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Iron metabolism in anoxic environments at near neutral pH

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Abstract

Anaerobic dissimilatory ferric iron-reducing and ferrous iron-oxidizing bacteria gain energy through reduction or oxidation of iron minerals and presumably play an important role in catalyzing iron transformations in anoxic environments. Numerous ferric iron-reducing bacteria have been isolated from a great diversity of anoxic environments, including sediments, soils, deep terrestrial subsurfaces, and hot springs. In contrast, only few ferrous iron-oxidizing bacteria are known so far. At neutral pH, iron minerals are barely soluble, and the mechanisms of electron transfer to or from iron minerals are still only poorly understood. In natural habitats, humic substances may act as electron carriers for ferric iron-reducing bacteria. Also fermenting bacteria were shown to channel electrons to ferric iron via humic acids. Whether quinones or cytochromes released from cells act as electron transfer components in ferric iron reduction is still a matter of debate. Anaerobic ferrous iron-oxidizing phototrophic bacteria, on the other hand, appear to excrete complexing agents to prevent precipitation of ferric iron oxides at their cell surfaces. The present review evaluates recent findings on the physiology of ferric iron-reducing and ferrous iron-oxidizing bacteria with respect to their relevance to microbial iron transformations in nature.

Keywords: Ferric iron-reducing bacteria; Ferrous iron-oxidizing bacteria; Anaerobic ferrous iron oxidation; Ferrihydrite; Humic substance

1. Introduction

Iron is the fourth most abundant element in the Earth's crust, and makes up by mass about 5.1%. Depending on the environmental conditions, iron can form stable compounds in both the divalent and trivalent state [1]. The redox change between Fe(II) and Fe(III) plays an important role in redox processes in anoxic soils and sediments. Fe(II), ferrous iron, forms minerals such as siderite, vivianite or iron sulfide only in anoxic habitats under weakly acidic to neutral conditions. In the presence of oxygen, ferrous iron is stable only under acidic conditions; at neutral pH it is rapidly oxidized to Fe(III) [1]. Fe(III), ferric iron, forms minerals that are widespread in both oxic and anoxic habitats. Today a total of 16 different ferric iron oxides, hydroxides or oxide hydroxides are known, and they are often collectively referred to as iron oxides [1,2]. The biogeochemical aspects of ferrous and ferric iron are rather complex and were recently reviewed in detail [3].

Dissimilatory ferric iron-reducing bacteria gain energy by coupling the oxidation of organic compounds or hydrogen to the reduction of ferric iron oxides, and microbial ferric iron reduction has been known as a phenomenon for decades (reviewed by [3–5]). However, the biogeochemical significance of microbial ferric iron reduction was recognized only in the last 10 years. Sediments and soils can contain iron minerals at quantities in the range of several 10 mmol per kg dry matter, and ferric iron is by mass the most important electron acceptor in such environments [2,3]. Only in marine sediments, the high sulfate content (28 mM in seawater) counterbalances the dominance of ferric iron as electron acceptor. Numerous dissimilatory ferric iron-reducing bacteria belonging to different phylogenetic groups have been isolated from marine and freshwater sediments [3,5]. Further novel strains of ferric iron-reducing bacteria were obtained from the deep terrestrial subsurface [6], a petroleum reservoir [7], a continental hot spring [8], and from a hydrothermal vent system [9].

Aerobic oxidation of ferrous iron minerals by lithotrophic acidophilic and neutrophilic bacteria has also been recognized many decades ago (reviewed by [10,11]). Anaerobic ferrous iron oxidation was discovered only recently, with the isolation of phototrophic purple, non-sulfur bacteria that were able to utilize ferrous iron as electron donor in the light [12]. Subsequently, nitrate-re-

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Table 1
Redox potentials of various redox couples relevant to iron metabolism at pH 7.0 and 25°C[a]

System	E_0' (mV)	Reference
$[Fe(CN)_6]^{3+}/[Fe(CN)_6]^{4+}$	+430	[39]
Fe(III) NTA/Fe(II) NTA[b]	+385	[3]
Fe(III) citrate/Fe(II) citrate	+372	[3]
Fe(III) EDTA/Fe(II) EDTA	+96	[40]
Ferrihydrite/Fe^{2+}	−100 to +100	[3,12,41]
γ-FeOOH (lepidocrocite)/Fe^{2+}	−88	[3]
AQDS/AHQDS[c]	−184	[42]
Humic substances[d]	−200 to +300	A. Kappler, personal communication
α-FeOOH (goethite)/Fe^{2+}	−274	[3]
α-Fe_2O_3 (hematite)/Fe^{2+}	−287	[3]
Fe_3O_4 (magnetite)/Fe^{2+}	−314	[3]

[a]Redox potentials depend strongly on pH value, temperature, concentration of reactants, and thermodynamic data chosen for calculations. For details see related references.
[b]Nitrilotriacetate.
[c]2,6-Anthraquinone disulfonate, 2,6-anthrahydroquinone disulfonate.
[d]Humic substances are complex organic polymers with redox-active moieties (esp. quinones) reduced or oxidized in the given range.

ducing bacteria were isolated which gain energy for growth by oxidizing ferrous iron anaerobically [13].

Despite the importance of iron metabolism in the environment, our knowledge of its physiology and biochemistry is still very limited. Most physiological studies on ferrous iron oxidation have been carried out with aerobic acidophilic bacteria, e.g. *Thiobacillus ferrooxidans* [14], which grow in media of pH 1–2 in which ferric and ferrous iron exist as dissolved ions, i.e. Fe^{3+} and Fe^{2+}. At neutral pH, however, substrates and products of iron metabolism are barely soluble, rendering physiological studies rather difficult. Moreover, the energetics of iron reduction and oxidation at neutral pH differ substantially from those in the acidic range: whereas at pH values below 2.5, the standard redox potential of the redox pair Fe^{3+}/Fe^{2+} is +770 mV, iron transformation at neutral pH involves several different iron species of various redox potentials (Table 1). Therefore, it is not surprising that iron reduction and iron oxidation at acidic and at neutral pH are carried out by different organisms because they deal with basically different chemical species as redox substrates.

To circumvent the difficulties with essentially non-dissolved iron species at neutral pH, in many studies chelators were applied which keep ferric and ferrous iron in solution. Although this appears to be an elegant solution, it bears several problems:

1. Most iron chelates do not occur as such in the environment and exhibit redox potentials quite different from those of ferric iron oxides found in nature (Table 1). Thus, such complexes may act only as models for the natural situation, and it is necessary to know the limitations of each experimental model system.
2. Iron is very reactive and changes readily between the divalent and trivalent state once the solubility problem has been overcome. Complexed iron species, therefore, often react quite unspecifically with any kind of electron-releasing or -accepting biological system. For example, potassium hexacyanoferrate can be used as an electron acceptor for assays of nearly every respiratory enzyme.
3. Chelators like EDTA and NTA interact with divalent cations such as Ca^{2+} or Mg^{2+}, and may cause an imbalance in the supply of these ions that might be detrimental, especially for Gram-negative bacteria which depend on such cations in their outer membranes.
4. Chelators such as citrate are excellent substrates for many bacteria and hence can be applied only in pure cultures unable to degrade them.

The following survey will summarize recently published papers on iron-reducing and iron-oxidizing bacteria from anoxic environments of near neutral pH, with emphasis on bioenergetic considerations and substrate availability.

2. Reduction of ferric iron oxides

There is a growing awareness of the difficulties in interpreting data on iron-reducing capacities that were obtained only with chelated forms of ferric iron. Therefore, most studies today include ferrihydrite which is one of the ferric iron oxides widespread in nature. Ferrihydrite is a reddish–brown mineral of poorly ordered crystal structure, and is also termed amorphous iron oxide, poorly crystalline Fe(III) oxide, ferric oxyhydroxide, or hydrous ferric oxide [2]. Bacteria reported to reduce ferrihydrite always also reduced chelated iron forms, but this is not necessarily true vice versa (Table 2). For example, new psychrophilic sulfate-reducing bacteria from Arctic sediments grew with Fe(III) citrate as electron acceptor but were unable to utilize ferrihydrite [15].

Although ferrihydrite is commonly found in surface environments and often makes up about 20% of the total iron phase in a sediment [3], it is also necessary to include, in future studies on iron-reducing microorganisms, other biogeochemically relevant ferric iron oxides such as goethite, hematite, lepidocrocite, or magnetite. Dissimilatory reduction of iron oxides other than ferrihydrite has only been documented for a few strains (Table 2). In addition, the ability to use various ferric iron oxides as electron acceptors might be useful as a marker for the physiological differentiation of iron-reducing bacteria. Furthermore, comparative studies might help to elucidate the features that make ferric iron minerals accessible to microbial reduction, and might help to understand the underlying mechanisms of ferric iron reduction in general.

Humic substances are complex organic polymers with redox-active moieties, and are ubiquitous in terrestrial

Table 2
Examples of ferric iron-reducing bacteria and their capacities to utilize different ferric iron forms as electron acceptors

Strain	Chelated ferric iron forms reduced	Mineral ferric iron oxides reduced	Reference
Desulfuromusa kysingii	Fe(III) citrate, Fe(III) NTA	No data	[43,44]
'*Geospirillum barnesii*'	Fe(III) NTA	No data	[44,45]
Rhodobacter capsulatus	Fe(III) citrate, Fe(III) NTA	No data	[46]
Desulfofrigus oceanense	Fe(III) citrate	None[a,b]	[15]
Desulfotalea psychrophila	Fe(III) citrate	None[a,b]	[15]
G. metallireducens	Fe(III) citrate	Ferrihydrite[b]	[27]
G. sulfurreducens	Fe(III) citrate	Ferrihydrite[b]	[47]
S. putrefaciens now: *S. oneidensis*	Fe(III) citrate	Ferrihydrite[b], magnetite[c]	[26,48]
S. alga	Fe(III) citrate	Ferrihydrite[b], goethite	[49,50]
Strain Dfr2	Fe(III) citrate	Ferrihydrite, akaganeite	[38]

[a]Only ferrihydrite tested.
[b]Also termed amorphous iron oxide, poorly crystalline Fe(III) oxide, ferric oxyhydroxide, iron(III) oxyhydroxide, or hydrous ferric oxide.
[c]Magnetite reduction is thermodynamically unfavorable at pH 7 but is feasible at pH values between 5 and 6 [48].

and aquatic environments [16]. Humic substances and in particular humic acids can chemically reduce ferric iron oxides [17]. Coupling of microbial humic acid reduction to chemical reduction of ferrihydrite was first demonstrated in cultures of *Geobacter metallireducens* and *Shewanella alga* [18]. This observation is intriguing because humic substances may constitute a natural carrier system that transfers electrons from bacterial cells to barely soluble electron acceptors. Moreover, fermenting bacteria such as *Propionibacterium freudenreichii*, *Lactococcus lactis*, and *Enterococcus cecorum* are capable of channelling electrons from anaerobic oxidations, via humic acids, towards iron oxide reduction [19]. Thus, bacteria that reduce humic substances might play a significant role in iron reduction although they do not reduce ferric iron oxides in a typical substrate utilization test.

Different strategies for electron transfer to insoluble ferric iron minerals appear to be taken by *Shewanella putrefaciens* and *Geobacter sulfurreducens*. *S. putrefaciens* releases a menaquinone-related redox-active small molecule into the medium, and genetic evidence suggests that this molecule is involved in transfer of electrons to ferrihydrite [20]. In cultures of *G. sulfurreducens*, substantial amounts (several 10 nM) of a small periplasmic *c*-type cytochrome are detectable in the surrounding medium, and this cytochrome is rapidly oxidized in vitro by various ferric iron forms [21]. The midpoint redox potential of this cytochrome was determined to be −167 mV, just at the right level to reduce efficiently ferrihydrite and ferric iron chelates like Fe(III) citrate and Fe(III) NTA. Since the cytochrome also reduces many other electron acceptors such as humic acids, elemental sulfur, or manganese dioxide, it may act as a rather unspecific electron mediator to any kind of suitable electron acceptor, including ferric iron. In addition, the cytochrome can mediate an electron transfer from *G. sulfurreducens* to electron-consuming partner bacteria such as *Wolinella succinogenes* [22].

At first sight, release of a quinone or cytochrome to act as an extracellular electron carrier appears to be a very expensive and dangerous strategy, taking into account that this carrier could be degraded by other organisms [23]. In nature, however, ferric iron-reducing bacteria probably live in surface-associated communities such as biofilms. Under these conditions, the release of few such carrier molecules per cell might be affordable, compared to the benefit it provides for the whole community.

3. Physiological aspects of ferric iron oxide reduction

The biochemistry of microbial ferric iron reduction is still only poorly understood. The best studied organism is *S. putrefaciens* which can use a wide range of electron acceptors including oxygen, and contains a complex multicomponent electron transport system [24]. Ferric reductase activity is detectable only in anaerobically grown cells, and is predominantly found in the outer membrane [25]. In the transfer of electrons to ferric iron oxides, *c*-type cytochromes are involved that are localized mainly in the outer membrane and in the periplasmic space [24,26]. Also in *G. metallireducens* ferric reductase activity is localized in the membrane fraction and cytochromes are thought to be involved in electron transfer to ferric iron oxides [27]. In *G. sulfurreducens*, ferric reductase is a peripheral protein in the outer face of the outer membrane, and its activity is associated with *c*-type cytochromes which are oxidized by ferrihydrite [28].

Acetate is oxidized by *G. metallireducens* through the tricarboxylic acid cycle [29], and the same is true for *G. sulfurreducens* (A. Galushko, personal communication). The oxidative steps are coupled to electron transfer through NAD(P) and a further low potential electron carrier such as ferredoxin, which both could deliver electrons through a proton-translocating respiratory mechanism to a quinoid carrier and from there to the periplasmic cytochrome system. Only the electrons released in succinate oxidation arise at a potential too positive for direct cytochrome *c* reduction, and might require a reversed electron

transport. Thus, the net ATP yield (including substrate activation) would be in the range of 1–2 ATP per acetate oxidized.

4. Anaerobic oxidation of ferrous iron by phototrophic bacteria

Oxygen-independent biological oxidation of ferrous iron was recognized first in cultures of anoxygenic phototrophic bacteria [12], indicating for the first time that ferrous iron can be reoxidized within anoxic environments. Iron-oxidizing phototrophic bacteria were isolated from freshwater and marine sediments, and were affiliated to different genera of purple or green phototrophic bacteria [30–33]. Ferrous iron oxidation by anoxygenic phototrophic bacteria is plausible in terms of energetics. The redox potential of the redox pair ferric/ferrous iron in bicarbonate-containing environments is approximately +100 mV at pH 7.0. Hence, electron transfer to the photosystems of purple or green bacteria, with midpoint potentials around +450 mV or +300 mV, respectively, is feasible [12,34].

The various ferrous iron-oxidizing phototrophic bacteria differ substantially in their efficiency to oxidize ferrous iron. Some strains such as *Rhodovulum robiginosum*, the purple non-sulfur phototroph strain SW2, or the green phototrophic sulfur bacterium *Chlorobium ferrooxidans* strain KoFox oxidized substantial amounts of ferrous iron (8–10 mM) and deposited the ferric iron oxide produced outside the cells, which was identified as ferrihydrite [30,32,33]. In contrast, *Rhodomicrobium vannielii* oxidized 1–4 mM ferrous iron and encrusted itself in a thick layer of ferric iron oxides which impeded further metabolic activities severely [12,31]. Similar differences were observed with different strains of nitrate-dependent ferrous iron-oxidizing bacteria (see below). These differences may be attributed to the production of low molecular mass compounds that solubilize iron. Spent media of strains SW2 and *C. ferrooxidans* KoFox contained dissolved ferrous and ferric iron at concentrations far higher than expected from the solubility constants, whereas medium of the self-encrusting *R. vannielii* contained little dissolved iron, only slightly more than that detectable in uninoculated medium (Table 3, R. Warthmann, personal communication). Unfortunately, the iron-solubilizing compounds in these spent media have not yet been identified.

Table 3
Concentrations of dissolved ferrous and ferric iron in culture supernatants of phototrophic ferrous iron-oxidizing bacteria

Sample	Ferrous iron (μM)	Ferric iron (μM)
Uninoculated medium	<200	<30
R. vannielii strain BS-1	300	30
Strain SW2	6200	300
'*C. ferrooxidans*' strain KoFox	2800	150

Since the ferrihydrite produced during phototrophic ferrous iron oxidation precipitates outside the cell, electrons have to be delivered from ferrous iron outside the outer membrane through the periplasmic space to the photosynthetically active membrane apparatus in or at the cytoplasmic membrane. Electron transfer components that transfer electrons across the periplasmic space have been postulated [30], but have not yet been described or detected.

5. Anaerobic oxidation of ferrous iron by nitrate-reducing bacteria

Ferrous iron can be oxidized anaerobically also in the dark, with nitrate as the electron acceptor [13,35]. At pH 7.0, all redox pairs of the nitrate reduction pathway are much more positive (E_0' values: NO_3^-/NO_2^-, +430 mV; NO_2^-/NO, +350 mV; NO/N_2O, +1180 mV; N_2O/N_2, +1350 mV; [36]) than the redox pair ferric/ferrous iron (Table 1) and are therefore favorable electron acceptors for ferrous iron oxidation. The oxidation of ferrous iron could as well be coupled to the reduction of nitrate to ammonium (E_0' mean value: NO_3^-/NH_4^+, +360 mV; calculated after Thauer et al. [36]), but so far no ferrous iron-oxidizing enrichment, Most-probable-number or pure culture has produced ammonium from nitrate [13,35,37]. In all cases, oxidation of ferrous iron was coupled to stoichiometric reduction of nitrate to N_2, with traces of N_2O formed as side product by one strain [13,35]. The ferric iron oxide produced was identified as ferrihydrite and was shown to be an excellent electron acceptor for dissimilatory ferric iron-reducing bacteria [38]. Further studies in gradient cultures revealed that nitrate-reducing strains were also able to oxidize ferrous iron with molecular oxygen [35]. Thus, the novel strains may play an important role not only in nitrate-dependent anaerobic but also in aerobic oxidation of ferrous iron.

Since nitrate-dependent oxidation of ferrous iron is not restricted to areas exposed to sunlight, it is probably more important on a global scale than its anaerobic oxidation by phototrophic bacteria. Enrichment cultures inoculated with freshwater or marine sediments were initiated with nitrate as electron acceptor and ferrous iron as sole electron donor. However, after several transfers, ferrous iron was oxidized in these cultures nearly exclusively in the presence of an organic cosubstrate such as acetate [13,35,37]. Subsequent characterization of iron-oxidizing nitrate-reducing bacteria revealed that most isolates depend on organic cosubstrates for synthesis of cell components [13,35]. Most-probable-number counts suggested that such lithoheterotrophic ferrous iron-oxidizing bacteria account for at most 0.8% of the total nitrate-reducing community, and are substantially more frequent than strictly lithoautotrophic iron oxidizers [37]. Of course, cell numbers alone do not allow conclusions on the actual rate of ferrous iron oxidation, and should be combined

with rate measurements and with analyses of chemical parameters such as concentrations of oxygen, nitrate, ferrous and ferric iron in situ. Nonetheless, the recently discovered nitrate-dependent ferrous iron oxidation and the facultatively anaerobic bacteria involved might contribute significantly to the overall electron flow in an anoxic environment, and might also increase the ecological importance of ferric iron reduction, either by classical ferric iron-reducing or by fermenting bacteria that transfer electrons to ferric iron oxides through, e.g., humic compounds as intermediary carriers.

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