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Review

Insight into the evolution of the iron oxidation pathways[☆]

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ABSTRACT

Iron is a ubiquitous element in the universe. Ferrous iron (Fe(II)) was abundant in the primordial ocean until the oxygenation of the Earth's atmosphere led to its widespread oxidation and precipitation. This change of iron bioavailability likely put selective pressure on the evolution of life. This element is essential to most extant life forms and is an important cofactor in many redox-active proteins involved in a number of vital pathways. In addition, iron plays a central role in many environments as an energy source for some microorganisms. This review is focused on Fe(II) oxidation. The fact that the ability to oxidize Fe(II) is widely distributed in *Bacteria* and *Archaea* and in a number of quite different biotopes suggests that the dissimilatory Fe(II) oxidation is an ancient energy metabolism. Based on what is known today about Fe(II) oxidation pathways, we propose that they arose independently more than once in evolution and evolved convergently. The iron paleochemistry, the phylogeny, the physiology of the iron oxidizers, and the nature of the cofactors of the redox proteins involved in these pathways suggest a possible scenario for the timescale in which each type of Fe(II) oxidation pathways evolved. The nitrate dependent anoxic iron oxidizers are likely the most ancient iron oxidizers. We suggest that the phototrophic anoxic iron oxidizers arose in surface waters after the *Archaea/Bacteria*-split but before the Great Oxidation Event. The neutrophilic oxic iron oxidizers possibly appeared in microaerobic marine environments prior to the Great Oxidation Event while the acidophilic ones emerged likely after the advent of atmospheric O₂. This article is part of a Special Issue entitled: The evolutionary aspects of bioenergetic systems.

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Iron is a relatively abundant element in the universe and is present in the sun and in many stars. In the Earth's crust, iron is the fourth most abundant element and the second metal after aluminium, with an abundance estimated to be ~5% while it is believed to be the main constituent of the Earth's core.

Iron is not only important for human activities (the processing of iron for industrial purposes accounts for 95% of worldwide metal production), but is also a crucial element for living cells from all three domains because it is incorporated as a cofactor in many metalloproteins involved in vital metabolic pathways. We will briefly survey general concepts on this element (Section 1) and will discuss its importance as cofactor in biology (Section 2). This review will then focus on another key role of iron, that is, its use as electron donor or acceptor for energy purposes by some microorganisms. The iron metabolism is thought to be quite ancient but the evolution of the systems involved is largely unknown. Based on what is known today on the paleo geochemistry of iron on Earth (Section 3.1), on the emergence of the redox cofactors (Section 3.2), on the distribution of the Fe(II) oxidizers on the phylogenetic tree of the prokaryotes (Section 4) and on the ferrous iron

oxidation pathways deciphered up to now (Section 5), we will discuss a possible scenario of the time intervals in which each type of Fe(II) oxidation pathways evolved (Section 6). However, this model is far from being complete and important questions remain to be answered (Section 7). We hope that this review will elicit experimental investigations to validate or invalidate this model or parts thereof and to advance our knowledge on when and how Fe(II) oxidation pathways appeared and evolved. Many of the topics discussed here have been reviewed previously and the readers will be referred to these articles, including references therein.

1. Iron, an element with unique properties: an overview of its (geo)chemistry

Iron is a transition metal; its chemical symbol is Fe from the Latin name, *ferrum*. The melting point of iron is 1536 °C, its boiling point is about 3000 °C and its density is 7.87 g cm⁻³. Iron can exist in various oxidation states (from -2 to +6), the principal forms that occur naturally however are either ferrous or ferric iron (Fe(II) or Fe(III), respectively). As

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biologists, we will not go into the details of iron chemistry; nevertheless, we would like to emphasize the reactivity of this element depending on its surroundings (temperature, pH, the nature of the complexing ligands, etc.). Not surprisingly, Fe(II) is more abundant in anoxic environments whereas, in an oxygen-containing environment, iron is readily oxidized from the Fe(II) to the Fe(III) state. Iron solubility and reactivity also strongly depend on pH as spontaneous chemical oxidation of iron can be rapid at neutral pH whereas at low pH this abiotic oxidation occurs very slowly. Fe(III) ions have a very low solubility (about 10^{-17} M), making iron less bioavailable at circumneutral pH than at acidic pH [1]. Acidophilic microorganisms are then more suited to use soluble Fe(II) than neutrophiles as we will describe below in Section 5.

Iron is not found as free metal in nature. It tends to coordinate with organic and inorganic ligands forming a wide number of minerals that play critical roles in environmental chemistry due to their high abiotic reactivity. This property can, for example, be used in chromate detoxification as iron is used as a potent reducing agent to reduce and therefore precipitate chromate [2]. Iron is known to react with oxygen (O_2) in water or air moisture to form various insoluble iron oxide compounds described commonly as rust; there are sixteen known iron oxides and oxyhydroxides [3]. The most common iron oxides are ferrihydrite ($Fe_2O_3 \cdot 0.5H_2O$), hematite (Fe_2O_3) and magnetite (Fe_3O_4). Depending on the environment, iron not only complexes with oxygen ligands, but also with a lot of different compounds such as carbonate and sulfur by abiotic or biotic reactions. Such complexes can be found naturally in the environment such as siderite (iron carbonate: $FeCO_3$), pyrite (FeS_2), schwertmannite ($Fe_8O_8(OH)_6(SO_4)$) etc. This list is not exhaustive but allows to realize the large diversity of iron compounds available for industry as well as for living organisms.

The redox potential of the Fe(II)/Fe(III) couple presents an extreme variability that can be finely tuned by the ligands (see Table 1). In natural and anthropogenic acidic environments, Fe(III) and sulfate ions are complexed leading to a decrease in the redox potential of the Fe(II)/Fe(III) couple from +0.77 V to +0.697 V [4]. Furthermore, depending on the chelating agent, the redox potential is different (as exemplified in Table 1). From these values, we can clearly see that at low pH values (acid environment), only O_2 can be used as an electron acceptor to gain enough energy for growth (O_2/H_2O redox potential at pH 2 is +1.12 V). However, at neutral pH the variation in redox potential allows some microorganisms to use Fe(II) as electron donor in anoxic conditions with nitrate as electron acceptor (nitrate/nitrite redox potential is +0.42 V) because the redox potential of the Fe(II)/Fe(III) couple is below +0.385 V depending on the complexing agent (Table 1) [5,6]. Similarly, the ligands involved in iron binding in proteins will strongly impact the redox properties of the metals making it well suited for many different pathways in biological system (as described in Section 2). Iron cofactors cover almost the entire biological range of redox potentials, from about -0.5 V to +0.6 V [7].

2. Importance of iron in biology

Iron is essential to most life forms. To date, the only organisms that do not depend on iron belong to the *Lactobacillus* spp. [8]. Many bacteria require near-millimolar concentrations of intracellular iron and the human body contains 4 to 5 g of iron. Disorders that perturb iron balance are among the most prevalent human diseases. Iron deficiency is the most common type of anemia and can be related to severe pathologies while some diseases like hemochromatosis can be due to iron overload (for a complete overview on the large impact of iron on health see for example the book "Iron and Human Disease" [9]). The U.S. Recommended Daily Allowance (USRDA) for iron is 18 mg. An example to show the key role of iron for bacteria has been evidenced by Flo et al. [10] who demonstrate that the immune system has developed lipocalin 2, a protein that sequesters iron in response to bacterial infection, to limit bacterial proliferation.

This metal is an integral part of a number of proteins and enzymes. The two oxidation states of iron (Fe(II) and Fe(III)) make it suitable for numerous biochemical reactions. Iron is an important cofactor in several proteins required for a large range of metabolic processes like (i) the transport, storage and activation of molecular oxygen, (ii) the activation and decomposition of peroxides, (iii) the reduction of ribonucleotides and dinitrogen and (iv) the electron transfer *via* a variety of electron carriers with a wide range of redox potentials. Inorganic iron involved in redox reactions is found in the heme group of the cytochromes, in the iron-sulfur clusters of many enzymes (such as nitrogenase and hydrogenase), but also as a dinuclear iron center (as for example in methane monooxygenase, ribonucleotide reductase and bacterioferritin) and a mononuclear iron center in the sulfur oxygenase reductase.

After the rise of O_2 in the atmosphere, the availability of iron changed as it was oxidized and precipitated as Fe(III) (see Section 3.1). Even though iron is less available under present day oxic conditions, a variety of proteins still contain iron. The fact that nature kept iron in a number of proteins throughout evolution highlights the unique and crucial chemical and physical properties of this metal. However, due to its low solubility at neutral pH, iron acquisition poses a problem for neutrophilic organisms. Two distinct molecular mechanisms have been characterized whereby environmental iron is solubilized and transported into the cytosol. Most prokaryotes produce siderophores, that have an extremely high affinity for iron [11]. Siderophores form soluble ferric chelates, that are taken up by the cell *via* high affinity receptors. In the case of mammalian cells, iron is acquired by a process involving transferrin that shows strong similarity with siderophores. The second mechanism for solubilizing iron, well-characterized in yeast, involves a reductase oriented toward the outside, that reduces Fe(III) into the more soluble Fe(II) [12,13]. Another example of a key player in iron homeostasis is the globular protein complex ferritin [14]. Each ferritin molecule can hold as many as 4,500 iron atoms inside its spherical structure formed from 24 subunits allowing intracellular iron-storage in *Bacteria* and *Eukarya* and keeping iron in a soluble and non-toxic form. Indeed, not only these mechanisms allow the uptake of iron, it also prevents it to be free in the cells where it can generate potent oxidizing hydroxyl radicals that are toxic [15,16]. Therefore, after uptake, the level of cellular iron must be carefully regulated. Within the cells, protein networks involving transporters, metal sensing and metal-storage proteins are required to maintain the proper subcellular concentrations of iron [17], and, not surprisingly, any perturbation can cause distinct pathological disorders [18]. The positive biological effects of iron are consequently

Table 1

Redox potentials of iron couple and other compounds of interest in this review.

Reduction pair	Eenv (volts)
O_2/H_2O (pH 2)	+1.12
ClO_4^-/Cl^- (pH 7)	+0.873
O_2/H_2O (pH 7)	+0.8
Fe^{3+}/Fe^{2+} (pH 2)	+0.77
$Fe(SO_4)_2^-/Fe^{2+}$ (pH 3)	+0.72
$Fe(SO_4)_2^-/Fe^{2+}$ (pH 1)	+0.697
ClO_3^-/Cl^- (pH 7)	+0.616
NO_3^-/NO_2^- (pH 7)	+0.42
Fe(III)-citrate/Fe(II)-citrate (pH 7)	+0.385
Fe(III)-NTA/Fe(II)-NTA (pH 7)	+0.372
$Fe(OH)_3/Fe(II)_{aq}$ (pH 7)	+0.014
γ -FeOOH lepidocrocite/ $Fe(II)_{aq}$ (pH 7)	-0.088
FeOOH/FeCO ₃ siderite (pH 7)	-0.05
α -FeOOH goethite/ $Fe(II)_{aq}$ (pH 7)	-0.274
α -Fe ₂ O ₃ hematite/ $Fe(II)_{aq}$ (pH 7)	-0.287
Fe ₃ O ₄ magnetite/ $Fe(II)_{aq}$ (pH 7)	-0.314

Reduction potential of non-iron compounds mentioned in this paper is represented in grey.

NTA: nitrilotriacetic acid.

Adapted from Refs. [4,6,60].

dose-dependent, excessive concentrations of free iron as well as untreated iron deficiency are both actually detrimental to cells [19].

We emphasize in this paragraph the importance of iron as a cofactor for metalloproteins; however iron also plays a central role as electron acceptor (Fe(III)) for heterotrophic bacterial growth and as electron donor (Fe(II)) for chemotrophic and phototrophic growth of *Bacteria* and *Archaea*. In Section 5, we will describe how organisms use iron as electron donor.

3. Iron and evolution of Earth

Since the earliest forms of life on Earth, the bioessential elements were selected according to four basic principles: (i) their abundance, (ii) their efficiency, (iii) their suitability for a given task and (iv) the evolutionary pressure (i.e. the environmental factors that influence the direction of natural selection, such as the bioavailability of essential elements) [20]. This has been exemplified by the metals present as cofactor in redox proteins, in particular in the case of iron. The paleochemistry of iron on Earth will be briefly described. The implication for the early biochemistry, and therefore for the evolution of life, will then be discussed.

3.1. Paleogeochimistry of iron on Earth

It is universally acknowledged that Earth evolved from an O₂ free environment, to a fully oxygenated biosphere through at least three redox stages (see among others [21–28]) (Fig. 1A). The two major irreversible oxygenation events likely occurred ~2.4 billion years and ~542 million years ago. However, how Earth's redox state evolved during the middle stage is still a matter of debate [27].

The first build-up of O₂ in the atmosphere, known as the Great Oxidation Event, was suggested to occur ~2.4 billion years ago and was likely due to the conversion of water to O₂ by cyanobacterial photosynthesis (Fig. 1A). Until recently, it was generally assumed that at the mid-Proterozoic stage, a slightly oxygenated atmosphere overlaid euxinic (anoxic and sulfidic) ocean and that H₂S, produced by hydrothermal sources and sulfate reducers, would have precipitated Fe(II) as insoluble iron sulfides, essentially pyrite (FeS₂). However, a recent paper [27] provides evidence for the coexistence of sulfidic and ferruginous (anoxic and Fe(II)-rich) conditions beneath oxic surface waters during this period (Fig. 1C). It is only after the second oxygenation event (Phanerozoic: the last ~542 million years) that a major change in the redox state of a number of bioessential elements would have occurred (Fig. 1B). During this period, ocean waters likely became oxygenated. Because bioessential elements such as sulfur, nickel, copper, zinc, molybdenum and iron are sensitive to environmental redox conditions, their abundance in the ocean has certainly changed (Fig. 1B). From an anoxic environment rich in Fe(II) and H₂S but poor in copper, zinc and molybdenum, present as insoluble sulfide minerals, the ocean was proposed to turn to generally oxic and oxygenated conditions. This likely causes the oxidation of these elements and therefore: (i) the decrease in iron (Fig. 1B) due to Fe(III) (hydr)oxides precipitation and the loss of the iron bioavailability since the concentration of soluble iron changed from 10⁻⁶ M Fe(II) to 10⁻¹⁷ M Fe(III) [21]; (ii) the increase in copper, zinc and molybdenum as soluble oxides (Fig. 1B); and (iii) the disappearance of H₂S in favor of sulfate (SO₄²⁻) (Table 2). Due to the solubilization of these elements from sulfide minerals, it was argued that the redox potential has risen from ~-0.4 V in the original ocean to ~+0.4 V [22,29].

3.2. Implications for the early biochemistry

The environmental conditions, in particular the bioavailability of essential elements in the ocean, put selective pressure on the evolution of life and altered biological molecules with respect to the

elements used as cofactors. It is now widely acknowledged that biochemistry coevolved with geochemistry. On that basis, it was speculated that the protein domains could reflect important geochemical events. Indeed, more and more data indicate that redox protein evolution follows paleochemistry on Earth (see [30–34]). As mentioned above, the original iron concentration decreased drastically, after the advent of O₂, making iron availability very low [21]. However, demand for iron remained high because iron-containing cofactors could have redox potential as low as -0.2 V. The organisms therefore developed systems to scavenge iron and to avoid its toxicity (see Section 2). On the other hand, the oxygenation of the ocean created the need for new redox active metals with higher redox potential.

This is illustrated by the cofactors of electron carriers and in this section, we will focus on the evolution of the ones shown, or predicted, to be involved in the Fe(II) oxidation pathways (see Section 5). Most of the assumptions presented below have been proposed based on the distribution of the prokaryotes containing proteins with the same cofactor on the 16S rRNA-based phylogenetic tree and/or on the phylogeny inferred from the alignments of such proteins/domains.

3.2.1. Iron–sulfur clusters

Based on chemistry and biochemistry of metals [20], on fold-domain mapping patterns [33] and on phylogenomic analysis of protein structure [34], it was concluded that the formation of the metallic cofactors coincides with the availability of the corresponding metals in the primitive sea. In the ancient ocean, Fe(II) and sulfide were abundant and soluble allowing the formation of the [4Fe–4S] clusters most likely followed by the [2Fe–2S] clusters [35]. This proposal is sustained by the fact that (i) among the low potential iron–sulfur proteins, the [4Fe–4S] clusters outnumber the [2Fe–2S] ones; (ii) the [4Fe–4S] clusters are more stable, flexible and versatile than the [2Fe–2S] ones [35]. Obviously, these properties help them to assemble spontaneously on protein templates in the reducing O₂-free primordial ocean [21,34–37]. The primitive clostridial 2 [4Fe–4S] ferredoxin fold, which is probably the most widespread iron–sulfur protein fold and the one that has undergone the most extensive modifications, was inferred to be one of the most ancient protein folds altogether [35]. Most of the [Fe–S] clusters have low redox potential, in agreement with the prevalent conditions in the primitive sea. Simple modifications in the surrounding polypeptide context enabled rapid diversification and improvement. Such changes would have allowed the adaptation to the increase of the ocean redox potential due to the oxygenation events by producing the high-potential [4Fe–4S] iron–sulfur proteins (HiPIP) (redox potential: from +0.05 to +0.5 V) [35]. Other arguments in favor of the late apparition of the HiPIP are that they are found only in *Alpha*-, *Beta*- and *Gamma* subgroups of *Proteobacteria* (with the exception of *Rhodothermus marinus*), known (i) to have appeared after the Great Oxidation Event, (ii) to contain ubiquinone (see below Section 3.2.4) and (iii) to respire O₂ [38].

3.2.2. Heme

Shortly after life's appearance on Earth, iron was also probably recruited for the formation of heme. This cofactor consists of a porphyrin ring surrounding an atom of iron and is present in all cytochromes. Porphyrin ring synthesis has been proposed to start from hydrogen cyanide chemically produced in the early Earth from methane and ammonia [21]. The most ancient heme domain is the globin-like fold [33] and was certainly present in LUCA, the Last Universal Common Ancestor.

The cytochrome *c* domains appeared in the *Bacteria* branch of the tree of life, and were eventually transferred to *Archaea* (through horizontal gene transfer) and *Eukarya* (through the endosymbiotic events that led to eukaryotic organelles) [39].

The main difference between cytochrome *b* and *c* is related to the binding of the heme that is covalently attached to the polypeptide *via*

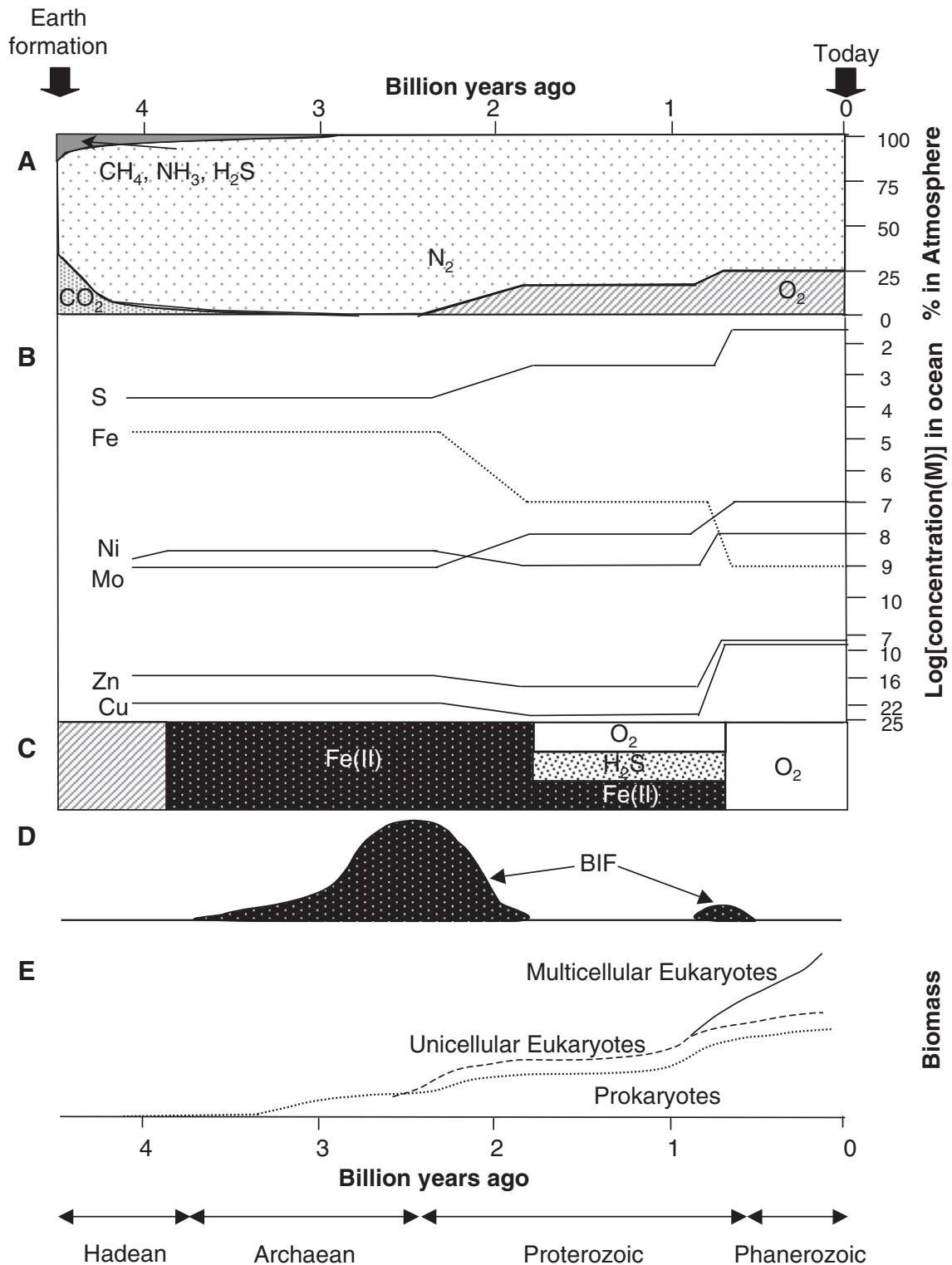


Fig. 1. Biogeochemical evolution of Earth. Abundance of some elements in the atmosphere (A) and in the ocean (B); chemical composition of the deep ocean (C); banded iron formation (BIF) deposits in the ocean (D); evolution of life from prokaryotes to multicellular Eukaryotes (E). Adapted from Refs. [21,26,27]. See the text for more details.

two thioether bonds in cytochrome *c* but not in cytochrome *b*. By incubating *in vitro* apocytochrome with ferrous heme in reducing conditions, both to avoid formation of a disulfide bond within the —CXXCH— motif and to maintain the heme in the ferrous state, a *b*-type cytochrome was formed followed by the formation of the thioether bonds to give the

c-type product, without the action of any biogenesis proteins [40]. These data show that cytochromes *b* and *c* can form in reducing conditions without the action of any biogenesis proteins and therefore are likely to assemble spontaneously in the primitive ocean. However, cytochrome *c* biogenesis *in vivo* is not facile because it requires the

Table 2

Available soluble concentrations of some key elements in the sea before and after the rise in atmospheric oxygen (adapted from Ref. [22]).

Metal ion	Original conditions (M)	Aerobic conditions (M)
Fe	$\sim 10^{-7}$ (Fe(II))	$\sim 10^{-19}$ (Fe(III))
Co(II)	$< 10^{-9}$	$\sim 10^{-9}$
Ni(II)	$< 10^{-9}$	$< 10^{-9}$
Cu	$< 10^{-20}$ (Cu(I))	$< 10^{-10}$ (Cu(II))
Zn(II)	$< 10^{-12}$	$< 10^{-8}$
Mo	$< 10^{-10}$ (MoS_4^{2-} , Mo(OH)_6)	$< 10^{-8}$ (MoO_4^{2-})
W	$\sim 10^{-9}$ (WS_4^{2-})	10^{-9} (WO_4^{2-})
H ⁺	Low pH (6.5?)	pH 7.6–8.2
H ₂ S	10^{-2}	Low (SO_4^{2-} : 10^{-2})

heme and the unfolded apo-cytochrome *c* to be delivered from the cytoplasm (reducing conditions) to the periplasm (oxidizing environment) and the subsequent heme attachment. Five systems differing between various cell types have been described for the biogenesis of cytochrome *c* [41–44]. The transfer of the genes encoding cytochrome *c* domains from *Bacteria* to *Archaea* and *Eukarya* co-occurred with the genes encoding the biogenesis machinery [45].

The cytochrome *bc* complexes, that represent the only complex common to almost all respiratory and photosynthetic electron transfer chains, probably existed in the common ancestor of *Bacteria* and *Archaea* [46–48] as a [2Fe–2S]-Rieske protein and cytochrome *b* complex. It is only later that this functional and evolutionary core may have captured the cytochrome *c* as indicated by (i) the genetic organization of the *petABC* operons encoding the *bc*₁ complex with *petC* (cytochrome *c*), when present, found upstream from *petA* (Rieske subunit) or downstream from *petB* (cytochrome *b*) and (ii) the absence of cytochrome *c* in the archaeal *bc*₁ complex [48,49]. Furthermore, in contrast to the Rieske and cytochrome *b* subunits, the type (mono-, di- or tetraheme) and the sequence of the cytochromes *c* are not conserved suggesting a polyphylogenetic origin [48,49]. This is a perfect example of the “redox protein construction kit” proposed by Baymann et al. [46].

3.2.3. Molybdenum cofactor

Molybdenum utilization is very likely an ancient trait present in LUCA because (i) it is utilized by almost all phyla of *Archaea* and *Bacteria* and (ii) a number of molybdo-enzymes, including the arsenite oxidase, the formate dehydrogenase, the nitrate reductase and the polysulfide reductase, have been predicted to have existed before the *Archaea/Bacteria* divergence [46,50–53]. However, the concentration of molybdenum in the primitive ocean was presumably low and increased likely only after the oxygenation of the Earth (Table 2 and Fig. 1) suggesting its poor bioavailability at the origin of life [54]. On the contrary, tungsten, that has the same chemical properties as molybdenum, was supposed to be soluble (Table 2) and could have been used instead in early life. Another possibility is that molybdenum has been supplied by alkaline hydrothermal vents [51]. As soon as molybdenum became soluble, and therefore more available than tungsten (Table 2 and Fig. 1), it might have taken over [52].

The synthesis of the molybdo-pterin cofactor is a conserved multi-step pathway that is present in molybdenum-utilizing organisms in all three domains of life (see Ref. [52] for review and references therein) and therefore might have been present in LUCA.

3.2.4. Quinones

Important components of the chemiosmotic energy-converting mechanisms are the lipophilic quinones that can diffuse in the lipid bilayer and connect the redox enzymes. Menaquinones are widely used in *Prokaryotes* in general and specifically in all the deep branching prokaryotic phyla (Schoepp-Cothenet et al., this issue) and consequently have been proposed to be present already in LUCA [55]. Whatever their exact chemical nature, they all present low redox potential, in

agreement with the proposed reducing archaeal environment. When the environmental oxidation state increased after the Great Oxidation Event, menaquinones appear to have been substituted by higher redox potential quinones, such as ubiquinones (*Proteobacteria*), plastoquinones (*Cyanobacteria*) and caldariellaquinones (*Sulfolobales*) ($E_m = +0.1$ V) [48,55]. The distribution of the quinone types on the prokaryote phylogenetic tree agrees with the emergence of menaquinones prior to ubiquinones (Schoepp-Cothenet et al., this issue). Another argument supporting this proposal is the phylogeny of the genes involved in quinone biosynthesis (see Schoepp-Cothenet et al., this issue).

3.2.5. Copper

It has been proposed that copper was solubilized from sulfide minerals to soluble Cu(II) form after the Great Oxidation Event (Table 2). Copper was then certainly bioavailable and has a higher potential than Fe(II)/Fe(III). It began to participate as cofactor in enzymatic reactions. Copper proteins have consequently likely appeared early after O₂ accumulation. They represent nearly 3000 members distributed over all domains. However they are mainly confined to the aerobic organisms [29]. To adapt to this new cofactor and to exploit the new environmental conditions, the cupredoxin fold seems to have appeared during this time period [33]. From bioinformatic analysis using the MANET (molecular ancestry network) database, the most ancient enzyme carrying this domain has been proposed to be the cytochrome *c* oxidase (EC.1.9.3.1) that catalyzed the reduction of O₂ to water [56]. The evolution of this superfamily is still a matter of debate. Based on phylogenetic, enzymatic and geochemical results, Ducluzeau et al. [57] proposed that it arose from nitric oxide reductase already present in the earliest Archaeon while, according to phylogenetic and distribution analysis, Gribaldo et al. [58] concluded that NO reduction is not ancestral but a derived feature in this family.

3.3. Conclusion

To summarize, the order of appearance of the cofactors and the corresponding domains present in the extant redox proteins is in agreement with the modification of the elements' aqueous solution chemistry during Earth's history. Before the oxygenation events [Fe–S] clusters, hemes and menaquinones likely appeared while copper and ubi-, plasto- and caldariella-quinones have certainly evolved after the oxygenation events. In addition, HiPIP would have appeared after [4Fe–4S] and [2Fe–2S] proteins, hemes B containing proteins would have emerged before cytochromes *c*, and tungsten- before molybdenum-enzymes. These data may help deciphering the evolution of the energy pathways, and we used them to discuss the potential evolution of the iron oxidation pathways (see below Section 6).

4. Physiology and phylogeny of ferrous iron oxidizers

Biodiversity of iron oxidizing prokaryotes has been extensively reviewed [4,59–63] and will be briefly addressed here. These microorganisms have wide metabolic diversities: they can be phototrophs or chemotrophs, autotrophs or heterotrophs, aerobes or anaerobes, acidophiles or neutrophiles, psychrophiles, mesophiles or thermophiles. The physiological characteristics of the Fe(II) oxidizers in which the Fe(II) oxidation pathway has been studied are summarized in Table 3. According to Hedrich et al. [59], they can be divided in four physiological groups: (1) the acidophilic aerobes, (2) the neutrophilic aerobes, (3) the neutrophilic photosynthetic anaerobes and (4) the neutrophilic anaerobes dependent on nitrate, perchlorate or chlorate reduction.

The aerobic acidophiles are the more widespread among the Fe(II) oxidizers and are found in Gram negatives (*Nitrospira*, *Acidobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*), Gram positives (*Firmicutes*

Table 3

Physiological characteristics of the microorganisms in which Fe(II) oxidation pathways are discussed in this review.

Eubacteria and Archaea	Domain	Phylum	pH ^a	Temperature ^b	Energy source ^c	Fe(II) electron acceptor ^d	Carbon source ^e	Ferrous iron oxidation pathways based on:					
								Molecular biology	(Meta) genomics	Transcriptomics differential display	(Meta) proteomics	Biochemistry	Mutant/heterologous complementation
<i>Acidithiobacillus ferrooxidans</i>	Eubacteria	γ -Proteobacteria	A	M	I	O ₂	I	+	+	+	–	+	–
<i>Acidithiobacillus ferrivorans</i>	Eubacteria	γ -Proteobacteria	A	Pt	I	O ₂	I	+	+	–	–	–	–
<i>Leptospirillum</i> group II (<i>L. ferriphilum</i> and " <i>L. rubarum</i> ")	Eubacteria	Nitrospira	A	M	I	O ₂	I	–	+	–	+	+	–
<i>Leptospirillum</i> group III (" <i>L. ferrodiazotrophum</i> ")	Eubacteria	Nitrospira	A	M	I	O ₂	I	–	+	–	+	+	–
<i>Sulfobacillus sibiricus</i>	Eubacteria	Firmicutes	A	Tm	I, O	O ₂	I, O	–	–	–	–	+	–
" <i>Thiobacillus prosperus</i> "	Eubacteria	γ -Proteobacteria	A	M	I	O ₂	I	+	–	+	–	–	–
" <i>Ferroplasma acidarmanus</i> "	Archaea	Euryarchaeota	A	Tm	I, O	O ₂	I, O	–	+	–	+	+	–
<i>Metallosphaera sedula</i>	Archaea	Crenarchaeota	A	T	I, O	O ₂	I, O	–	+	+	–	–	–
" <i>Metallosphaera yellowstonensis</i> "	Archaea	Crenarchaeota	A	T	I, O	O ₂	I, O	+	+	–	–	–	–
<i>Sulfolobus metallicus</i>	Archaea	Crenarchaeota	A	T	I	O ₂	I	+	–	+	+	–	–
<i>Sulfolobus tokodaii</i>	Archaea	Crenarchaeota	A	T	I, O	O ₂	O	–	+	–	–	–	–
<i>Mariprofundus ferrooxydans</i>	Eubacteria	ζ -Proteobacteria	N	M	I	O ₂	I	–	+	–	–	+	–
<i>Sideroxydans lithotrophicus</i>	Eubacteria	β -Proteobacteria	N	M	I	O ₂	I, O?	+	–	–	–	+	+
<i>Rhodospseudomonas palustris</i>	Eubacteria	α -Proteobacteria	N	M	I, O	RC	I, O	+	–	–	–	–	+
<i>Rhodobacter capsulatus</i>	Eubacteria	α -Proteobacteria	N	M	I, O	RC	I, O	+	–	–	–	–	+
<i>Dechlorosoma suillum</i>	Eubacteria	β -Proteobacteria	N	M	I, O	NO ₃ /ClO ₄	O	–	–	–	–	+	–
<i>Dechloromonas agitata</i>	Eubacteria	β -Proteobacteria	N	M	I, O	NO ₃	O	–	–	–	–	+	–
<i>Pseudogulbenkiania</i> sp.	Eubacteria	β -Proteobacteria	N	M	I, O	NO ₃	I, O	–	–	–	–	+	–

nd: not determined; ^aA: acidophile, N: neutrophile; ^bM: mesophile, Pt: psychrotolerant, T: thermophile, Tm: moderate thermophile; ^cI: inorganic, O: organic; ^dO₂: oxygen, NO₃: nitrate, ClO₄: perchlorate, RC: reaction center.

Box shade corresponds to the four physiological groups of the Fe(II) oxidizers: the acidophilic aerobes (white), the neutrophilic aerobes (light grey), the neutrophilic photosynthetic anaerobes (middle grey) and the neutrophilic nitrate/perchlorate/chlorate reducing anaerobes (dark grey).

and *Actinobacteria*) and in *Archaea* (*Crenarchaeota* and *Euryarchaeota*) [59,62]. On the contrary, all currently known neutrophilic aerobic Fe(II) oxidizers have only been identified in *Proteobacteria*, with the ones living in fresh water belonging to the *Betaproteobacteria* while the marine ones cluster in a new class, the “*Zetaproteobacteria*” [61]. The anaerobic Fe(II) oxidizers are phylogenetically diverse. The nitrate/perchlorate/chlorate-dependent ones are encountered in *Bacteria* (*Alpha*-, *Beta*-, *Gamma*- and *Deltaproteobacteria*) as well as in *Archaea* (*Crenarchaeota*) while phototrophs have been detected in *Chlorobia*, *Alpha*- and *Gammaproteobacteria* but thus far not in *Archaea* [59,60]. A number of phylogenetic trees based on the 16S rRNA gene of the iron oxidizers have been recently inferred and the readers are referred to the corresponding papers (Fe(II) oxidizers and Fe(III) reducers [60], lithotrophic and heterotrophic Fe(II) oxidizers [61], Fe(II) oxidizing *Proteobacteria* [59] and the acidophilic Fe(II) oxidizers [62]).

The fact that the ability to oxidize Fe(II) is widely distributed in *Bacteria* and *Archaea* suggests that the dissimilatory Fe(II) oxidation may be an ancient energy metabolism. It has even been proposed that the anoxic Fe(II) oxidizers might have been responsible for the early Banded Iron Formation deposition that were formed in the anoxic biosphere early during Earth history (see Fig. 1D) [64].

5. Different strategies developed by microorganisms to oxidize ferrous iron

5.1. Ferrous iron oxidation, a true challenge for microorganisms

While a number of Fe(II) oxidizers have been identified, the understanding of the Fe(II) oxidation pathways lags far behind. This is mainly due to the poor cell yield with Fe(II) as an electron donor and to the lack of an efficient genetic system in these microorganisms. Furthermore, even if the capability to use Fe(II) for energy purposes is widespread, its oxidation is a real challenge for both acidophiles and neutrophiles due to the iron chemistry [4,6,59,62]. First, at $\text{pH} \geq 5$, Fe(II) is rapidly oxidized by O_2 while it is soluble and rather stable at $\text{pH} < 4$ even in oxic conditions (see Section 1). Therefore, the neutrophiles have to compete with the spontaneous oxidation of free Fe(II) and to be able to oxidize the various forms of complexed Fe(II) (ligand or mineral bound) that have different reduction potentials (see Section 1 and Table 1) [6,59], while Fe(II) is readily available for acidophiles. The second problem encountered by the iron oxidizers is the insolubility at neutral pH of Fe(III) which precipitates rapidly as ferric (hydr)oxides. If oxidation occurs at the neutral pH of the cytoplasm, this implies that (i) the Fe(III) produced will precipitate clogging and acidifying the cytoplasm and (ii) the reaction of Fe(II) with O_2 will generate free radicals inducing oxidative stress. The third critical point is the redox potential of the Fe(II)/Fe(III) couple that is dependent on pH and on the complexing agents (see Section 1 and Table 1). Higher redox potentials occur at acidic pH where both species are soluble, $\sim +0.720$ V at pH 3 when complexed with sulfate (the most frequent complexing agent in acid mine/rock drainage waters) [4]. This means that the only electron acceptor that acidophiles can use is O_2 with a redox potential of $\sim +1.12$ V at low pH values. Because of the weak difference in the mid-point redox potential between Fe(II)/Fe(III) and $\text{O}_2/\text{H}_2\text{O}$ couples, Fe(II) oxidation is at the thermodynamic limit and is an energy dearth for these microorganisms and, as a result, a considerable amount of Fe(II) has to be oxidized to sustain the growth and poor cell yield is obtained. In addition, the reducing power (NAD(P)H) has to be reconstituted for anabolic processes such as CO_2 and N_2 fixation while the redox potential of the $\text{NAD(P)}^+/\text{NAD(P)H}$ couple is -0.32 V at cytoplasmic pH. Consequently, energy is required to push the electrons “uphill” from Fe(II) to NAD(P)^+ against the redox potential gradient. This means that in the autotrophs, the electrons from Fe(II) oxidation have to be conducted not only “downhill” to O_2 for energy gain but also “uphill” to NAD(P) via an endergonic pathway. It has to be

noted, however, that this reasoning is based on mid-point redox potentials that are determined under standard conditions, that is under the assumption of equilibrium conditions, that is certainly not the case in the environment. In mixotrophic and heterotrophic neutrophilic iron oxidizers, the reducing equivalents can be generated by carbon source oxidation.

5.2. Ferrous iron oxidation pathways, models described so far

Although the components involved in the oxidation of Fe(II) differ significantly from one organism to the other, the overall organization seems to be conserved. First, while the redox potential difference between Fe(II)/Fe(III) and $\text{O}_2/\text{H}_2\text{O}$ is small, the number of redox proteins is surprisingly high, in particular in Gram negative bacteria. Second, instead of being organized “horizontally” with the redox proteins located along the cytoplasmic membrane as in most respiratory chains, the topography of the electron carriers in the iron oxidizers is vertical allowing a nanowire to be formed between the outside medium and the cytoplasm. The high number of electron carriers enables this “vertical” topography across the cell membrane(s). This organization allows (i) to maintain iron outside of the cell to avoid Fe(III) precipitation at the neutral pH of the cytoplasm and (ii) to escape from oxidative stress due to Fe(II) reacting with O_2 . Therefore, Fe(II) oxidation is taking place in a different subcellular compartment than the O_2 reduction. Furthermore, in acidophiles, this organization enables to keep a neutral pH in the cytoplasm by consuming the protons entering the cell matrix due to the proton gradient between outside and inside the cell by reduction of O_2 to H_2O .

The known Fe(II) oxidation pathways have been reviewed recently (Refs. [6,62,65,66] and references therein). They are summarized in this section and schematized in Fig. 2 (acidophiles) and Fig. 3 (neutrophiles). Experimental approaches used to define each individual models are summarized in Table 3.

5.2.1. Aerobic iron oxidation

5.2.1.1. Acidophiles. The electron transfer chain between Fe(II) and O_2 of *Acidithiobacillus* (*At.*) *ferrooxidans* is the best studied case [6,62,65,67–71]. This pathway involves outer membrane, periplasmic and inner membrane components that constitute a super-complex spanning the outer and the inner membranes conducting the electrons from sulfide minerals (such as FeS_2) to the terminal electron acceptor located in the cytoplasm [67,69,72,73] (Fig. 2A). This electron wire consists of the outer membrane embedded cytochrome *c* *Cyc2* which contains a domain facing the external environment and where Fe(II) oxidation occurs [69,73], the periplasmic copper protein rusticyanin [74], the membrane-bound cytochrome *c* *Cyc1* [75] and the integral inner membrane *aa*₃ cytochrome oxidase [76] that catalyzes O_2 reduction. From rusticyanin, the electrons can also take an “uphill” pathway driven energetically by the proton motive force to the NADH-1 complex catalyzing the reduction of NAD^+ on the cytoplasmic side of the inner membrane via the membrane-bound cytochrome *c* *CycA1*, the integral cytoplasmic membrane *bc*₁ complex functioning in reverse and the membrane associated ubiquinones [77–80].

The iron oxidizing *Acidithiobacillus* spp. likely comprise four species among which *At. ferrooxidans* and *At. ferrivorans* [81,82]. In two of them, including *At. ferrivorans*, two different Fe(II) oxidation pathways exist: one via the archetypal rusticyanin *RusA* as in *At. ferrooxidans* (Fig. 2A) and the second likely through the high potential iron–sulfur protein (HiPIP) *Iro* and possibly an isozyme of rusticyanin, *RusB* (Fig. 2B) [81].

In the salt-tolerant *Gammaproteobacterium* “*Thiobacillus prosperus*,” redox proteins presenting significant similarities to all but one of the electron carriers involved in the electron transfer from Fe(II) to O_2 in *At. ferrooxidans* have been identified: the outer membrane cytochrome *c*, the copper protein and the four subunits of an *aa*₃-type cytochrome

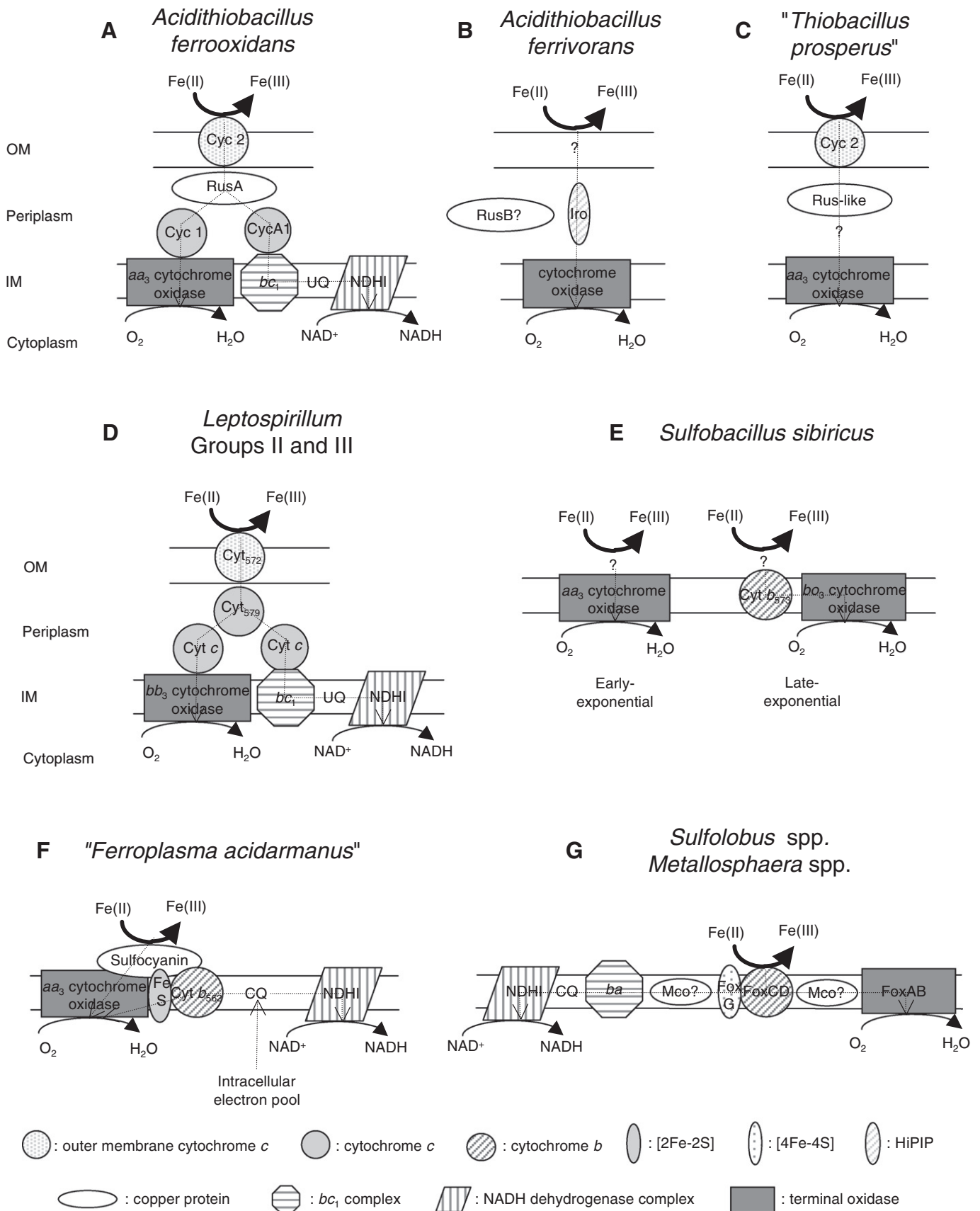


Fig. 2. Iron oxidation pathways in the acidophilic microorganisms. A: *Acidithiobacillus ferrooxidans* [65–73]; B: *Acidithiobacillus ferrivorans* [81]; C: "*Thiobacillus prosperus*" [83]; D: *Leptospirillum* Groups II and III [86–88]; E: *Sulfobacillus sibiricus* [92]; F: "*Ferroplasma acidarmanus*" [57,88,93]; G: *Sulfolobus* spp. and *Metallosphaera* spp. [94–96]. Each redox protein family is schematized with a different shape and a different motif as indicated below the Figure. The electron flow is indicated as a dashed line (see the text for more details).

oxidase [83]. The only electron carrier that was not detected is the membrane-bound cytochrome *c* Cyt*c*. It has been proposed that the electrons are transferred directly between rusticyanin and the terminal

oxidase (Fig. 2C). However, it is also possible that another electron transfer protein yet to be discovered is involved in this intermediary electron transfer [83].

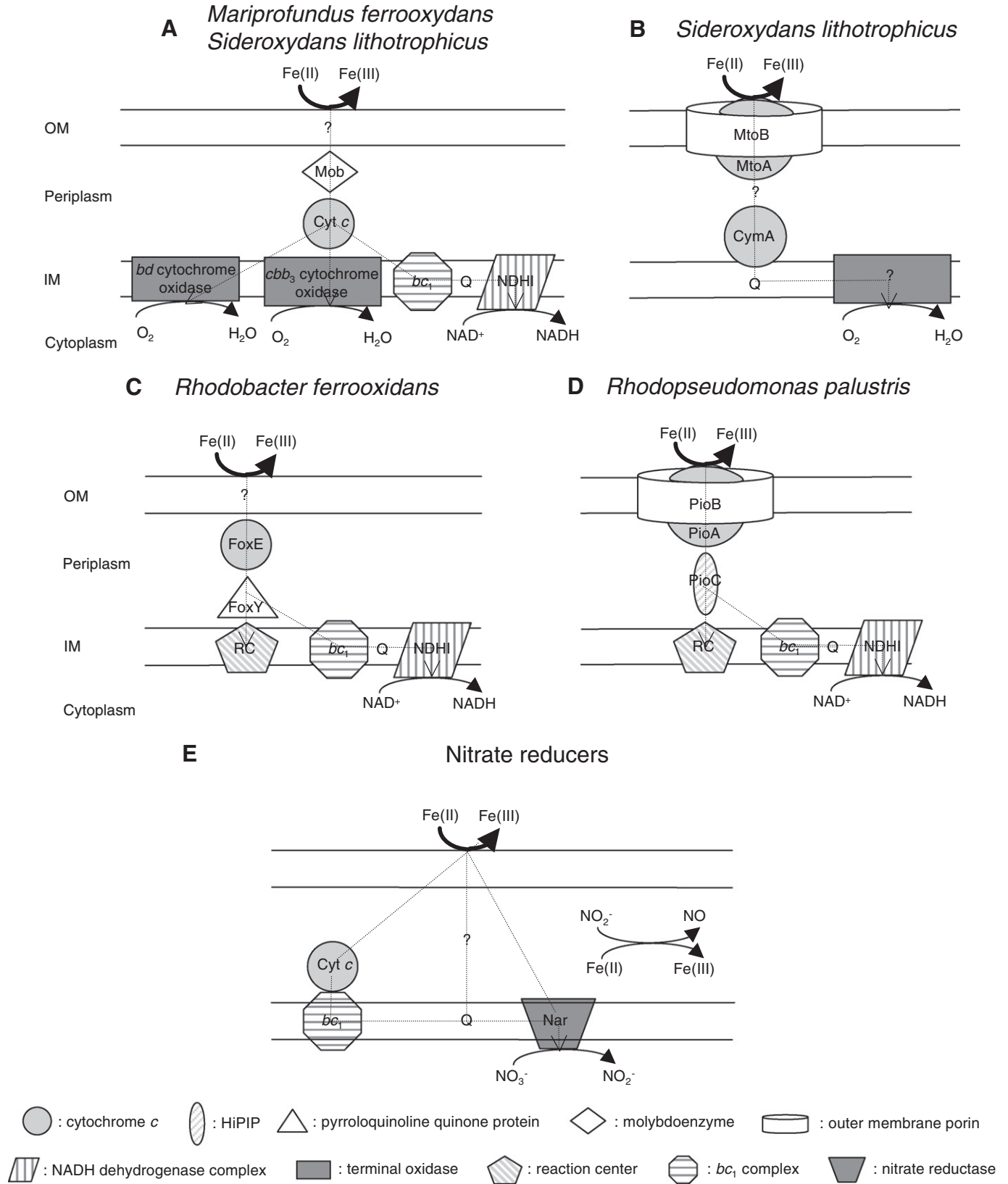


Fig. 3. Iron oxidation pathways in the neutrophilic microorganisms. A: *Mariprofundus ferrooxydans* [99]; B: *Sideroxydans lithotrophicus* [101]; C: *Rhodobacter ferrooxidans* [106]; D: *Rhodopseudomonas palustris* [108]; E: nitrate reducers [113]. Each redox protein family is schematized with a different shape and a different motif as indicated below the Figure. The electron flow is indicated as a dashed line (see the text for more details).

For the *Leptospirillum* spp. (at least for Groups II and III) belonging to the *Nitrospira* class, it has been proposed [84–88] that the outer membrane cytochrome *c* *Cyc*₅₇₂ [89] is the direct oxidant of Fe(II) and electrons are then passed through the periplasmic cytochromes *c* *Cyc*₅₇₉ [90,91] and periplasmic cytochromes *c* not yet identified to a *cbb*₃ oxidase or to the NADH-1 complex via a *bc*₁ complex and the quinol pool (Fig. 2D).

Electron transfer from Fe(II) to O₂ in the Gram positive *Sulfobacillus sibiricus* has been proposed to occur via an *aa*₃-type oxidase in early-exponential growth phase and via the membrane-bound cytochrome *b*₅₇₃ and a *bo*₃-type oxidase in late-exponential growth phase [92] (Fig. 2E).

Concerning the *Euryarchaeota* “*Ferroplasma acidarmanus*” [93] and *Ferroplasma* type II [88], the copper protein sulfocyanin and the cytochrome *cbb*₃ oxidase seem to be involved in the electron transfer chain between Fe(II) and O₂ (Fig. 2F). However, no archaeal *cbb*₃ oxidases were detected by phylogenetic analysis of the heme copper oxidase family [57]. From this study, it seems more likely that an *aa*₃ type oxidase is involved in these *Archaea*.

A completely different pathway occurs in the *Crenarchaeota*. In *Sulfolobus* (*S.*) *metallicus* and *S. tokodaii* [94] on the one hand, and in *Metallosphaera* (*M.*) *sedula* [95] and in “*M. yellowstonensis*” [96] on the other hand, a similar cluster of genes more highly expressed in the presence than in the absence of Fe(II) has been identified. The gene order and content in this cluster, referred to as the *fox* gene cluster, vary between the four species. Most of the *fox* genes encode membrane proteins among which two putative cytochromes *b*, the predicted subunits I and II of a heme copper oxidase, ferredoxins and others proteins with [Fe–S] binding domains. In the two *Metallosphaera* species, the *soxNL-cbsABA* cluster encoding a cytochrome *ba* complex, analogous to the *bc*₁ complex [97], also has been shown to be triggered by Fe(II). A tentative model has been proposed for “*M. yellowstonensis*”: electrons are extracted from Fe(II) by the cytochromes *b* FoxCD and then a multicopper oxidase (Mco in *Metallosphaera*) transports electrons either “uphill” to the cytochrome *ba* complex for anabolic processes requiring NAD(P)H or “downhill” to the heme copper oxidase FoxAB for O₂ reduction (Fig. 2G).

5.2.1.2. Neutrophiles. The only way aerobic neutrophilic iron oxidizers are able to compete with the chemical oxidation of Fe(II) is by living in microoxic niches such as anoxic–oxic transition zones. Such is the case for *Mariprofundus* (*M.*) *ferrooxydans* and *Sideroxydans* (*S.*) *lithotrophicus* for which a Fe(II) oxidation pathway model has been recently hypothesized.

The marine *M. ferrooxydans* excretes a stalk that was proposed to direct iron oxidation formation to prevent cell encrustation [98]. The predicted Fe(II) oxidation pathway involves a non-identified iron oxidase located in the outer membrane, a periplasmic Fe–S molybdopterin oxidoreductase, periplasmic cytochromes *c* that deliver the electrons to a *cbb*₃ and/or *bd* oxidase where O₂ is reduced (“downhill” pathway) and to a *bc*₁ complex (“uphill” pathway) for NADH synthesis [99] (Fig. 3A).

Unlike *M. ferrooxydans*, *S. lithotrophicus* does not form stalks or sheaths but rather produces amorphous iron oxyhydroxides which coat the cells [100]. The Fe(II) oxidation pathway proposed in *M. ferrooxydans* seems to be present also in *S. lithotrophicus*. In addition, two components homologous to MtrA and MtrB described for the extracellular reduction of Fe(III) in *Shewanella oneidensis* and for the extracellular oxidation of Fe(II) in *Rhodospseudomonas palustris* (see below) have been suggested: a decaheme cytochrome *c* MtoA, buried into the outer membrane protein MtoB, is proposed to oxidize Fe(II) and to transfer electrons to the quinone pool via an unknown periplasmic protein and the membrane-anchored tetraheme cytochrome *c* CymA [101] (Fig. 3B).

5.2.2. Anaerobic iron oxidation

As mentioned earlier, Fe(II) oxidation in anoxic conditions is feasible only in neutrophilic conditions in which this reaction is thermodynamically favorable. Indeed, at near-neutral pH, the redox potential of the Fe(II)/Fe(III) couple (<+0.385 V depending on the complexing agent) is lower than that of the photosystem I (+0.45 V) and of the nitrate/nitrite couple (+0.43 V) (see Refs. [6,59]). However, in some photosynthetic [102] and nitrate reducing [103] bacteria, Fe(II) oxidation has been suggested to be a detoxification mechanism to alleviate Fe(II) toxicity rather than an energy yielding pathway.

5.2.2.1. Phototrophs. The Fe(II) oxidation pathway has been deciphered in two phototrophs, namely *Rhodobacter* (*Rb.*) *ferrooxidans* [64] and *Rhodospseudomonas* (*Rp.*) *palustris* [104].

The *Rb. ferrooxidans* strain SW2 grows autotrophically by using soluble Fe(II) as a sole electron donor source for anoxygenic photosynthesis [64,105]. The *foxEYZ* operon is necessary for this phototrophic Fe(II) oxidation. It encodes a bihemic cytochrome *c* (FoxE), a periplasmic protein containing the redox cofactor pyrroloquinoline quinone (FoxY) and an inner membrane transport protein (FoxZ) [106]. FoxE has been shown very recently to be thermodynamically and kinetically able to oxidize Fe(II) [107]. A tentative model has been proposed in which the electrons extracted from Fe(II) are transferred to FoxE, then to FoxY and from there to the reaction center or to the *bc*₁ complex [6] (Fig. 3C).

In *Rp. palustris*, the *pioABC* operon has been shown to be essential for phototrophic Fe(II) oxidation [108]. It encodes a decaheme cytochrome *c*, an outer membrane and a HiPIP protein. PioAB are homologs of MtrAB of the Fe(III) reducing bacterium *S. oneidensis*. MtrAB were shown to form a complex that transfers the electrons across the outer membrane from periplasmic electron carriers to cytochrome *c* located in the outer membrane on the exterior surface (see Ref. [6] for review and references therein). It is therefore likely that the cytochrome *c* PioA inserted in the outer membrane porin-like PioB transfers the electrons from Fe(II) to the periplasmic HiPIP PioC which hands them on to the reaction center or to the *bc*₁ complex [6,108] (Fig. 3D).

5.2.2.2. Nitrate reducers. Some microbes are able to grow by using nitrate, nitrite, chlorate, or perchlorate as a terminal electron acceptor when oxidizing Fe(II). However, it has to be pointed out that most of them do not grow lithoautotrophically with Fe(II) as the sole electron donor and require an additional electron donor or organic carbon as an energy source [60]. The components of these pathways have not been studied. Nevertheless, the cytochromes *c* of *Dechloromonas agitata* [109], *Dechlorosoma suillum* [110] and of *Pseudogulbenkiania* sp. strain 2002 [60] were reduced *in vivo* in the presence of Fe(II) and chlorate or nitrate suggesting that at least one cytochrome *c* is involved in the electron transfer from Fe(II) to the respiratory chain. In addition, the Fe(II) oxidation takes place likely outside the cell as shown by the microbially catalyzed nitrate-dependent oxidation of solid-phase Fe(II) [111,112]. Four possible mechanisms have been recently suggested [113] that can be summarized as follows: (i) a dedicated Fe(II) oxidoreductase, (ii) an aspecific activity of the nitrate reductase, (iii) the *bc*₁ complex that accepts the electrons from Fe(II) and reduces the quinone pool and (iv) an abiotic reaction between Fe(II) and NO₂[−] [112,114] (Fig. 3E). We hope that future experiments will address these predictions.

6. Evolution of the Fe(II) oxidation pathways

It is clear (i) that iron oxidizers are ubiquitous and have been identified in *Bacteria* as well as in *Archaea* from various environments and with quite different physiologies (Table 3) and (ii) that the Fe(II) oxidation pathways are diverse (Figs. 2 and 3). Can any information be drawn from the sections presented above to help deciphering the history of the biotic Fe(II) oxidation? First, a scenario on the

evolution of the Fe(II) oxidation pathways will be presented based on the iron paleochemistry on Earth (Section 3.1) and on the distribution of the iron oxidizers on the prokaryote phylogenetic tree (Section 4). Another one will be hypothesized from the data we now have on the proposed redox cofactors emergence (Section 3.2) and on the redox proteins involved in the Fe(II) oxidation pathways characterized so far (Section 5). Both scenarios will then be compared and the points of disagreement will be discussed.

Due to the chemical weathering of the continental crust and/or subsurface hydrothermal convection processes, soluble Fe(II) was certainly sufficiently abundant in the ocean during the first two billion years of Earth (Fig. 1) to constitute an important substrate for microbial metabolism. It was likely the most widespread source of reducing power and as such was supposed to be used as electron donor in respiratory pathways since the earliest forms of life. Because the ocean likely became oxic only 1 to 0.54 billion years ago, oxic Fe(II) oxidation may have emerged after oxygen-independent Fe(II) oxidation. It was proposed that anoxygenic nitrate-dependent Fe(II) oxidation might have evolved in the primordial deep sea water that was rich in NO_3^- due to the N_2 conversion during lightning discharge on the early Earth [115] while the anoxygenic phototrophic Fe(II) oxidation was operative only at surface water where sunlight was available [110]. Noteworthy, the nitrate-dependent Fe(II) oxidizers are widespread and encountered in the Bacteria (*Alpha*-, *Beta*-, *Gamma*- and *Deltaproteobacteria*) as well as in Archaea (*Crenarchaeota*) while the phototrophs remain confined to *Chlorobia*, *Alpha*- and *Gammaproteobacteria*. The limited phylogenetic distribution of the photosynthetic iron oxidizers is likely due to the fact that all the bacteria containing reaction center I and/or II cluster together in a common region of the phylogenetic tree that correspond to late radiations of bacterial clades, shortly before the Great Oxidation Event [116–119]. By contrast, the membrane-attached nitrate reductase Nar was likely a pre-LUCA enzyme [53]. Therefore, the nitrate-dependent Fe(II) oxidizers represent most likely the most ancient dissimilatory Fe(II) metabolism. Based on the microbial community analysis of microbial mats heavily loaded with Fe(III), it has been proposed that *Cyanobacteria*, that have invented oxygenic photosynthesis, may oxidize Fe(II) [120,121]. However such iron oxidizers have never been isolated and are not likely to exist (D. B. Johnson, personal communication). Indeed, oxidative photosynthesis is known to increase the pH and the O_2 concentration which could well cause Fe(II) to oxidize spontaneously. Therefore, *Cyanobacteria* likely play an indirect role in Fe(II) oxidation by increasing the pH and the oxygen concentration in the mats. The Great Oxidation Event provided the acidophiles with the opportunity to use Fe(II) as an energy source since O_2 is the only electron acceptor thermodynamically relevant in that case. The acidophilic iron oxidizers are widespread and fall into different phyla of both Bacteria and Archaea but their Fe(II) oxidation pathways are diverse. This suggests that the capacity to oxidize Fe(II) in these prokaryotes appears independently more than once, possibly from systems that adapted to O_2 , followed by diversification and dissemination by lateral gene transfer. The neutrophilic aerobic iron oxidizers are restricted to the *Beta*- and *Zetaproteobacteria* classes, suggesting a later emergence in fresh and marine water, respectively. Based on the paleochemistry of Earth, the phylogeny and the physiology of the iron oxidizers, the following timescale of Fe(II) oxidizers' evolution can be proposed. First, the nitrate-dependent anoxygenic iron oxidizers arose early in the ancient deep ocean and might be involved in the formation of Fe(III) oxides, magnetite and hematite in anoxic sediments, as shown with extant microorganisms [110,122,123], and could consequently be at the origin of the Banded Iron Formation deposition during the late Archaean to the early Proterozoic (Fig. 1). Second, the phototrophic anoxygenic iron oxidizers arose in surface waters after the Archaea and Bacteria split but before the Great Oxidation Event and may reflect a transition from anoxygenic to oxygenic photosynthesis.

Then, the O_2 produced by *Cyanobacteria* catalyzed Fe(II) oxidation to Fe(III) which deposited as the second Banded Iron Formation (e.g. at ~0.9 billion years ago). Third, after the advent of O_2 , the acidophilic and the neutrophilic aerobic Fe(II) oxidizers emerged.

Is this time frame of the dissimilatory iron oxidizer evolution in agreement with the biochemistry of their Fe(II) oxidation pathways described so far? If we accept that protein cofactors reflect Earth's paleochemistry, as pointed out in Section 3.2, then the cofactors of the redox proteins involved in the Fe(II) oxidation pathway might help to understand when these proteins, and likely their pathway, occurred relative to the rise of O_2 . As shown in Section 3.2, the copper proteins (including the heme copper oxidases), the HiPIP, the ubiquinone (UQ) and the caldariellaquinone (CQ) appear after the Great Oxidation Event while the redox proteins carrying iron–sulfur, heme, molybdenum cofactor and menaquinone were already present in LUCA. Based on these observations, we propose that the pathways in which a copper protein (rusticyanin A or B, rusticyanin-like, sulfocyanin, Mco, *cbb*₃, *bb*₃, *aa*₃ or *bo*₃ heme copper oxidase), a HiPIP (Iro and PioC), ubiquinone (UQ) or caldariellaquinone (CQ) is present, likely arose after the Great Oxidation Event. By contrast, the ones with only iron–sulfur protein(s) ([4Fe–4S] or [2Fe–2S]), cytochrome(s) (cytochromes *b* or *c*), molybdoenzyme (Mob) or menaquinone (MQ) may have evolved earlier and been present in LUCA, or pre-LUCA. Accordingly, we suggest that the pathways of *Rb. ferrooxidans* (Fig. 3C) and of the nitrate reducers (Fig. 3E) could have arisen before the Great Oxidation Event while those of *At. ferrooxidans* (rusticyanin A, *aa*₃ cytochrome oxidase and ubiquinone, Fig. 2A), *At. ferrivorans* (Iro and/or rusticyanin A and/or B, Fig. 2B), “*T. prosperus*” (rusticyanin-like and *aa*₃ cytochrome oxidase, Fig. 2C), *Leptospirillum* Groups II and III (ubiquinone and *bb*₃ cytochrome oxidase, Fig. 2D), *S. sibiricus* (*aa*₃ and *bo*₃ cytochrome oxidases, Fig. 2E) “*Ferroplasma acidarmanus*” (sulfocyanin and *aa*₃ cytochrome oxidase, Fig. 2F), *Sulfolobus* spp. (FoxAB, Fig. 2G), *Metallosphaera* spp. (Mco, FoxAB, Fig. 2G), *S. lithotrophicus* and *M. ferrooxidans* (*cbb*₃ cytochrome oxidase, Fig. 3A) and *Rp. palustris* (PioC, Fig. 3D) were acquired after the rise of O_2 .

It has to be pointed out that the latter microorganisms, except *Rp. palustris*, are acidophilic oxic iron oxidizers that have no choice than to use O_2 as terminal electron acceptor when oxidizing Fe(II) due to thermodynamic constraints (see Section 5.1). Why has *Rp. palustris*, which oxidizes Fe(II) in anoxygenic phototrophic conditions, a HiPIP protein (PioC) in its Fe(II) oxidation pathway to the reaction center? A possible explanation is brought by the analysis of a *pioC* in frame deletion mutant. This mutant has only a partial defect in Fe(II) oxidation and it has been proposed that PioC could be substituted by other unidentified small soluble electron carriers [108]. The *pioC* is the last gene of the *pio* operon and could have been acquired by horizontal gene transfer and fused to *pioAB*. An argument that supports this hypothesis is that *S. lithotrophicus* has a homolog of the *pioAB* operon, the *mtaAB* cluster, that is devoid of a HiPIP encoding gene but instead has the *cymA* gene encoding a membrane-anchored cytochrome *c* (Fig. 3B) [101]. Other apparent discordances between the redox cofactors evolution and the phylogeny of the Fe(II) oxidizers concern *S. lithotrophicus* and *M. ferrooxidans* (Fig. 3B) in which only “ancient” cofactors (heme C and molybdenum cofactor) are present while these bacteria use O_2 as terminal electron acceptor. One possibility is that this pathway is indeed ancient and existed before the O_2 appearance and has been acquired by horizontal gene transfer from anoxygenic phototrophic iron oxidizers such as *Rp. palustris*. Another not exclusive explanation is that the proposed pathway is incomplete and that some other electron carriers have not yet been identified. An interesting possibility is that the systems described in *M. ferrooxydans* and in *S. lithotrophicus* constitute indeed the complete Fe(II) oxidation pathway with MtoAB delivering the electrons from Fe(II) to the molybdoenzyme that then transfers the electrons via CymA to the respiratory chain (*bd* or *cbb*₃ oxidase) and to the *bc*₁ complex. Interestingly, the PioAB system [101,108] presents similarities to MtrAB shown to be involved in Fe(III) reduction in *S. oneidensis* [124,125]. A quick survey of

the non-redundant databanks with MtrA/PioA and MtrB/PioB sequences has shown that the genes encoding these proteins are present in a number of *Bacteria*, in particular in known Fe(II) oxidizers and Fe(III) reducers. These include, for Fe(III) reducers, *Alphaproteobacteria* (*Magnetospirillum magneticum*), *Betaproteobacteria* (*Rhodospirillum rubrum*), *Gammaproteobacteria* (*Ferrimonas balearica*, *Nitrosococcus halophilus*, *Vibrio vulnificus* and a number of *Shewanella* species) as well as *Deltaproteobacteria* (*Geobacter uraniireducens*). Fe(II) oxidizers harboring these genes belong to the *Alphaproteobacteria* (*Rhodospirillum rubrum*, *Rhodospirillum rubrum*), *Betaproteobacteria* (*Dechloromonas aromatica*, *Gallionella capsiferriformans*, *S. lithotrophus*) and *Gammaproteobacteria* (*Rhodospirillum rubrum*). This suggests that the PioAB/MtrAB system originated likely in the ancestor of the *Proteobacteria*, that is, before the Great Oxidation Event. However, it cannot be concluded from this phylogenetic distribution whether the oxidative or the reductive pathway appeared first. Nevertheless, from the iron paleochemistry arguments (Section 3.1), we favor an evolutionary adaptation of the Fe(II) oxidation pathway to operate in the reductive direction when the Fe(II) present in the Ocean was suggested to be oxidized to Fe(III). Overall, the analysis of the cofactors of the redox proteins predicted to be involved in the Fe(II) oxidation pathway agrees with the terminal electron acceptor used, that is, the oxic Fe(II) oxidizers appear after the anoxic ones.

As shown in Figs. 2 and 3, Fe(II) oxidation pathways with dissimilar electron carriers have evolved not only in different biotopes (compare the acidophiles and the neutrophiles Figs. 2 and 3, or the sea- and freshwater bacteria Fig. 3A and B) but also in distinct species living in the same habitats. This is particularly well illustrated by the case of the acidophiles: for example, *At. ferrooxidans*, *At. ferrivorans*, *Leptospirillum* spp., *S. sibiricus* and *Ferroplasma* spp., frequently encountered in the same ecosystem, i.e. acid mine drainage waters or heap bioleaching processes, nevertheless have clearly different respiratory chains (Fig. 2A, B, D, E and F). Noteworthy also, *At. ferrivorans* likely has a pathway not present in the closely related *At. ferrooxidans* [81]. In few cases, the electron carriers and their order in the respiratory chain seem to be conserved: namely in *At. ferrooxidans* and “*T. prosperus*” (Fig. 2A and C) and in *Sulfolobus* spp. and *Metallosphaera* spp. (Fig. 2G). However, the similarities between putative homologs are relatively weak [83,94] precluding phylogenetic analysis. For example, the identity of the outer membrane cytochrome *c* *Cyc2* from *At. ferrooxidans* is only 37% with *Cyc2* from “*T. prosperus*” [83] and 15% to *Cyt₅₇₂* from *Leptospirillum* [89]. This could be due to early horizontal transfers followed by divergence. Another intriguing observation is the heterogeneity of the terminal heme copper oxidase: *aa₃* oxidase in *At. ferrooxidans*, “*T. prosperus*,” *S. sibiricus* and “*Ferroplasma acidarmanus*,” *bb₃* oxidase in *Leptospirillum* and *ccb₃* oxidase in *M. ferrooxidans*, *FoxAB* in *Sulfolobus* and *Metallosphaera* spp. (see Figs. 2 and 3), suggesting again diversification of the Fe(II) pathways.

Nevertheless, the global spatial arrangement of these respiratory chains is the same: the electrons are extracted from the Fe(II) substrates at the surface of the cell and transferred to a terminal oxidase the catalytic site of which is located inside the cell (Figs. 2 and 3). This organization allows the spatial separation of Fe(II) from O₂ thus avoiding oxidative stress and Fe(III) precipitation inside the cell. Another common “theme” is the presence of cytochromes, cytochromes *c* in *Bacteria* and cytochromes *b* in *Archaea*. In conclusion, it looks like the Fe(II) oxidation pathways arose independently more than once in evolution and evolved convergently.

7. Concluding remarks and open questions

The dissimilatory Fe(II) oxidation is very likely an ancient energy metabolism as shown by the widespread distribution of the iron oxidizers on the phylogenetic tree of prokaryotes. However, the Fe(II) oxidation pathways are quite diverse suggesting that the ability to oxidize Fe(II) arose independently more than once in evolution. Based on iron paleochemistry, the phylogeny and the physiology of the

iron oxidizers, as well as the nature of the cofactors of the redox proteins involved in these pathways, we propose that the nitrate dependent anoxic iron oxidizers might be the most ancient iron oxidizers. The phototrophic anoxic iron oxidizers likely arose next in surface waters after the *Archaea/Bacteria*-split but before the Great Oxidation Event. The neutrophilic oxic iron oxidizers possibly appeared in microaerobic marine environments prior to the Great Oxidation Event while the acidophilic ones emerged after the advent of atmospheric O₂. This model so far remains very speculative since our understanding of the neutrophiles, and in particular of the nitrate reducers, is in its infancy.

Several questions are still open and we hope that in the near future, with the growing number of pure isolates and of genome sequences, more Fe(II) oxidation mechanisms will be deciphered eventually providing insights into their evolution. A non-exhaustive list of open questions is listed below and suggestions are provided to test the proposed model.

- The isolation of novel iron oxidizers and their distribution on the phylogenetic tree of prokaryotes may improve our understanding of the timeframe during which iron oxidation evolved. In particular, one may wonder whether *Cyanobacteria* able to oxidize Fe(II) do in fact exist. To test this hypothesis, it might be worthwhile to search for such bacteria using buffered medium to maintain a constant pH and therefore to prevent abiotic Fe(II) oxidation. Up until now, the only described electron acceptors involved in Fe(II) oxidation are O₂, nitrate or the reaction center in anaerobic phototrophs. The redox potential of some ferrous iron containing compounds (goethite, hematite and magnetite, see Table 1) should be sufficiently low (~ -0.27 V) to be used by neutrophiles with other electron acceptor such as dimethyl sulfoxide (+0.16 V), fumarate (+0.3 V), or sulfur compounds (tetrathionate (+0.24 V), trithionate (+0.225 V) which were likely to be present in the ancient ocean. To isolate and to characterize such microorganisms is a real challenge but undoubtedly could bring new information on the evolution of Fe(II) oxidation pathways.
- Another aspect that absolutely needs to be addressed to validate/invalidate the model proposed in this review is to decipher the Fe(II) oxidation pathways in nitrate reducers.
- Two Fe(II) oxidation systems seem to be more widespread than the other ones: the *FoxABCDG* system described in *Euryarchaeota* and in *Crenarchaeota* and the *PioAB/MtrAB* system detected in a number of Fe(II) oxidizing and Fe(III) reducing *Proteobacteria*. The genes encoding the *PioAB/MtrAB* systems have been detected in the genome of several microorganisms and it might be interesting to determine whether they oxidize or reduce iron. As more Fe(II) oxidizers and Fe(III) reducers sharing the same system are available, it could be worthwhile to perform a phylogenetic analysis on their key proteins. In particular, this could help deciphering which one came first, the *PioAB* system involved in Fe(II) oxidation or the *MtrAB* system involved in Fe(III) reduction.
- Concerning the evolution of the molybdenum enzyme, it is still not clear whether tungsten was used as cofactor before the Great Oxidation Event. The biochemistry of such enzymes thought to have been present before the advent of O₂ could be informative, notably, the comparison of their activity *in vitro* and *in vivo* with tungsten and with molybdenum.
- To advance our knowledge on when and how Fe(II) oxidation pathways appeared and evolved, geochemical experiments are required on Fe(III) rich sediments, such as the Banded Iron Formations. These experiments include iron and nitrogen isotope fractionation, hopanes and steranes detection. Indeed, (i) distinct iron isotope fractionations seem to be produced by different oxidative pathways [126]; (ii) nitrogen isotopic compositions of old sedimentary formation could record biotic ammonium oxidation/nitrification/denitrification processes [127,128]; (iii) hopanes are

recognized as molecular fossils that are used as markers of cell biological processes [129] and (iv) steranes have been used as proxy for oxygenation [130].

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