mineral phases (Law et al. 2010). Thus, a reduction from Np(V) to Np(IV) potentially leads to immobilisation of Np within the subsurface.

Direct enzymatic reduction of soluble Np(V) species has been demonstrated for the metal-reducing bacterium *S. oneidensis* (Lloyd et al. 2000b; Icopini et al. 2007). The former study, using paper chromatography with phosphorimaging techniques for Np oxidation state separation, suggested Np(V) to Np(IV) bioreduction after inoculation with *S. oneidensis,* with H_2 as the electron donor. The majority of the resulting Np(IV) remained in solution, but in combination with an extracellular phosphate producing *Citrobacter* sp., 95 % of Np(IV) was removed, presumably as an Np(IV) phosphate. Then in the latter study (Icopini et al. 2007), however, *S. oneidensis* was able to simultaneously reduce and precipitate Np(IV) from a Np(V) solution, using lactate as the electron donor. The same study failed to replicate this reduction with the bacterium *G. metallireducens*, and it has been shown previously that *G. sulfurreducens* is also unable to reduce Np(V) (Renshaw et al. 2005). *G. metallireducens* did show Np reduction when Np(V) was present as a citrate complex, which was inferred to reduce its toxic effects upon the cell. A further study was able to reduce and precipitate Np(V) to Np(IV), utilising either pyruvate or H_2 as electron donors (Rittmann et al. 2002). This was achieved using a consortium of bacteria, containing several sulphate reducers of the *Desulfovibrio* genus, isolated from creek sediments. When pyruvate was used as the electron donor, complexation of Np(IV) with fermentation intermediates was suggested to prevent complete precipitation. The contribution of enzymatic Np(V) reduction to total reduction within complex environmental media, such as sediments, is difficult to determine. A recent study tackled this challenge and attributed reduction in sediment microcosms to abiotic reduction by Fe(II) coupled to microbial Fe(III) reduction (Law et al. 2010).

4.4 Reduction of Pu(V) and Pu(VI)

The release of Pu into the environment is of particular concern due to its long half-life (2.4×10^4 years) and its high toxicity (Boukhalfa et al. 2007). Its biogeochemical behaviour is more complex than the other actinides as it can occur in the environment as four valence states; Pu(III), Pu(IV), Pu(V) and Pu(VI) (Panak and Nitsche 2001). The oxidised forms, Pu(V) and Pu(VI), generally occur as relatively more soluble species than the Pu(IV) species (Neu et al. 2006). The solubility of Pu(III) however is often controlled by the presence of complexing ligands which increase its solubility (Icopini et al. 2009). The action of humics in natural

systems can also reduce Pu(V) and Pu(VI) to Pu(IV) thus effectively decreasing the solubility of Pu (Choppin et al. 2001). The Pu(IV) valence state is easily hydrolysed, causing adsorption to particles and generally maintains its low solubility at circum-neutral pH (Choppin et al. 2001).

Due to the complexities of Pu chemistry only a few studies have approached microbial Pu reduction (Neu et al. 2006). These have focussed upon the intensely studied metal-reducing bacteria of *S. oneidensis* (Boukhalfa et al. 2007; Icopini et al. 2009; Renshaw et al. 2009), *S. alga* (Reed et al. 2007; Deo et al. 2011), *G. metallireducens* (Boukhalfa et al. 2007; Icopini et al. 2009) and *G. sulfurreducens* (Renshaw et al. 2009). Enzymatic reduction of higher valence Pu(V) and Pu(VI) to Pu(IV) was observed for *S. oneidensis* and *G. metallireducens* (Icopini et al. 2009). The former exhibiting the highest reduction rates with near complete reduction of $\sim$0.4 mM within 5 h using a cell density of 5×10^8 cells/ml. The resulting Pu(IV) formed a crystalline nanoparticulate phase on the surface or within the cell walls of the bacteria thus immobilising Pu from solution. Importantly, no further reduction to the more soluble Pu(III) was noted. A further study demonstrated reduction of Pu(V) (PuO_2^+) via a Pu(IV) intermediate to Pu(III) as a $PuPO_4$ precipitate, using the bacterium *S. alga* (Deo et al. 2011). This was carried out in the presence and absence of Fe(III) and a nitrilotriacetic acid (NTA) ligand, although at higher NTA concentrations, some soluble Pu(III) complexes were observed. Reduction of Pu(VI) to Pu(IV) by *G. sulfurreducens* concurrent with a decrease of aqueous Pu has also been recorded (Renshaw et al. 2009). This study also noted limited further reduction to Pu(III), by *G. sulfurreducens* and *S. oneidensis*. This however did not cause an increase in the soluble Pu fraction, theorised to be the result of adsorption to the cell surface. Other studies have observed an increase in Pu solubility from the reduction of Pu(IV) to Pu(III) when in the presence of an EDTA ligand (Boukhalfa et al. 2007). This study however recorded no reduction of the amorphous $Pu(IV)(OH)_4$ starting material in the absence of complexing agents.

Despite the potential to mobilise Pu(IV), few studies have addressed the impact of stimulated bioreduction strategies on Pu solubility in environmental systems. However, a recent study using Pu contaminated soils from the UK Atomic Weapons Establishment (AWE) site, Pu occurring as Pu(IV) proved highly refractory during the bioreducing conditions (Kimber et al. 2012). Thus in sediments contaminated with a broad-range of redox active radionuclides, reductive immobilisation of uranium, neptunium and technetium would be expected, without the mobilisation of any Pu(IV) present.

5 Conclusions

The role of microbial metal reduction in the bioremediation of environments contaminated with metals and organics is now well established. Underpinning investigations range from those addressing a molecular-scale understanding of

microbial reduction to the complexities associated with field application. The emerging "omics" approaches to environmental microbiology, alongside other advanced analytical techniques, are increasingly being utilised to give greater insights into bioremediation processes. While many of these studies are currently at a "proof of concept" laboratory-scale, some notable exceptions have been implemented for field or industrial-scale problems. These can lead to the complete oxidation of toxic organics, to the irreversible reduction of toxic metals and radionuclides; see (Law et al. 2010; McBeth et al. 2007) for recent examples of reduced, insoluble radionuclide phases that are resistant to re-oxidation and re-mobilisation. It can be anticipated that further field-scale applications utilising microbial metal reduction as a bioremediation tool will emerge.

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Dissimilatory Metal Reducers Producing Electricity: Microbial Fuel Cells

Sven Kerzenmacher

Abstract This chapter provides an introduction to the application of dissimilatory metal reducers in microbial fuel cells. In this type of fuel cells, exoelectrogenic bacteria act as anodic electrode catalysts and enable the direct generation of electricity from e.g., waste waters and other organic carbon sources. The chapter covers the basic thermodynamic principles of electrochemical energy conversion and the interrelations between cell voltage, power density, and efficiencies. Furthermore, important aspects of fuel cell construction are discussed, including reactor design as well as suitable anode materials and catalysts for cathodic oxygen reduction. Special importance is given to fuel cell characterization techniques that allow researchers to evaluate the power output of a microbial fuel cell and distinguish the different loss mechanisms that govern its performance. The chapter closes with a comparison of typical application examples and a perspective on future challenges and trends in the field of microbial fuel cells, also regarding emerging applications beyond electricity generation.

1 Introduction

The observation that microorganisms can produce useful electricity dates back to the beginning of the twentieth century. As early as 1911, M. C. Potter, Professor of Botany at the University of Durham, reported on the "electro motive force" developed by cultures of *Saccharomyces cerevisiae* and *E. coli* growing in vicinity of a platinum electrode (Potter 1911). In 1931, Cohen expanded these observations by

S. Kerzenmacher (✉)
Laboratory for MEMS Applications, IMTEK—Department of Microsystems Engineering, University of Freiburg, Georges-Koehler-Allee 103, 79110 Freiburg, Germany
e-mail: sven.kerzenmacher@imtek.uni-freiburg.de

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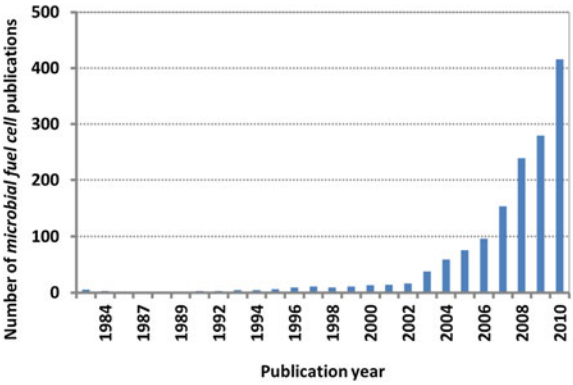

Fig. 1 Evolution of microbial fuel cell publications since 1983. Data extracted from an ISI Web of Science query with the search term topic = "microbial fuel cell"

demonstrating that also *Bacillus subtilis* and *Proteus vulgaris* function as an electrochemical half-cell and generate electrical currents (Cohen 1931). However, the concept found no immediate practical application and was thus quickly forgotten. Later, research activities in the field of microbial fuel cells were driven by space and military research during the 1960s, and the use of microbial fuel cells not only as power source but also as toxicity sensors and for the generation of oxygen from CO_2 for blood oxygenation were discussed (Thomson and Brady, 1963). Still, in 1966 Kenneth Lewis concluded at the *Annual Meeting of the American Society for Microbiology* that *the available results indicate that a direct biofuel cell in which the microbial cells are located at the electrode has little value […] the systems are not competitive with conventional power sources* (Lewis 1966).

Nevertheless, with the beginning of the twenty-first century research on microbial fuel cell systems has regained considerable and further growing interest, as illustrated by the number of publications on microbial fuel cells since 1983 shown in Fig. 1. This is not only related to the increasing necessity to develop environmentally friendly and renewable power sources, but also to the discovery of the direct electron transfer capability of *Shewanella putrefaciens* (*oneidensis*) and the consequent demonstration of the first mediator-less microbial fuel cell by Kim et al. in 1999 (Kim et al. 2002; Logan 2008). The following chapter of this book is intended to provide readers from different disciplines with a general overview on the overall concept, technology, design, characterization, and applications of microbial fuel cells, in which exoelectrogenic bacteria act as anodic electrode catalysts. For further reading on microbial fuel cells the detailed and comprehensive textbooks "Bioelectrochemical systems" (Rabaey et al. 2010) and "Microbial Fuel Cells" (Logan 2008) as well as the cited literature accompanying each section of this chapter are recommended.

1.1 Bacteria as Electrode Catalysts—Microbial Fuel Cells

In principle, any chemical reaction involves the transfer of electrons from an oxidizable species ("fuel") to a reducible reaction partner ("oxidant"). This exchange

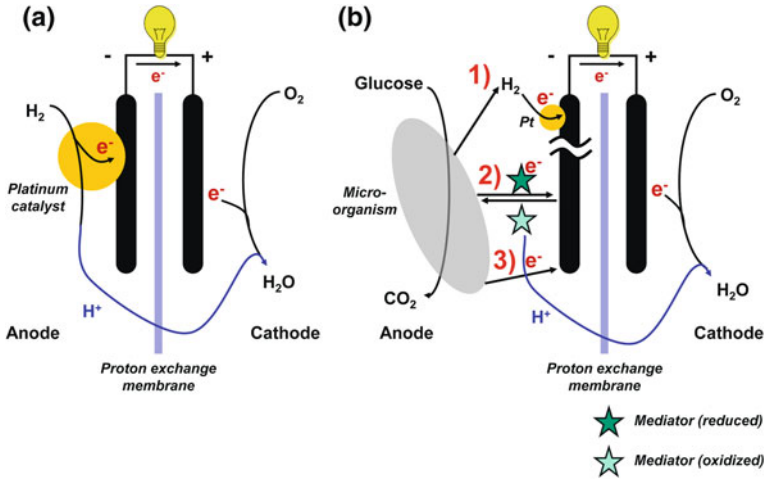

Fig. 2 Fuel cell principles. **a** Conventional H_2–O_2 fuel cell with platinum as anode catalyst. **b** Three different types of microbial fuel cells: (1) indirect, (2) mediated, and (3) mediator-less type. See text for explanations

of electrons can be transformed into a useful electrical current by means of a fuel cell. Here, oxidation and reduction reaction take place at two spatially separated electrodes, connected through an external electrical load circuit, as illustrated in Fig. 2. At the anode, the "fuel" is oxidized, releasing its electrons. These travel through the external load to the cathode, where an "oxidant" (usually oxygen) accepts these electrons and is, thus, reduced. The driving force for the electron flow from anode to cathode is the difference in redox potential between the oxidation and reduction reaction. To enable the electrode reactions of a fuel cell, catalysts are required. As illustrated in Fig. 2, not only noble metals such as platinum, but also the enzymatic systems of living microorganisms can act as electrode catalysts. In practice, three types of microbial fuel cells can be distinguished:

- In *indirect microbial fuel cells* microbes break down a complex fuel into smaller molecules [e.g. glucose into H_2, for example by *clostridium butyricum* (Karube et al. 1977)] which can be easily oxidized on e.g., a platinum electrode. The microbes are not necessarily in direct contact with the electrode and do not directly exchange electrons with it. The disadvantage of these systems is the need for costly noble metal catalysts to enable the oxidation of the microbial fermentation products.
- *Mediated microbial fuel cells* require a mediator or electron shuttle to transfer metabolic electrons from the microorganism to the anode. Examples are the use of *E. coli* or *Saccharomyces cerevisiae* (Bennetto et al. 1983). Typically, mediators are small redox-active molecules [e.g. thionine, viologens, and methylene blue (Chang et al. 2006)] which can diffuse into the microbial cell and can be reduced there. At the anode, they in turn release electrons and are thus oxidized. The main disadvantage of such systems is the cost and loss of mediators in flow-through systems.

- In *mediator-less microbial fuel cells* dissimilatory metal reducing bacteria are used, which possess the ability to transfer electrons using surface exposed proteins. Such microbes can directly live on an electrode and transfer respiratory electrons released upon metabolic oxidation of an organic fuel to the fuel cell's anode. Mediators or electron shuttles are not necessary.

As mentioned before, the first mediator-less microbial fuel cell was demonstrated by Kim et al. in 1999 (Kim et al. 2002) using *Shewanella oneidensis (putrefaciens)*. In the meantime, further exoelectrogenic microorganisms found practical application at the anode of microbial fuel cells. Example organisms are: *Aeromonas hydrophilia*, *Clostridium butyricum*, *Desulfobulbus propionicus*, *Enterococcus gallinarum*, *Geobacter sulfurreducens*, and *Rhodoferax ferrireducens* (Chang et al. 2006). The prime advantage of exoelectrogenic bacteria in the design of microbial fuel cells is the abdication of the otherwise required mediators. Mediators are not only potentially toxic, but can also become a cost factor (Chang et al. 2006). This becomes particularly relevant if large systems or flow-through setups e.g., operated with waste water streams are considered.

In addition to the direct electron transfer via membrane-bound proteins, some exoelectrogenic bacteria are also capable of secreting natural redox mediators (Gralnick and Newman 2007; Marsili et al. 2008), or transferring electrons throughout the biofilm by e.g., bacterial nanowires (Gorby et al. 2006). These capabilities enlarge the number of bacterial cells that are electrically connected to the electrode, and can thus increase the power output of a microbial fuel cell (see also "On the Role of Endogenous Electron Shuttles in Extracellular Electron Transfer" and "Humic Substances and Extracellular Electron Transfer").

1.2 Fuel Cell Voltage, Current, Power, and Efficiency

In general, a fuel cell system is characterized by its voltage, current and power density, as well as efficiency. In the following section, the thermodynamic and electrochemical principles behind these figures are introduced (the textbooks "Fuel Cell Systems Explained" by Larminie and Dicks (2000) and "Microbial Fuel Cells" by Logan (2008) are suggested for further reading).

Using acetate as exemplary *fuel* that is fully oxidized to carbon dioxide and oxygen as *oxidant* (or terminal electron acceptor), the electrode reactions of a microbial fuel cell are

$$\text{Anode: } C_2H_3O_2^- + 4H_2O \rightarrow 2HCO_3^- + 9H^+ + 8e^- \quad (1)$$

$$\text{Cathode: } 2O_2 + 8H^+ + 8e^- \rightarrow 4H_2O \quad (2)$$

$$\text{Overall: } C_2H_3O_2^- + 2O_2 \rightarrow 2\ HCO_3^- + H^+ \quad (3)$$

Table 1 Values for the standard free energy of formation ΔG_f^0 (Thauer et al. 1977) for different compounds of relevance in the microbial fuel cell context

Substance	ΔG_f^0 at 25 °C kJ mol^{-1}
O_2	0
H_2O	−237.178
H^+	0
HCO_3^-	−586.85
Acetate$^-$	−369.41
Lactate$^-$	−517.81

Let us first consider the *standard redox potential* (or *standard electromotive force*) ΔE_r^0 of the overall electrochemical fuel cell reaction. According to thermodynamics, this is the maximum voltage a single fuel cell can produce. It can be calculated from the *standard free energy* of the overall reaction ΔG_r^0

$$\Delta E_r^0 = -\frac{\Delta G_r^0}{n\mathrm{F}} \tag{4}$$

wherein $n = 8$ is the number of electrons transferred in the reaction and F = 96,500 C mol^{-1} is the Faraday constant (Mortimer 1996). The *standard free energy* of the overall reaction ΔG_r^0 can be calculated from tabulated values (see for instance Table 1) of the *standard free energy of formation* ΔG_f^0 using the relation (Thauer et al. 1977)

$$\Delta G_r^0 = \Sigma \Delta G_f^0\,(\text{Products}) - \Sigma \Delta G_f^0\,(\text{Educts}) \tag{5}$$

For reaction (3) the *standard free energy* ΔG_r^0 and the standard potential ΔE_r^0 thus calculate to

$$C_2H_3O_2^- + 2O_2 \rightarrow 2\ HCO_3^- + H^+$$

$$\begin{aligned}\Delta G_r^0 &= (2 \cdot (-586.85) + 0)\ \mathrm{kJmol^{-1}} - (-369.41 + 2 \cdot 0)\ \mathrm{kJmol^{-1}} \\ &= -804.29\,\mathrm{kJmol^{-1}}\end{aligned}$$

$$\Delta E_r^0 = -\frac{\Delta G_r^0}{n\mathrm{F}} = \frac{804.29\,\mathrm{kJ\,mol^{-1}}}{8 \cdot 96500\,\mathrm{C\,mol^{-1}}} = 1.042\,\mathrm{V}$$

In a similar way also the potentials (with reference to the standard hydrogen electrode SHE) of the half-cell reactions, and thus the individual electrodes can be calculated. Consider that according to the IUPAC convention (Logan 2008) the direction of the anode reaction Eq. (1) has to be reversed, so that the products are the reduced species (educts + e$^-$ → products). This leads to

$$\text{Anode: } 2\ HCO_3^- + 9H^+ + 8\ e^- \rightarrow C_2H_3O_2^- + 4\ H_2O$$

$$\begin{aligned}\Delta G^0_{\text{anode}} &= (-369.41 + 4 \cdot (-237.178)) \; - \; (2 \cdot (-586.85) + 9 \cdot 0) \; \text{kJ mol}^{-1} \\ &= \; -144.422 \, \text{kJ mol}^{-1}\end{aligned}$$

$$\Delta E^0_{\text{anode}} = -\frac{\Delta G^0_{\text{anode}}}{n\text{F}} = \frac{144.422 \, \text{kJ mol}^{-1}}{8 \cdot 96500 \, \text{C mol}^{-1}} = 0.187 \, \text{V vs. SHE}$$

$$\text{Cathode: } 2O_2 + 8H^+ + 8e^- \rightarrow \; 4H_2O$$

$$\begin{aligned}\Delta G^0_{\text{cathode}} &= \left(4 \cdot \left(-237.178 \, \text{kJ mol}^{-1}\right)\right) \; - \; (2 \cdot 0 + 8 \cdot 0) \\ &= \; -948.712 \, \text{kJ mol}^{-1}\end{aligned}$$

$$\Delta E^0_{\text{cathode}} = -\frac{\Delta G^0_{\text{cathode}}}{n\text{F}} = \frac{948.712 \, \text{kJ mol}^{-1}}{8 \cdot 96500 \, \text{C mol}^{-1}} = 1.229\text{V vs. SHE}$$

Since in biological systems the concentrations of the reaction partners are usually different from standard conditions (pH = 0; T = 298 K; concentration of all species at 1 mol L^{-1}) the potential of the overall reaction under non-standard conditions $\Delta E^{0\prime}$ has to be corrected for the actual concentrations of the reaction partners. Assuming a pH of 7, an oxygen partial pressure of 0.2 bar, and the concentrations of acetate and HCO_3^- being at 16.9 and 5 mM, respectively, (Logan 2008) the potential of the overall reaction under non-standard conditions calculates from the concentration of the products and educts to the power of their respective stoichiometric coefficients p and e according to

$$\begin{aligned}\Delta E^{0\prime}_r &= \Delta E^0_r - \frac{RT}{n\text{F}} \cdot \; \ln \frac{[\text{products}]^p}{[\text{educts}]^e} \\ &= \Delta E^0_r - \frac{RT}{n\text{F}} \cdot \; \ln \frac{[H^+]^1 \cdot [HCO_3^-]^2}{[C_2H_3O_2^-]^1 \cdot [O_2]^2} \\ &= 1.042 \, \text{V} \; - \frac{8.31 \cdot 298}{8 \cdot 96500} \, \text{JC}^{-1} . \; \ln \frac{[10^{-7}]^1 \cdot \; [0.005]^2}{[0.0169]^1 \cdot \; [0.2]^2} \\ &= 1.104 \, \text{V}\end{aligned} \tag{6}$$

This correction can also be applied to the individual electrode potentials.

$$\text{Anode: } C_2H_3O_2^- + 4H_2O \rightarrow 2HCO_3^- + 9H^+ + 8e^-$$

$$\begin{aligned}\Delta E^{0\prime}_{\text{anode}} &= \Delta E^0_{\text{anode}} - \frac{\text{RT}}{n\text{F}} \cdot \ln \frac{[C_2H_3O_2^-]^1}{[H^+]^9 \cdot \; [HCO_3^-]^2} \\ &= 0.187\text{V} \; - \frac{8.31.298}{8 \cdot 96500} \, \text{J C}^{-1} \cdot \ln \frac{[0.0169]^1}{[10^{-7}]^9 \cdot \; [0.005]^2} \\ &= \; -0.299\text{V vs. SHE}\end{aligned}$$

$$\text{Cathode : } \; 2O_2 + 8H^+ + 8e^- \rightarrow 4H_2O$$

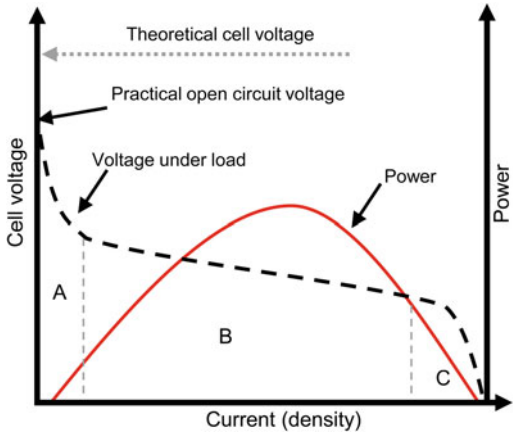

Fig. 3 Schematic representation of the polarization behavior of a complete fuel cell. The thermodynamic reversible potential is indicated by a *gray line* and differs from the practical open circuit potential. With increasing current (density) losses occur due to: activation overpotential (region *A*), ohmic resistances (region *B*), mass-transport limitations (region *C*)

$$\begin{aligned}\Delta E'^{0}_{\text{cathode}} &= \Delta E^{0}_{\text{cathode}} - \frac{RT}{nF}\cdot \ln\frac{[H_2O]^4}{[H^+]^8\cdot[O_2]^2}\\ &= 1.229\,\text{V} - \frac{8.31\cdot 298}{8\cdot 96500}\text{JC}^{-1}\cdot \ln\frac{[1]^4}{[10^{-7}]^8\cdot[0.2]^2}\\ &= 0.805\,\text{V vs. SHE}\end{aligned}$$

The above-derived theoretical potentials and the overall cell voltage are only an ideal point-of-view. Due to the irreversible losses that occur at the fuel cell electrode the practical cell voltage of the fuel cell is considerably lower than the theoretical thermodynamic voltage. This is illustrated in Fig. 3 and explained as follows.

Already under open circuit conditions (no current flowing) activation losses reduce the practical open circuit voltage of the fuel cell. These stem from the activation energy necessary to drive the electrochemical reactions or in the case of microbial fuel cells the electron transport chain of the bacterial metabolism (Logan and Regan 2006). When current is flowing the practical fuel cell voltage is further decreased by ohmic losses e.g., resulting from the resistivity of the electrolyte and electrodes, and mass-transfer losses e.g., due to the depletion of a reaction partner. The origin of these losses and their influence on fuel cell voltage are discussed in Sect. 3.2 in more detail. In principle, the cell voltage of the fuel cell ΔE_{FC} follows:

$$\Delta E_{\text{FC}} = \Delta E_{\text{ocp}} - IR_{\text{int}} \tag{7}$$

wherein ΔE_{ocp} *i*s the practical open circuit voltage, I is the current, and R_{int} is the sum of the fuel cells internal resistances. It depends nonlinearly on current density, and combines the linear ohmic resistances with the nonlinear polarization and mass-transfer resistances occurring at the electrodes. Furthermore, the electrical power output of the fuel cell at a given current can be calculated. As shown

schematically in Fig. 3, the power output goes through a maximum and follows the equation

$$P = \Delta E_{\mathrm{FC}} I \tag{8}$$

1.3 Calculating Power: Normalization Allows for Comparison

From an applicational point-of-view the power output of a microbial fuel cell is the prime figure of interest. It can be easily calculated as the product of fuel cell current and voltage. Since a fuel cell's power output clearly depends on its size some normalization is necessary to be able to compare the performance of several fuel cells that e.g., differ in their size, design, or in the type of electrode materials employed. For fuel cell designs of the essentially 2D nature (e.g. two electrodes laminated to an ion exchange membrane) normalization to the projected electrode area is obviously reasonable. However, in systems where one electrode is considerably more bulky than the other normalization to the volume of one electrode or even the overall fuel cell is a better way of reporting power density.

1.4 Fuel Cell Efficiency

An important characteristic number of a fuel cell is its efficiency, which relates the fuel's energy content to the obtained electrical energy. One way to calculate the efficiency of any energy conversion device is to relate the fuel cell's power output to the energy flow into the fuel according to

$$\begin{aligned} \eta &= \frac{(\text{electrical output power})}{(\text{enthalpy of the fuel}) \cdot (\text{mass flow})} \\ &= \frac{(\Delta E_{\mathrm{FC}} \cdot I)}{(\Delta H_{\mathrm{fuel}}) \cdot (\text{mass flow})} \end{aligned} \tag{9}$$

While this is a straightforward way of accounting for a fuel cell's efficiency, it requires knowledge about the fuels energy content and in particular about the fuel flow into the fuel cell. A more convenient way to estimate a fuel cell's efficiency is based on the comparison of theoretical to practical voltage. As shown in Eq. (4) only the free energy ΔG_r^o of a reaction is transformed into electricity in a fuel cell. However, the complete energy content of a fuel is described by its enthalpy. If we now assume that all the fuels' energy content (heating value or molar enthalpy) could be transformed into electricity, the corresponding theoretical voltage E_{th} would calculate to

$$\Delta E_{\text{th}} = \frac{-\Delta H_{\text{fuel}}}{n\text{F}} \tag{10}$$

For instance, with the *standard enthalpy* (or heating value, heat of combustion) ΔH^0_{fuel} (under standard conditions) of complete oxidation of acetic acid to water and CO_2 amounting to -874 kJ mol^{-1} (Lide 2001) this leads to

$$\begin{aligned}\Delta E_{\text{th}} &= -\frac{-874}{8 \cdot 96500}\,\text{kJ C}^{-1} \\ &= 1.132\,\text{V}\end{aligned}$$

With the practical fuel cell voltage ΔE_{FC} at its point of operation, a voltage efficiency η_v of the fuel cell system can then calculated according to

$$\eta_V = \frac{\Delta E_{\text{FC}}}{\Delta E_{\text{th}}} \tag{11}$$

In other words, the voltage efficiency η_v describes how much of the theoretical energy content of a fuel is actually converted into electricity, assuming that all of the fuel takes part in the electrochemical reaction. Considering that the operating voltage of an acetate-fed microbial fuel cell is in the range of 400 mV (Nevin et al. 2008), it is obvious that their efficiency cannot be better than 35 %, even when complete fuel utilization is assumed.

The fact that in a fuel cell side reactions occur and not all of the fuel is transformed into electrical current is expressed as coulomb-efficiency or fuel utilization coefficient

$$\eta_C = \frac{\text{coulombs recovered as electricity}}{\text{total coulombs in the substrate}} \tag{12}$$

Knowing these two numbers, the overall efficiency of a fuel cell system (neglecting balance of plant such as fans and pumps) can be calculated according to

$$\begin{aligned}\eta &= \eta_V \cdot \eta_C \\ &= \frac{\Delta E_{\text{FC}}}{\Delta E_{\text{th}}} \cdot \eta_C = \frac{\Delta E_{\text{OCP}} - IR_{\text{int}}}{\Delta E_{\text{th}}} \cdot \eta_C\end{aligned} \tag{13}$$

This simple equation implies an important characteristic of fuel cell systems: with increasing current the efficiency of the fuel cell is decreased. Consequently, high fuel efficiency requires a low current density. This translates into a larger fuel cell to deliver the same power output and thus a high capital cost and space demand of the overall fuel cell system. As a consequence, fuel cell optimization also means reducing the internal resistances of the fuel cell, to enable high current at minimized voltage losses and thus optimize power output and efficiency of the overall system. Important optimization points are the fuel cell reactor design, the choice of electrode materials and electrocatalysts, as described in the following section on fuel cell construction.

2 Fuel Cell Construction

2.1 Reactor Design

The easiest way to construct a microbial fuel cell is to simply place anode (anaerobic) and cathode (aerobic) in separate vessels, connected by an ion bridge. An example of this configuration is shown in Fig. 4a). Here, a proton conducting membrane is situated between the two horizontal tubes interconnecting both vessels, thus preventing crossover of fuel, bacteria, or oxygen between the compartments. While comparably easy to fabricate, the main drawback of this concept is the large distance between the electrodes, which translates into a high internal resistance due to ohmic losses in the electrolyte. Such systems are useful to study e.g., an individual electrode reaction (preferably with the help of a reference electrode, see also Sect. 3.1), but produce only little electrical power.

Often, the anode is comparably thicker in comparison to the cathode, since it must accommodate the bacterial biofilm. This for instance is reflected in the anode concept (Fig. 4b) where graphite fibers in the form of a brush serve as 3D electrode on which the anodic biofilm is formed (Logan et al. 2007). Similarly in the tubular fuel cell concept, a stable cylindrical structure is formed from e.g., an ion exchange membrane (Fig. 4c). The cylinder's interior volume is filled with the anodic matrix (e.g. graphite granules) through which the fuel solution is pumped. The cathode is realized as a relative thin layer e.g., from woven graphite mat laminated to the outside of the cylinder (Rabaey et al. 2005). A limitation of these concepts is the comparably large distance between anode and cathode, leading to large ohmic losses in the electrolyte and consequently reduced power density.

As with conventional hydrogen fuel cells, also with microbial fuel cells the ohmic losses in the electrolyte can be reduced by minimizing the distance between the electrodes, e.g., by placing or laminating the electrodes to the two faces of an ion conducting membrane (Min and Logan 2004). This concept is illustrated in Fig. 4d, and has been adopted by a number of researchers.

A major cost factor in the construction of microbial fuel cells can be the *ion exchange membrane* that serves as a separator between anode and cathode compartment. Typically, it prevents the diffusion of oxygen into the anode compartment, which would decrease the coulombic efficiency of the fuel cell due to unwanted side reactions (see also Sect. 1.4). It thus has to be gas tight, but at the same time allow for the transport of protons from the anode to the cathode compartment. Other functions of the membrane are the prevention of bacteria or fuel leakage (crossover) to the cathode, which may reduce fuel cell performance by unwanted side reactions and fouling. Historically, high-performance materials such as Nafion (a sulfonated Teflon-like polymer), which is used in hydrogen fuel cells due to its excellent proton transport capability, have been applied. However, at the relatively low current densities typical for microbial fuel cells the high proton conductivity of Nafion is not needed. Consequently, researchers have considered cheaper membranes of the cation or anion exchange type (Kim et al.

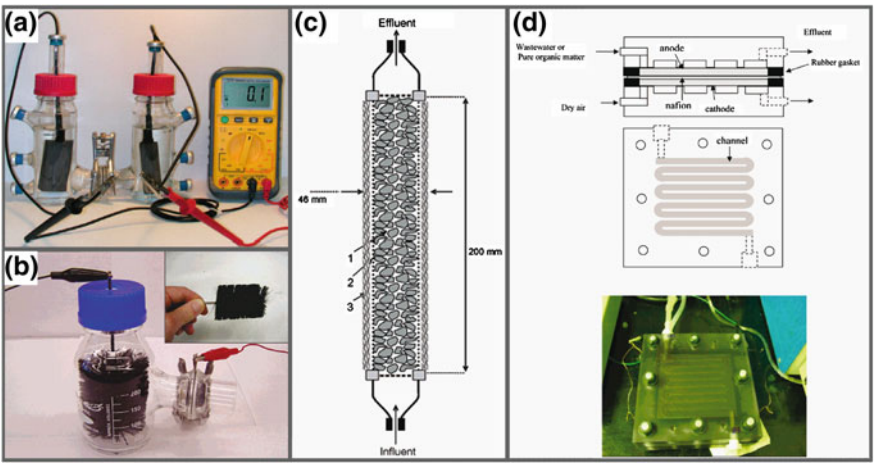

Fig. 4 Comparison of reactors from literature: **a** anode (anaerobic) and cathode (aerobic) in separate vessels each, interconnected by an ion bridge. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology (Lovley 2006), copyright (2006). **b** Three-dimensional graphite fiber brush anode with large ohmic resistance between anode and cathode. Reprinted with permission from (Logan et al., 2007), copyright (2007) American Chemical Society. **c** Tubular microbial fuel cell with graphite granule anode inside the tube, and a woven graphite mat as exterior cathode. Reprinted with permission from (Rabaey et al. 2005), copyright (2005) American Chemical Society. **d** Flat-plate type microbial fuel cell with minimized ohmic resistance between the electrodes. Reprinted with permission from (Min and Logan 2004), copyright (2004) American Chemical Society

2007), glass fiber mats (Zhang et al. 2011), or even omitted the membrane completely (Li et al. 2011; Liu and Logan 2004). One difficulty with the use of ion exchange membranes in microbial fuel cells is that at neutral pH the proton concentration in the typical electrolyte is much lower than the concentration of cations such as sodium. Consequently, e.g., sodium ions travel preferably over protons through the membrane to achieve charge balance, and the pH in the anodic compartment increases whereas the cathode becomes more alkaline (Rozendal et al. 2006).

For power generation, the use of oxygen from air as the electron acceptor at the *cathode* is almost exclusively considered. In principle, also other chemical compounds such as ferricyanide (Rabaey et al. 2004) can be reduced, which happens at low overpotential and yields comparably high power densities. However, once all the ferricyanide in a fuel cell is reduced it must be regenerated; the concept is thus hardly sustainable. With respect to the design of the oxygen (air) cathode in a microbial fuel cell, the question whether the cathode is operated in submerged or air-breathing configuration is of great importance. In *submerged* operation, the cathode is placed in an aqueous electrolyte containing dissolved oxygen. The advantage of this approach is that this way the electrode surface always is in intimate contact with the electrolyte, which is prerequisite for the electrochemical

reaction to take place. However, the main limitation of this type of electrode is the low (<200 μM) concentration of dissolved oxygen in aerated aqueous solutions. At higher current densities this leads to the increasing dominance of mass-transport limitations on cathode performance, which can also put an upper constraint on the total possible fuel cell current density and power output. Mass transport (or specifically oxygen transport) to the cathode electrode can be greatly augmented by the construction of *air-breathing* or *gas-diffusion cathodes* (Rismani-Yazdi et al. 2008), which is standard in case of conventional fuel cells. Here, the electrochemical reaction takes place at the triple phase boundary which is formed at the interface between electrolyte, electrode, and gas (air) phase.

2.2 Anode Materials

A suitable anode material for microbial fuel cells has to be electrically conductive, biocompatible, chemically inert, and allow for an efficient electron exchange with the microorganism. Furthermore, it has to be porous enough to enable sufficient mass transport to supply reactants and remove microbial reaction products. In the early works with indirect and mediated microbial fuel cells, platinum was the electrode material of choice due to its chemical inertness, electrical conductivity, and electrocatalytic activity toward the oxidation of small organic molecules. However, its drawback is the high cost and consequently alternative materials were sought for practical application of microbial fuel cells as electricity generators.

In practice, carbon-based materials such as graphite felts, fibers, or granules are nowadays dominant (Wei et al. 2011). New and promising materials are carbon fiber electrodes prepared as a 3D network by electrospinning (Chen et al. 2011) or textile fibers modified with carbon nanotubes (Xie et al. 2011). Carbon-based materials can be further improved by the introduction of surface functional groups, resulting in improved anode performance. Examples are the treatment with HNO_3 and ethylenediamine (Zhu et al. 2011) or ammonium gas treatment (Cheng and Logan 2007).

2.3 Electrocatalysts for Oxygen Reduction

Although it is consensus among researchers that the high cost of platinum prohibits its economic use as oxygen reduction catalyst in microbial fuel cells, platinum electrodes often serve as the benchmark in microbial fuel cells, against which other oxygen reduction catalysts can be compared (Birry et al. 2011; Harnisch et al. 2009b; Yu et al. 2009). However, it should be noted that platinum is far from being the optimal catalyst for oxygen reduction under the typical operation conditions of a microbial fuel cell. In particular, the near-neutral pH and interfering substances, such as e.g., chloride (Skou 1973), have a detrimental effect on its catalytic activity. Similarly, also fuel crossover effects, similar to the presence of methanol

at platinum cathodes in direct methanol fuel cells (Convert et al. 2001), can negatively affect the performance of a platinum cathode in a microbial fuel cell. Thus, not only the high cost of platinum, but also its sensitivity to interfering substances sparks new research on alternative catalyst materials (Harnisch and Schroder 2010), as outlined in the following.

Carbon materials are not only suitable as anode material, but also exhibit catalytic activity toward oxygen reduction (Kerzenmacher et al. 2008; Kozawa et al. 1970). In the field of microbial fuel cell cathodes, there are some examples where carbon materials have successfully been employed. For instance, Freguia et al. demonstrated the use of graphite granules as efficient oxygen reduction catalyst in a microbial fuel cell, and put special emphasis on the importance of further research on high surface area materials (Freguia et al. 2007). Zhang et al. demonstrated the use of activated carbon as efficient catalyst for air-breathing cathodes. In phosphate-buffered saline containing acetate as fuel this electrode even exhibited a slightly better performance than a platinum cathode (Zhang et al. 2009).

The catalytic activity of carbon materials can be further improved by chemical treatment, e.g. HNO_3 (Erable et al. 2009). Another possibility to increase the catalytic activity of carbon is the adsorption of *metal macrocycles* such as phthalocyanines (Harnisch et al. 2009a) or porphyrines (Zhao et al. 2005) to carbon materials, sometimes in conjunction with a pyrolysis step. Such catalysts can show performance comparable to platinum (Haoyu et al. 2007); however, their long-term stability demands further investigation. A different class of catalysts is based on *manganese oxide* (Roche and Scott 2009). These materials show only little electrical conductivity, and thus need to be dispersed on support materials such as carbon black. So far, these materials exhibited significantly lower oxygen reduction performance than platinum, but have the advantage of comparably low cost (Roche et al. 2010).

In principle, also a number of *enzymes* would be suitable as catalysts for the cathodic oxygen reduction in microbial fuel cells. Among the reported enzyme catalysts for cathodic oxygen reduction in microbial fuel cells is for instance laccase (Schaetzle et al. 2009). At pH 5, the oxygen-reducing laccase system exhibited a by approx. 50 % higher power density compared to ferricyanide reduction. In the context of conventional hydrogen and methanol fuel cells also the favorable *air-breathing* cathodes using laccase have been reported (Gellett et al. 2010; Shleev et al. 2010). Another option for air-breathing cathodes is *bilirubin oxidase* (Gupta et al. 2011), which compared to laccase has the advantage of higher tolerance toward chloride ions. This aspect is relevant for a number of practical applications where the presence of chloride ions cannot be circumvented, e.g., waste water or sediment fuel cells in the ocean floor. Furthermore, *bilirubin oxidase* has a wide operational pH range (5–8) (Gupta et al. 2011). One of the main limitations of all enzymatic catalysts is their short lifetime in the range of a few weeks, which at present hinders long-term application in fuel cells. Besides immobilization and structural optimization of the enzymes, the continuous production of active enzyme by microorganisms directly at the electrode is a promising approach to extend the lifetime of enzymes in bioelectrochemical systems (Rubenwolf et al. 2011, 2012). Recently, the use of the crude culture supernatant of the fungus *Trametes versicolor*

to supply laccase to a biofuel cell cathode has been demonstrated as a first step toward a self-regenerating enzymatic cathode (Sané et al. 2011).

Besides abiotic catalysts and enzymes, also microorganism can be used to facilitate oxygen reduction at the cathode. Several studies have demonstrated the feasibility of this concept (Clauwaert et al. 2007; Mao et al. 2010; Rabaey et al. 2008; Rismani-Yazdi et al. 2008), but the field is still in its infancy.

3 Fuel Cell Testing

Being a relatively new field of great dynamics, the current practice in microbial fuel cell research still lacks the standardization and established characterization procedures that are now common practice in e.g., the field of conventional fuel cells for automotive and power generation applications [see for instance "The Fuel Cell Testing and Standardisation Network" (Tsotridis et al. 2006)]. The following section aims at providing guidelines to obtain significant, meaningful, and comparable performance data of microbial fuel cells. Furthermore, some dedicated testing device architectures for microbial fuel cells are discussed. Besides the recording of polarization curves, other electrochemical methods such as cyclic voltammetry or impedance spectroscopy can provide more detailed insight into electrode processes and loss mechanisms. However, their description is beyond the scope of this chapter. For a detailed treatise on the application of these techniques, the reader is thus referred to specialized textbooks on electrochemistry (Hamann et al. 2007) and in particular the respective chapters in the book "Bioelecrochemical systems" (Rabaey et al. 2010).

3.1 Electrode Polarization: Insight into Loss Mechanisms

Besides the determination of the fuel cell's maximum power output, the detailed analysis of polarization curves also allows for further insight into the loss mechanisms that affect the power output of the complete fuel cell. Analysis of the polarization behavior of the individual electrodes, furthermore, allows for identification of the limiting electrode reaction, a prerequisite for systematic optimization.

In Fig. 5, the typical polarization behavior of a complete fuel cell and its individual electrodes (potential recorded against a reference electrode) are shown schematically. Starting from their respective open circuit potential (at zero current) the anode is polarized toward more positive potentials, whereas the cathode is polarized toward more negative potentials with increasing current. Consequently, the overall cell voltage (difference between cathode and anode potential) decreases. As shown in Fig. 5, the polarization curve can be divided into three regions, in which different loss mechanisms dominate:

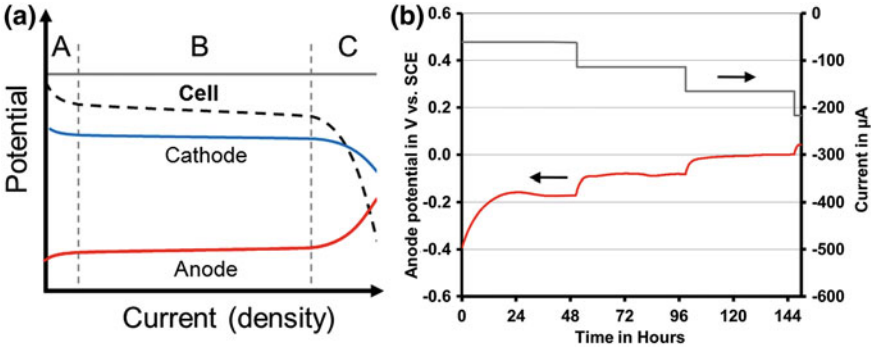

Fig. 5 **a** Schematic representation of the polarization behavior of a complete fuel cell and its individual electrodes. With increasing current (density) losses occur due to: A activation overpotential, B ohmic resistances, C mass-transport limitations. **b** Evolution of the anode potential (vs. the standard calomel electrode, SCE) during the recording of a polarization curve with stepwise increased load current. Activated carbon cloth anode with 2.25 cm^2 geometric area and *S. oneidensis* under anoxic conditions (Kipf et al. 2011)

- Region A: the pronounced voltage drop at low current densities is caused by the *activation overpotential* necessary to drive the electrochemical reactions at the electrode. These activation losses are related to the catalytic activity of the electrode (materials), e.g., a cathode with a better electrocatalyst will show less activation polarization.
- Region B: with increasing current *ohmic resistances* within the fuel cell dominate the overall polarization and the polarization curve shows a linear dependence between cell voltage and current. These ohmic resistances either result from the ionic resistance of the electrolyte (large spacing between the electrodes) or from electronic resistance within the electrodes or fuel cell connection cables. It can be lowered e.g., by reducing the space between the electrodes or using highly conductive electrode materials.
- Region C: with a further increase in current a sharp drop in cell voltage is observed, which is generally attributed to *mass-transfer* losses. These result from insufficient reactant supply due to limited diffusion or convection, or from the limited turnover number of an enzymatic reaction. The reactant supply at the cathode could be improved for instance by using *air-breathing* instead of *submerged* cathodes (see Sect. 2.1).

An important parameter in the recording of polarization curves is the load change rate. Although the slow load change response of microbial fuel cells is well documented in published experimental results (Fischback et al. 2006; Logan et al. 2006; Rhoads et al. 2005; Walker and Walker 2006), researchers often do not consider that under such conditions a too fast recording of polarization curves leads to an overestimation of performance. This can be prevented when the polarization curve is recorded in a stepwise technique (Kerzenmacher et al. 2009), as shown exemplarily in Fig. 5b for *Shewanella oneidensis* growing on a carbon

anode (Kipf et al. 2011). Due to the slow load change response, it can take up to several hours until the anode potential stabilizes. The stepwise recording of the polarization curve allows identifying the time period after which the fuel cell electrode potentials stabilize under load, and enables the assessment of actual performance values. Furthermore, the performance sustainability can be quantified by calculating the voltage drift (degradation) over a given time period.

3.2 Techniques for the Recording of Polarization Curves

A commonly employed technique to record polarization curves is the use of variable load *resistors*, sometimes combined with the measurement of individual electrode potentials against a reference electrode (Kerzenmacher et al. 2009; Logan et al. 2006). This technique can be easily implemented in any laboratory at low cost, but mandates that always a complete fuel cell with anode and cathode is assembled. This is usually no disadvantage when the characterization of a complete system is intended. However, for the optimization of single fuel cell components, such as the development of an optimized anode structure or the comparison of the current-generating capabilities of different exoelectrogenic organisms this can become time consuming and costly. Furthermore, with a passive resistor load the fuel cell can only be operated until the overall cell potential reaches zero. This implies that great care must be taken to prevent that other components of the fuel cell, e.g., membrane resistance or cathode performance do no limit overall performance before the operational limit of e.g., the anode is reached. This can be circumvented by using *potentiostats*, which enable the forced operation of half-cell electrodes against arbitrary counter electrodes. They allow for the precise control of either cell or electrode potential (potentiostatic mode) or load current (galvanostatic mode) to record polarization curves, both techniques leading to comparable results (Hamann et al. 2007). The galvanostatic technique is particularly useful to maintain a defined reactant consumption rate at the electrodes, and thus to investigate mass-transfer related effects. However, potentiostats provide more functionality than required for the automated recording of polarization curves to perform e.g., cyclic voltammetry or impedance spectroscopy experiments. Consequently, they are only available at comparably high cost, and most research budgets thus do not allow for the procurement of a large number of these devices to establish a parallel characterization environment (Kerzenmacher et al. 2009).

Nevertheless, most experiments with microbial fuel cells are time consuming. For time-efficient characterization and development it is, thus, essential to perform multiple experiments in a highly parallel fashion. One reason is their slow load change response which mandates that polarization curves are recorded at sufficiently slow scan rates to prevent performance overestimation (see Sect. 3.1). In addition, experimental times up to several weeks or months can be required until a stable biofilm or microbial consortium forms at the electrodes. Several research groups thus

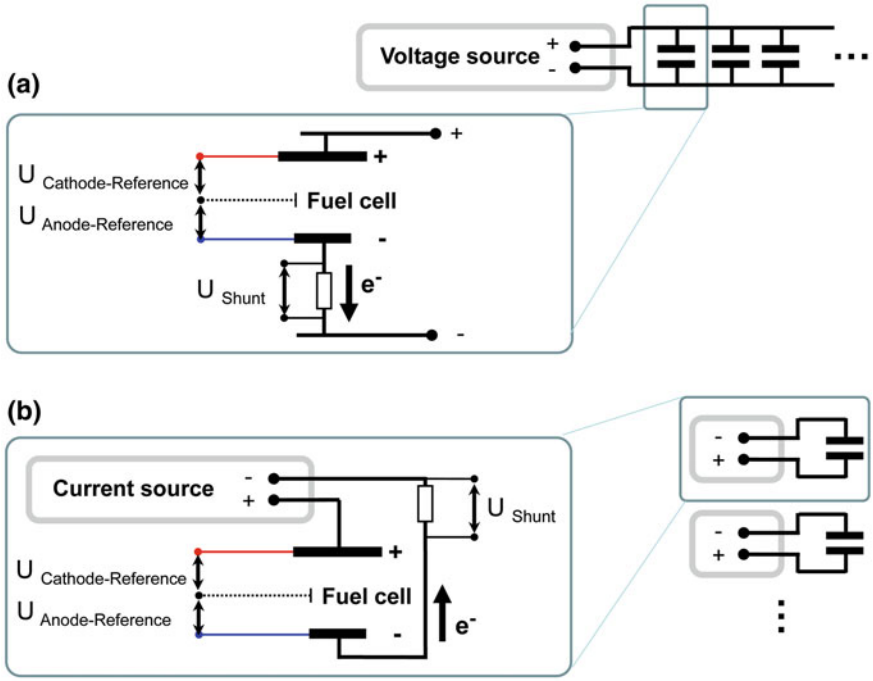

Fig. 6 Two concepts for highly parallel testing devices. **a** Multiple fuel cells are connected in parallel to a single power supply. **b** Each fuel cell is connected to its own current source (galvanostat). In both cases a reference electrode is used to measure the potentials of anode and cathode separately. The voltage drop across a shunt resistor in the circuit is used to measure the fuel cell current. See text for further explanations

developed dedicated testing setups, specifically designed for the intended task of performing many (microbial) fuel cell polarization experiments in parallel.

One of them is the setup for high-throughput material screening developed by Bruce Logan and co-workers (Call and Logan 2011). As shown in Fig. 6 a, it features a standard laboratory power supply as voltage source, to which a number of electrochemical cells (either fuel cells or half-cells) are connected in parallel. The cells themselves consist of small glass vials into which anode, cathode, and optionally a reference electrode are inserted. When a constant voltage is applied a current will flow through each of the cells connected in parallel. Its magnitude depends on the internal resistance of each cell, and is recorded by measuring the voltage drop over a shunt resistor. Polarization curves can be recorded by varying the external applied voltage and measuring the resulting electrode potentials against a reference electrode. The system can work with both, complete fuel cells or mere half-cells where only the electrode of interest is placed into the vial together with an arbitrary counter electrode. Its advantage is its high scalability at low cost: with a single power supply several thousand cells may be operated in

parallel, depending on its current capability (Call and Logan 2011). However, with all the electrochemical cells connected in parallel they are always subjected to the same experimental procedure. Furthermore, the system does not allow for keeping the load current through each cell at a constant value, which can be of relevance when investigating mass-transfer related phenomena.

A more flexible, but also more costly setup is depicted schematically in Fig. 6b (Kerzenmacher et al. 2009). It comprises a number of individually controllable electronic loads, through which a defined load current can be applied to complete fuel cells (or alternatively half-cells with arbitrary counter electrodes). A data acquisition unit is used to individually record the fuel cells' electrode potentials against reference electrodes. The system is fully computerized and features galvanic isolation between the individual channels, which ensures interference-free operation of multiple fuel cells immersed in a common testing solution. This can be of advantage when e.g., a high degree of comparability between the individual experiments is required or when the testing medium itself requires elaborate control mechanisms to keep parameters such as pH and substrate concentration constant.

4 Application Examples

In the following section, some typical application examples for microbial fuel cells are presented. The characteristics and power densities of some microbial fuel cells are compared in Table 2.

4.1 Waste Water Treatment

Waste water treatment combined with electricity generation—this application of microbial fuel cells is probably the most prominent and fascinating for both scientists and non-scientists. In the literature, a number of examples for the treatment of waste water are reported, including landfill leachate (Puig et al. 2011), rice mill waste water (Behera et al. 2010b), municipal sewage (Hays et al. 2011; Lefebvre et al. 2011), and even solid waste (Lee and Nirmalakhandan 2011). Pilot scale plants have already been realized for the application with waste water from a brewery (Logan 2010).

Usually, these systems do not operate with a pure culture or microorganism, but with naturally enriched consortia. Sometimes an inoculum from an already operating microbial fuel cell is used to speed up the formation of a stable biofilm. The anodic community of a microbial fuel cell can differ significantly depending on the type of waste water used (Kiely et al. 2011). In Fig. 7, the bacterial communities of microbial anodes operated with domestic waste and waste water from a winery are compared (Cusick et al. 2010).

Table 2 Some selected characteristics and power densities of microbial fuel cells

Reference	Electrodes	Substrate	Power density	Remarks
Pure culture systems				
(Watson and Logan 2010)	Anode: carbon fiber brush with *S. oneidensis* MR-1 Cathode: platinum on carbon	Lactate	332 mW m^{-2} (bottle reactor)	Power density normalized to the projected cathode area, anode much larger than cathode
(Xing et al. 2008)	Anode: carbon fiber brush with *Rhodopseudomonas palustris* DX-1 Cathode: platinum on carbon	Acetate	2,720 mW m^{-2} (86.6 W m^{-3})	Power density normalized to the projected cathode area, anode much larger than cathode
(Nevin et al. 2008)	Anode: solid graphite with *G. sulfurreducens* Cathode: platinum on carbon	Acetate	1,900 mW m^{-2} (43 W m^{-3}); Optimized anode: (2.15 kW m^{-3})	Power density normalized to the projected anode area, anode much smaller than cathode
Mixed consortia				
(Watson and Logan 2010)	Anode: carbon fiber brush with waste water inoculum Cathode: platinum on carbon	Lactate	559 mW m^{-2} (bottle reactor) 858 mW m^{-2} (cubic reactor)	Power density normalized to the projected cathode area, anode much larger than cathode
(Cheng et al. 2006)	Anode: carbon cloth with waste water inoculum Cathode: carbon cloth	Glucose	766 mW m^{-2}	Power density normalized to the projected cathode area, equally sized anode
(Shimoyama et al. 2008)	Anode: graphite felt with soil inoculum Cathode: platinum on carbon	Model organic wastewater	899 mW m^{-2} (129 W m^{-3})	Power density normalized to the projected anode area or volume anode, equally sized cathode (flat-plate type)

4.2 Energy-Autonomous Power Supply Systems

A promising application example is the realization of energy-autonomous sensor nodes, powered by benthic microbial fuel cells embedded within the marine sediment (Donovan et al. 2008; Nielsen et al. 2008; Tender et al. 2008). Recently, Tender et al. reported the first demonstration of such a microbial fuel cell in which sufficient electrical power to supply a meteorological buoy was generated (Tender et al. 2008). Their fuel cell was constructed from graphite-plate anodes embedded in the marine sediment and a graphite brush cathode positioned in the overlying water. It delivered 36 mW of continuous electrical energy (16 mW m^{-2} per geometric anode surface) and supplied a set of sensors (temperature, air pressure, and relative humidity) as well as a low-power line-of-sight RF transceiver, which transmitted the data in 5-min intervals. From an economical point-of-view, the concept is attractive, since even in the prototype state the microbial fuel cell's cost is comparable to the cost of changing a conventional battery once a year.

Microbial fuel cells have also been applied to power autonomous robots that feed from the environment. Kelly (2003) first presented their slugbot in 2003 as a robotic predator that autonomically collects snails and carries them to a central fermenter unit. Here, the snails are "digested" in a microbial fuel cell, and the generated electricity is in turn used to recharge the battery packs of the robots. While in this first design the microbial fuel cell had to be stationary due to its size and weight, a later robot called "Eco-BotII" was powered by several onboard microbial fuel cells operating on fuels such as sugar, fruit, and insects (Melhuish et al. 2006). The same research group also suggested the use of microbial fuel cells as power supply for energy-autonomous underwater robots (Ieropoulos et al. 2007).

4.3 Miniature Microbial Fuel Cells, Microbial Sensors and Biobatteries

At present, also a number of miniature microbial fuel cells in mL and μL scale are being developed (Biffinger et al. 2007). Potential applications include e.g., on-chip power supply for lab-on-a-chip systems and microfluidic devices (Wang et al., 2011). Researchers also envision the development of body-implantable microbial fuel cells, situated either in the human body tissue (Wang et al. 2011) or the intestine (Han et al. 2010). However, biocompatibility issues and the associated risk of infection are clearly obstacles for the practical realization of these concepts.

In future, microbial fuel cell technology may be used for the development of biobatteries, intended as power source for mobile devices or distributed sensor networks or in general as an alternative to today's chemical batteries. Biobatteries may also be constructed from fully biodegradable, nontoxic, and low-price materials. Together with advances in biodegradable electronics (Bettinger and Bao

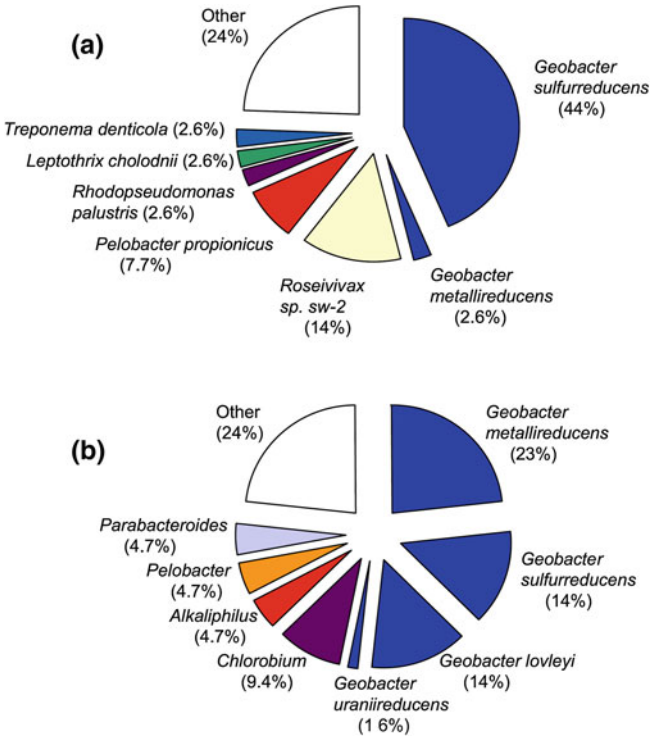

Fig. 7 Anodic bacterial communities of microbial anodes operated with **a** winery waste water and **b** domestic waste water (Cusick et al. 2010). Reprinted from International Journal of Hydrogen Energy 35 (17), Roland D. Cusick, Patrick D. Kiely, Bruce E. Logan, A monetary comparison of energy recovered from microbial fuel cells and microbial electrolysis cells fed winery or domestic wastewaters, 8855–8861, Copyright (2010), with permission from Elsevier

2010) this may pave the way for environmentally friendly disposable distributed sensors ("smart dust") that automatically dissolve after their intended time of operation, and thus do not pollute the environment.

Microbial fuel cells can also serve as sensors (Su et al. 2011) for toxic substances or parameters such as organic carbon or biological oxygen demand (Chang et al. 2004; Di Lorenzo et al. 2009; Kim et al. 2009; Peixoto et al. 2011).

5 Challenges and Future Trends

5.1 Materials, Design, and Testing

Cost is still a major issue when it comes to the practical and commercially successful application of microbial fuel cells. In a recent study, the total acceptable

cost for microbial fuel cell system to become an economically viable option was estimated to be below ~4,000 € per kW. Assuming 2 W m^{-2} as feasible power density of microbial fuel cells in waste water, this corresponds to a maximum cost of ~8 € per m^2, including electrodes, membranes, and casings, as well as auxiliaries such as pumps (Sievers et al. 2010). When comparing this figure to the price of a Nafion membrane in the range of several hundred € per m^2, it becomes clear that new and less costly concepts to construct microbial fuel cells with improved performance are needed. This is not only limited to finding new materials for electrodes and membranes, but also casings, cables, and pumps have to be considered.

Furthermore, a microbial fuel cell is worthless without the bacteria actually doing the job. Besides finding new, more powerful organisms and consortia it is necessary to gain a better understanding of the metabolic principles behind microbial electron transfer and electricity generation (Bucking et al. 2010; Nevin et al. 2009; Newton et al. 2009; Schuetz et al. 2009). This may at some point allow researchers to develop synthetic organisms optimized for electricity generation (Nevin et al. 2009; Rosenbaum et al. 2010) from a variety of substrates in a microbial fuel cell. Last but not least, low-cost fuel cell design, operation strategies for improved performance and long-term stability, as well as power conditioning to step up the relatively low fuel cell voltage to grid-compatible levels will be gaining importance on the road toward practical application. Examples are the low-cost microbial fuel cell made from an earthen pot in place of the costly proton exchange membrane (Behera et al. 2010a), or the use of oxygen at the anode to boost current generation by *Shewanella oneidensis* (Rosenbaum et al. 2010).

Probably most important, some standardization is necessary to ensure that meaningful and significant data is obtained that allow for a critical comparison between the results obtained in different laboratories (see Sect. 3). In a nutshell: *use reference electrodes, report polarization data for the individual electrodes, and record polarization curves with a sufficiently low scan rate to prevent overestimation of performance.*

5.2 Beyond Power: Other Applications of Bioelectrochemical Systems

Besides the generation of electricity, microbial fuel cells or more generally bioelectrochemical systems are increasingly considered for other applications (Lovley and Nevin 2011). These include microbial electrolysis cells (Cheng and Logan 2011; Cusick et al. 2011), where an additional voltage is supplied to the fuel cell so that instead of oxygen protons are reduced at the cathode, leading to the production of hydrogen gas. Also desalination cells based on microbial fuel cell concepts have been successfully used to generate fresh water with a lower energy demand than conventional technologies (Kim and Logan 2011). Furthermore, a

bioelectrochemical system can be used to e.g., reduce or immobilize pollutants such as nitrate or uranium in soil or ground water (Gregory et al. 2004; Gregory and Lovley 2005), or to fixate CO_2 and produce valuable organic compounds (Nevin et al. 2011).

In summary, a number of researchers are now of the opinion that successful future application of microbial fuel cells will not only depend on the power output of these systems, but also on additional benefits and added values.

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