Review

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The iron-oxidizing proteobacteria

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The 'iron bacteria' are a collection of morphologically and phylogenetically heterogeneous prokaryotes. They include some of the first micro-organisms to be observed and described, and continue to be the subject of a considerable body of fundamental and applied microbiological research. While species of iron-oxidizing bacteria can be found in many different phyla, most are affiliated with the Proteobacteria. The latter can be subdivided into four main physiological groups: (i) acidophilic, aerobic iron oxidizers; (ii) neutrophilic, aerobic iron oxidizers; (iii) neutrophilic, anaerobic (nitrate-dependent) iron oxidizers; and (iv) anaerobic photosynthetic iron oxidizers. Some species (mostly acidophiles) can reduce ferric iron as well as oxidize ferrous iron, depending on prevailing environmental conditions. This review describes what is currently known about the phylogenetic and physiological diversity of the iron-oxidizing proteobacteria, their significance in the environment (on the global and micro scales), and their increasing importance in biotechnology.

Introduction

The 'iron bacteria' were among the first prokaryotes to be observed and recorded by pioneer microbiologists, such as Ehrenberg and Winogradsky, in the 19th century. They were originally considered to be bacteria that catalysed the oxidation of iron II (Fe^{2+} , ferrous iron) to iron III (Fe^{3+} , ferric iron), often causing the latter to precipitate and accumulate as extensive, ochre-like deposits (Supplementary Fig. S1a), although the definition of what constitutes an 'iron bacterium' has been extended to include prokaryotes that, like *Geobacter* spp., catalyse the dissimilatory reduction of ferric to ferrous iron. Iron-oxidizing prokaryotes have continued to be the focus of a considerable body of research, due to not only the perceived importance of these micro-organisms in the global iron cycle and industrial applications (chiefly biomining), but also discoveries over the past 20 or so years of novel genera and species that catalyse the dissimilatory oxidation of iron at circum-neutral pH in micro-aerobic and anaerobic environments (Emerson *et al.*, 2010). While classified species of iron-oxidizing bacteria occur in a number of phyla within the domain Bacteria, including the Nitrospirae and the Firmicutes, the majority are included within the largest bacterial phylum, the Proteobacteria. Within this phylum are found iron-oxidizing bacteria that have different physiologies in terms of their response to oxygen (obligate aerobes, facultative and obligate anaerobes) and

pH optima for growth (neutrophiles, moderate and extreme acidophiles). These bacteria are the subject matter of this review. Other related reviews that have focused on particular groups and aspects of iron-oxidizing proteobacteria and other bacteria include those by Straub *et al.* (2001) (anaerobic iron oxidizers), Weber *et al.* (2006) (anaerobic iron oxidizers), Johnson & Hallberg (2008) (acidophilic species) and Emerson *et al.* (2010) (environmental and genomic aspects).

Biogeochemistry of iron

Iron is the most abundant element (by weight) in planet earth, and the second most abundant metal (after aluminium) in the lithosphere, where it is present at a mean concentration of 5% (Lutgens & Tarbuck, 2000). It occurs in a number of mineral phases, including oxides, carbonates, silicates and sulfides. Banded iron formations (BIFs; oxidized deposits of Pre-Cambrian age) are the largest accumulations of iron in the lithosphere (Nealson, 1983), containing about 28% (by weight) iron. Laterites are surface deposits of oxidized iron, and are important as they contain significant reserves of metals of economic value, such as nickel and cobalt (Elias, 2002). Iron is an essential nutrient for all known life forms, with the seeming exception of *Lactobacillus* spp. (Archibald, 1983). It is usually required only in trace amounts (i.e. it is a micro-nutrient), although in some exceptional cases such as the magnetotactic bacteria, cellular iron contents are up to 11.5-fold greater than in more 'typical' bacteria (Chavadar & Bajekal, 2008).

Two supplementary figures are available with the online version of this paper.

In nature, iron occurs mostly in two oxidation states (+2 and +3). Which form of iron predominates depends greatly on prevailing environmental physicochemical parameters, such as pH, oxygen concentration and redox potential (Stumm & Morgan, 1996). Ferrous iron is stable in anoxic environments, but is susceptible to spontaneous chemical oxidation by molecular oxygen. The rate at which this occurs depends on temperature, and on the concentrations of protons (hydronium ions), dissolved oxygen and ferrous iron, as shown in equation [1] (Stumm & Morgan, 1996):

$$\frac{-d[\text{Fe(II)}]}{dt} = \frac{k[\text{O}_2]}{[\text{H}^+]^2} [\text{Fe(II)}] \quad [1]$$

where k is a temperature-dependent constant ($3 \times 10^{-12} \text{ mol l}^{-1} \text{ min}^{-1}$ at 20°C). In most environments, rates of spontaneous chemical oxidation of ferrous iron are very low at $\text{pH} < 4$, though these become appreciably greater at higher pH values. Although ferric iron is thermodynamically stable in aerated waters, its strong tendency to hydrolyse (react with water) means that it is present at extremely low concentrations in most water bodies, though in the presence of complexing agents (e.g. chelating organic acids and humic colloids), concentrations of soluble ferric iron can be significantly greater. However, the solubility of non-complexed ferric iron is also pH-related, and extremely acidic waters (pH of ~ 2.5 or less) may contain highly elevated concentrations of this ionic species, which is a powerful oxidizing agent.

Another important aspect of iron chemistry, which greatly affects its use by prokaryotes as a source of energy, is the redox potential of the ferrous/ferric couple. This is again dictated by (i) solution pH, as this affects both ferric iron solubility and, to a smaller extent, that of ferrous iron, and (ii) the presence (or not) of complexing agents. As shown in Fig. 1, the most positive redox potential of the ferrous/ferric couple is observed in extremely acidic liquors where both species are soluble, while at circum-neutral pH the redox potential of typical non-soluble ferrous/ferric phases that exist under such conditions (ferrous carbonate and ferric hydroxide) is much less positive. Soluble ferrous/ferric complexes also tend to have less positive redox potentials than that of the free forms of the ions (Fig. 1).

The combination of abiotic ferrous iron oxidation, iron solubilities and the redox potentials of the ferrous/ferric couple has an overwhelming bearing on microbiological strategies that have evolved to convert the energy available from iron oxidation into usable energy. Micro-organisms that live in extremely acidic environments (acidophiles) are faced with thermodynamic constraints that mean that only molecular oxygen can act as electron acceptor for iron oxidation, if energy is to be conserved (Fig. 1). Although perchlorate (ClO_4^-) can in theory act as an alternative electron acceptor for ferrous iron oxidation in extremely low pH liquors, its scarcity in the environment and the

general toxicity of anions (other than sulfate) to acidophiles (Ingledew & Norris, 1992) mean that this is not a feasible alternative to molecular oxygen. The redox potential of the oxygen/water couple (equation [2]) is pH-dependent, since protons are involved in the net reaction:

At pH 2, the redox potential of the oxygen/water couple is some 300 mV more positive than at pH 7 (Fig. 1), making oxygen a more energetically favourable electron acceptor for acidophiles than it is for neutrophiles (Ferguson & Ingledew, 2008). However, ferrous iron oxidation is inevitably a low energy-yielding reaction ($\sim 30 \text{ kJ mol}^{-1}$ at pH 2), and the autotrophic acidophile *Acidithiobacillus ferrooxidans* (*At. ferrooxidans*) has been estimated to oxidize about 71 moles of ferrous iron to fix one mole of CO_2 (Kelly, 1978). On the other hand, at pH 7, the ferrous carbonate/ferric hydroxide couple redox potential is sufficiently low to allow other compounds, such as nitrate and nitrite, to be used as alternative electron acceptors to oxygen (Fig. 1). Iron oxidation at circum-neutral pH can also be coupled to photosynthesis by phototrophic purple bacteria, since the midpoint potential of photosystem I is $\sim 450 \text{ mV}$ (Widdel *et al.*, 1993). This means that, in contrast to acidic environments, dissimilatory iron oxidation can be mediated in anoxic as well as in oxic environments in circum-neutral pH situations. However, the redox differential between electron donor and acceptor (230 mV when iron oxidation is coupled with the reduction of nitrate to nitrite) again limits the amount of energy available for anoxic metabolism. Coupling iron oxidation to oxygen reduction at pH 7 is much more energetically favourable, but the downside here is the potential for spontaneous oxidation of iron by molecular oxygen, as noted above. However, as indicated in equation [1], spontaneous iron oxidation is much slower in micro-aerobic than in more oxygenated waters, so that bacteria that are able to exploit such environmental niches have the potential to maximize the energy yield available from oxidizing iron.

Phylogenetic and physiological diversity of iron-oxidizing proteobacteria

Bacteria that catalyse the dissimilatory oxidation of iron can be subdivided into four main physiological groups: (i) acidophilic, aerobic iron oxidizers; (ii) neutrophilic, aerobic iron oxidizers; (iii) neutrophilic, anaerobic (nitrate-dependent) iron oxidizers; and (iv) anaerobic photosynthetic iron oxidizers. With three of these groups, most species so far identified fall into one class within the phylum Proteobacteria, though this is not the case with the nitrate-dependent iron oxidizers (Fig. 2). The phylogenetic tree shown in Fig. 2 does not include the numerous iron-oxidizing isolates whose 16S rRNA gene sequences clearly place them within the phylum Proteobacteria, but for which limited physiological data have been published.

Fig. 1. Different redox potentials of soluble and insoluble ferrous/ferric couples, as well as those of other couples that serve as electron acceptors for iron-oxidizing proteobacteria (Langmuir, 1997; Madigan *et al.*, 2002; Thamdrup, 2000; Thauer *et al.*, 1977; Weber *et al.*, 2006).

Acidophilic, aerobic iron-oxidizing proteobacteria

The acidophilic iron-oxidizing proteobacteria have been the focus of a great deal of research since the discovery of the first species, *At. ferrooxidans*, in the late 1940s (Colmer *et al.*, 1950), primarily because of their importance in biotechnology (predominantly biomining) and in environmental pollution (their role in generating acidic and metal-enriched mine drainage waters). Acidophilic prokaryotes have a 'ready-made' pH differential across their cell membranes that allows them to produce ATP via the F_1F_0 ATP synthase, though the influx of protons that drives this needs to be counterbalanced with electrons derived from, for example, the oxidation of ferrous iron. While most acidophiles can obtain energy from the oxidation of ferrous iron alone when this is coupled to the reduction of molecular oxygen, most described species are in fact facultative anaerobes that can also couple the oxidation of reduced sulfur compounds (and in some cases hydrogen) to the reduction of ferric iron in anoxic environments.

The most widely studied of all iron oxidizers is the acidophile *At. ferrooxidans*. However, caution has to be exercised in describing all of the physiological traits attributed to this bacterium over the past 50 years, as the identification of isolates as strains of *At. ferrooxidans* on the

basis of iron and sulfur oxidation at very low pH is now recognized to be somewhat tenuous. Harrison (1982) was the first to highlight that major phylogenetic differences existed among many of the '*At. ferrooxidans*' strains that he examined, a theme that was later also emphasized by Selenska-Pobell *et al.* (1998) and Karavaiko *et al.* (2003). Hallberg *et al.* (2010) described a novel iron-oxidizing species, *Acidithiobacillus ferrivorans* (*At. ferrivorans*), that differed in some significant physiological traits (most notably in being psychro-tolerant) from the type strain of *At. ferrooxidans*. It appears that *At. ferrooxidans* and *At. ferrivorans* also use very different pathways to oxidize ferrous iron (Amouric *et al.*, 2011; Hallberg *et al.*, 2010). Based on multi-locus sequence analysis of 21 strains of iron-oxidizing acidithiobacilli (together with limited published DNA:DNA hybridization data), Amouric *et al.* (2011) have suggested that iron-oxidizing acidithiobacilli comprise at least four distinct species, rather than the two currently validated, and that these 'species' use at least two different pathways to oxidize ferrous iron (Fig. 3).

Acidithiobacillus spp. were among the bacteria first named as species of *Thiobacillus* on the basis that they are rod-shaped Gram-negative bacteria that can oxidize reduced forms of sulfur. Most acidophilic species were reclassified as *Acidithiobacillus* spp. following phylogenetic (16S rRNA

Fig. 2. Phylogenetic tree showing the relationship of acidophilic (red), neutrophilic aerobic (green), nitrate-dependent (black) and phototrophic iron-oxidizing (blue) proteobacteria. The tree is a maximum-likelihood tree based on the small-subunit (16S) rRNA gene, showing bootstrap values (out of 1000 replicates). Topologies of phylogenetic trees calculated using other methods were similar to the maximum-likelihood tree: only the phylogenetic position of *Thermomonas* sp. BrG3 (U51103) seemed to change between the Beta- and Gammaproteobacteria. GenBank accession numbers for sequences are given in parentheses. The tree was rooted with the 16S rRNA gene sequence from the type strain of *Desulfovibrio desulfuricans* (M34113; not shown).

gene sequence) analysis (Kelly & Wood, 2000), but two 'species' of iron-oxidizing acidithiobacilli could not be affiliated to the new genus. One of these, '*Thiobacillus ferrooxidans*' strain m-1, had previously been highlighted by Harrison (1982) as a probable distinct species. Many years later, it was fully described as the type strain of the novel genus and species *Acidiferrobacter thiooxydans* (*Af. thiooxydans*) (Hallberg *et al.*, 2011). *Af. thiooxydans* shares many physiological characteristics with the type strain of *At. ferrooxidans* (*At. ferrooxidans*^T), including being a facultative anaerobe that oxidizes iron and reduced sulfur (though not hydrogen). Differences include a requirement for reduced sulfur by *Af. thiooxydans*, and the fact that the latter is more tolerant of extreme acidity and moderately high temperatures (growth occurs at up to 47 °C) than *At. ferrooxidans*^T. The situation with the other iron-oxidizing '*Thiobacillus*' sp. ('*Thiobacillus prosperus*') still awaits resolution. The main distinguishing feature of this acidophile is its tolerance to salt, being able to grow in up to 3.5% (0.6 M) sodium chloride, whereas most iron-oxidizing acidithiobacilli are inhibited by 1% salt (Nicolle *et al.*, 2009). Both the original strain and a novel strain (V6) have

been shown to grow optimally in the presence of 1–2% sodium chloride and also, like *Af. thiooxydans*, require reduced sulfur (e.g. tetrathionate) for rapid oxidation of ferrous iron in liquid media (Nicolle *et al.*, 2009).

The 16S rRNA gene sequences of *Af. thiooxydans* and '*T. prosperus*' clearly place them within the Gammaproteobacteria, a class with which *Acidithiobacillus* spp. have generally been considered to be affiliated. However, Williams *et al.* (2010), using data obtained from multi-protein analysis, have challenged the generally accepted view that the order Acidithiobacillales is affiliated to the Gammaproteobacteria, and suggested that these bacteria, and the neutrophilic iron-oxidizing marine bacterium *Mariprofundus ferrooxydans*, diverged after the establishment of the Alphaproteobacteria and before the separation of the Betaproteobacteria/Gammaproteobacteria. A more obvious divergence from the pattern that all acidophilic iron-oxidizing proteobacteria are gammaproteobacteria is the named, but as yet non-validated, betaproteobacterium '*Ferroplasma myxofaciens*' ('*Fv. myxofaciens*') (Kimura *et al.*, 2011). Like the other iron oxidizers described in this section,

Fig. 3. Proposed organization of the genes involved in ferrous iron oxidation by *Acidithiobacillus* spp. and corresponding biochemical pathways: (a) the *rus* operon and ferrous iron oxidation in *At. ferrooxidans* ATCC 23270^T ('group I') and ATCC 33020 ('group II'); (b) Iro and proteins proposed to be involved in ferrous iron oxidation in *Acidithiobacillus* 'group IV' strain JCM 7811. Solid arrows represent transcriptional units, and broken arrows represent electron transport. Most strains of *At. ferrooxidans* ('group III') possess only *rusB* genes, and the Iro protein is considered to have a more direct involvement in ferrous iron oxidation in this species, as in 'group IV' strains (Amouric *et al.*, 2011).

'*Fv. myxofaciens*' is an extreme acidophile, though with a pH optimum of 3.0 and a pH minimum of ~2, it is less acidophilic than *Acidithiobacillus* spp., *Af. thiooxydans* and '*T. prosperus*'. Uniquely among these bacteria, it appears to oxidize ferrous iron only, and is an obligate aerobe. '*Fv. myxofaciens*' is widely distributed in acidic, iron-rich streams and rivers, where it is frequently observed as macroscopic streamer growths (Supplementary Fig. S2c) (Hallberg *et al.*, 2006). It has also been identified as the major iron-oxidizing bacterium colonizing a pilot-scale mine water treatment plant designed to oxidize and precipitate iron from contaminated ground water (Heinzel *et al.*, 2009).

Other bacteria, classified as 'moderate acidophiles' (pH optima for growth of 3–5) have also been occasionally reported to catalyse the dissimilatory oxidation of iron under aerobic conditions. These include some *Thiomonas* spp. (mixotrophic sulfur-oxidizing betaproteobacteria) that have been reported to precipitate ferric iron when grown in liquid and on solid media (Battaglia-Brunet *et al.*, 2006). However, great care has to be taken to differentiate biological and abiotic iron oxidation, as noted above, particularly as small changes in the culture pH of moderate acidophiles can induce rapid chemical oxidation of iron. Slyemi *et al.* (2011) reported that *Thiomonas* strains that deposited ferric iron in shake flasks and on solid media did not oxidize iron in pH-controlled bioreactors, leading to the conclusion that *Thiomonas* spp. probably do not directly catalyse ferrous iron oxidation.

Neutrophilic, aerobic iron-oxidizing proteobacteria

Currently, all known oxygen-dependent, neutrophilic, lithotrophic iron oxidizers are proteobacteria (Emerson *et al.*, 2010). Interestingly these appear to be readily divided into freshwater species, all of which are betaproteobacteria, and marine species, which have mostly been affiliated to the proposed (*Candidatus*) class 'Zetaproteobacteria' (Emerson *et al.*, 2007). Although this group includes one of the earliest described bacteria (*Gallionella*), most neutrophilic aerobic iron-oxidizing bacteria have been isolated and characterized only relatively recently. Because of the potential for rapid abiotic oxidation of ferrous iron in oxygen-rich, pH-neutral waters, aerobic, neutrophilic iron oxidizers often colonize the interface between aerobic and anoxic zones in sediments and ground waters, and have often been described as 'gradient' organisms. Techniques used to isolate these bacteria have usually attempted to mimic these environmental conditions *in vitro*, such as incubating in micro-aerobic atmospheres and using 'gradient tubes' (e.g. Druschel *et al.*, 2008; Emerson & Floyd, 2005; Hallbeck *et al.*, 1993). In contrast to their acidophilic counterparts, neutrophilic iron oxidizers do not have pre-existing pH gradients across their membranes that can drive ATP synthesis and, although the redox potentials of the ferrous/ferric couple(s) are lower at pH 7 than at pH 2, so is that of the oxygen/water couple (Fig. 1). For these reasons, lithotrophic, iron-oxidizing bacteria

have been described as living on the 'thermodynamic edge' (Neubauer *et al.*, 2002).

Gallionella ferruginea was first described by Ehrenberg in 1838, and more is known about this bacterium than any other neutrophilic iron oxidizer. *G. ferruginea* can grow autotrophically or mixotrophically using ferrous iron as electron donor (Hallbeck & Pedersen, 1991). It forms bean-shaped cells with characteristic long, twisted stalks of ferrihydrite-like precipitates (Hanert, 1981). Stalk formation, which has been claimed to have a protective role against free oxygen radicals formed during iron oxidation (Hallbeck & Pedersen, 1995), appears, however, not to be constitutive. Stalks are not observed when the bacteria are grown at pH <6, or under micro-aerobic conditions (Hallbeck & Pedersen, 1990). In addition, Emerson & Moyer (1997) isolated a closely related strain (ES-2) that did not form stalks when grown on ferrous iron at pH >6. *G. ferruginea* remains as the only classified species of this genus, although 16S rRNA genes of bacteria occurring in iron- and CO₂-rich well waters (pH 5.8) have revealed two clades related to, but still distinct from, *G. ferruginea* (Wagner *et al.*, 2007). In addition, bacteria that appear to be distinct species of *Gallionella* have been found in clone libraries of DNA extracted from acidic (pH 2.6–3.0) iron-rich water bodies (Hallberg *et al.*, 2006; Heinzl *et al.*, 2009; Kimura *et al.*, 2011), raising the possibility that acidophilic or acid-tolerant *Gallionella* spp. exist and await isolation and characterization.

While the situation regarding dissimilatory iron oxidation by *Gallionella* is unambiguous, this is not the case with sheath-forming *Leptothrix* spp. (Supplementary Fig. S1b, c) and *Sphaerotilus natans* (Emerson *et al.*, 2010). *Leptothrix* currently comprises four recognized species, three of which (*Leptothrix discophora*, *Leptothrix cholodnii* and *Leptothrix mobilis*) are obligate heterotrophs, while the other (*Leptothrix ochracea*) has not been cultivated and studied in the laboratory in pure culture, or subjected to thorough phylogenetic analysis. While all four species (and *S. natans*, which is also a heterotroph) can accumulate ferric iron and/or manganese (IV) on their sheaths, the only species for which there is circumstantial evidence for autotrophic growth using energy derived from iron oxidation is *L. ochracea*. It is conceivable that the accumulation of ferric iron deposits by heterotrophic, sheath-forming betaproteobacteria is serendipitous, and derives from the breakdown of organic iron complexes by these bacteria, with the sheath acting as a focal point for the hydrolysis and precipitation of the ferric iron released.

Two novel genera of aerobic, neutrophilic iron-oxidizing betaproteobacteria have been proposed more recently. The proposed genus '*Sideroxydans*' currently includes two species, '*Sideroxydans*' sp. ES-1 (Emerson & Moyer, 1997) and '*Sideroxydans paludicola*', while '*Ferritrophicum radicum*' ('*Ft. radiculicola*') is currently the only known species of the genus '*Ferritrophicum*' (Weiss *et al.*, 2007). While the genus '*Sideroxydans*' belongs to the same bacterial order as

G. ferruginea (the Gallionellales), '*Ft. radiculicola*' is currently the sole representative of a new betaproteobacterial order, the '*Ferritrophicales*'. '*Sideroxydans*' spp. and '*Ft. radiculicola*' share the physiological traits of being unicellular rods that do not form sheaths or stalks, and all three species are obligate aerobes (micro-aerophiles) that appear to use ferrous iron as sole energy source, and are autotrophic (Emerson *et al.*, 2010). Interestingly, '*Sideroxydans*' sp. ES-1 was also the dominant phylotype detected in gene libraries obtained from a ferrous iron-oxidizing/nitrate-reducing enrichment culture by Blöthe & Roden (2009). Another, though as yet unclassified, neutrophilic and autotrophic iron oxidizer (strain TW2) was isolated from freshwater sediments by Sobolev & Roden (2004). This bacterium, which appears to represent a novel genus, also belongs to the Betaproteobacteria, though in contrast with most other aerobic and neutrophilic iron oxidizers, strain TW2 can grow both as an autotroph and as a mixotroph (Sobolev & Roden, 2004). Strain TW2 is related to the (per)chlorate reducer *Azospira oryzae*, described below.

Marine waters are typically pH 8.3 to 8.4, and the half-life of ferrous iron in seawater (pH ~8.0) is ~2 min (Millero *et al.*, 1987). As with freshwaters, micro-aerophilic iron-oxidizing proteobacteria have recently been isolated from iron mats in submarine geothermal areas and characterized *in vitro* (Emerson *et al.*, 2007). *M. ferrooxydans* is a marine, mesophilic, autotrophic iron oxidizer that has close morphological similarity (bean-shaped cells and stalk formation) to *G. ferruginea*, though phylogenetically it is very distant from the freshwater bacterium, and is affiliated with the *Candidatus* class 'Zetaproteobacteria' (Emerson *et al.*, 2010). Another strain of *M. ferrooxydans* has recently been isolated from a near-shore marine environment (McBeth *et al.*, 2011). In addition to *M. ferrooxydans*, other (as yet unclassified) marine strains of iron-oxidizing proteobacteria were described by Edwards *et al.* (2003). These were psychrophilic facultative anaerobes that were related to alphaproteobacteria and gammaproteobacteria. More recently, Sudek *et al.* (2009) reported that heterotrophic *Pseudomonas/Pseudoalteromonas*-like gammaproteobacteria isolated from a volcanic seamount could also catalyse ferrous iron oxidation under micro-aerobic conditions, and therefore contribute to the formation of iron mats in the deep oceans.

The status of other neutrophilic, aerobic iron oxidizers (e.g. *Siderocapsa*, *Metallogenium* and *Crenothrix*) has long been brought into question, given the conflicting and sometimes sparse information on some of these bacteria. This issue has been eloquently addressed by Emerson *et al.* (2010).

Neutrophilic iron-oxidizing proteobacteria that respire on nitrate

The fact that some bacteria are able to catalyse the dissimilatory oxidation of ferrous iron in anaerobic as well as in aerobic environments has been recognized only since

the early 1990s (Straub *et al.*, 1996; Widdel *et al.*, 1993). Two distinct metabolisms are known: one in which iron oxidation is used as a source of electrons by some photosynthetic bacteria, and one that is a variant of anaerobic respiration, in which ferrous iron is used as electron donor and nitrate as electron acceptor. The feasibility of the latter being an energy-yielding reaction depends on the redox potential of the ferrous/ferric couple being more negative than that of the nitrate/nitrite couple (+430 mV; Fig. 1), which restricts this metabolic lifestyle to environments that have circum-neutral (and higher) pH values, and where the redox potential of the ferrous/ferric couple(s) is much lower (about +200 mV) than in acidic liquors (+770 mV; Fig. 1). Iron-oxidizing/nitrate-reducing bacteria have been found in marine, brackish and freshwaters, and in anaerobic sediments (Benz *et al.*, 1998; Kappler & Straub, 2005; Straub & Buchholz-Cleven, 1998). In contrast to other groups of iron-oxidizing proteobacteria described in this review, the anaerobic nitrate-reducing iron oxidizers are not found exclusively or predominantly in one class of the Proteobacteria, and the (relatively few) bacteria described are randomly affiliated to the classes Alpha-, Beta-, Gamma- and Deltaproteobacteria.

Bacteria that couple iron oxidation and nitrate reduction in anaerobic environments can be divided into those that are autotrophic, and those that use organic materials as carbon sources and can also grow as heterotrophs. In the first report of this form of metabolism, Straub *et al.* (1996) isolated three Gram-negative bacteria from an active enrichment culture containing ferrous iron and nitrate, all of which could grow on organic acids using either nitrate or oxygen as electron acceptor. While all three isolates could also oxidize ferrous iron in anaerobic, nitrate-containing media, rates of iron oxidation were relatively slow when no organic acid (acetate or fumarate) was provided, and even then iron oxidation by pure cultures of the isolates was never as rapid as observed with the enrichment culture. The end product of nitrate reduction in all three cases was predominantly nitrogen gas, and small amounts of nitrous oxide (N₂O) were also detected. Ferric iron was deposited as the mineral ferrihydrite, and the overall reaction that occurred is depicted in equation [3]:

Later, Straub *et al.* (2004) identified one of these isolates as a strain of *Acidovorax* and another as a strain of *Aquabacterium* (both betaproteobacteria). A third anaerobic iron-oxidizing isolate was most closely related to the gammaproteobacterial genus *Thermomonas*. Kappler *et al.* (2005) also isolated a strain of *Acidovorax* (strain BoFeN1) from a freshwater lake sediment, and this strain could couple iron oxidation to nitrate reduction. Like the isolate of Straub and co-workers, this *Acidovorax* strain was a mixotroph, and could only oxidize ferrous iron effectively in the presence of an organic acid, such as acetate (Muehe

et al., 2009). *Acidovorax* sp. strain BoFeN1 could also reduce nitrite, nitrous oxide and oxygen. Another heterotrophic betaproteobacterium, isolated from a swine-waste lagoon (*Dechlorosoma suillum* strain PS, subsequently renamed as *A. oryzae* strain PS), was found to oxidize ferrous iron using either nitrate or chlorate as electron acceptor, with acetate as co-substrate (Chaudhuri *et al.*, 2001).

In circum-neutral pH environments, ferrous iron oxidation can also, in theory, be coupled to the reduction of nitrate to ammonium (E^0 of +360 mV; Fig. 1). Weber *et al.* (2006) reported that oxidation of ferrous iron correlated with the appearance of ammonium in a nitrate-containing anaerobic enrichment culture containing *Geobacter* and *Dechloromonas* spp., though no iron-oxidizing bacterium that could reduce nitrate to ammonium in pure culture was identified.

Anaerobic, nitrate-dependent oxidation of iron by autotrophic bacteria is also known. One of the first indications of this was an observation by Straub *et al.* (1996) that *Thiobacillus denitrificans* oxidized ferrous sulfide (FeS) in the presence of nitrate. *T. denitrificans* is a strictly autotrophic betaproteobacterium that is best known for coupling the oxidation of various reduced inorganic sulfur compounds (or elemental sulfur) to the reduction of nitrate. *T. denitrificans* has also been implicated in the anaerobic oxidation of pyrite in anoxic sediments (Jørgensen *et al.*, 2009). Whether the sulfide or ferrous iron moiety (or both) is the primary electron donor in this context is unclear, though in the absence of molecular oxygen pyrite oxidation is mediated by ferric iron, implying that *T. denitrificans* does indeed oxidize ferrous iron.

Weber *et al.* (2006) obtained an isolate (*Pseudogulbenkiania* strain 2002) from a freshwater lake sediment that could oxidize ferrous iron and reduce nitrate while growing as an autotroph, though it was also reported to grow heterotrophically on a variety of organic compounds (Weber *et al.*, 2009). Analysis of its 16S rRNA gene sequence showed that this isolate (a facultative anaerobe) was very closely related (99.3% sequence similarity) to the betaproteobacterium *Pseudogulbenkiania subflava* (Weber *et al.*, 2009). A different approach to enrich for nitrate-dependent iron oxidizers was used by Kumaraswamy *et al.* (2006), who included EDTA-complexed ferrous iron as the electron donor. As shown in Fig. 1, the redox potential of the EDTA-complexed ferrous/ferric couple is much less positive than that of the non-complexed metal and also less positive than that of the inorganic (carbonate/hydroxide) couple frequently quoted at circum-neutral pH values. These authors obtained an isolate that was related to species of the alphaproteobacterial genus *Paracoccus*. A new species designation ('*Paracoccus ferrooxidans*') was proposed for the isolate, which was described as a facultative autotroph capable of growth in both aerobic and anoxic conditions. Intriguingly, there has been at least one report

describing nitrate-dependent ferrous iron oxidation by the strict anaerobe *Geobacter metallireducens* (Finneran *et al.*, 2002). Whether this deltaproteobacterium could use the energy from this reaction to support its growth was not ascertained, but given the widespread abundance of *Geobacter* spp. in anaerobic sediments (Lovley, 1991), the possibility exists that these anaerobic iron-reducing bacteria can also oxidize iron when nitrate is available.

Phototrophic iron-oxidizing proteobacteria

Phototrophic purple proteobacteria provided the first evidence that micro-organisms could oxidize ferrous iron in anaerobic environments. Following the pioneering work by Friedrich Widdel and colleagues (Ehrenreich & Widdel, 1994; Widdel *et al.*, 1993), iron-oxidizing phototrophs have been isolated from a variety of freshwater and marine environments (Croal *et al.*, 2004b; Heising & Schink, 1998; Jiao *et al.*, 2005; Straub *et al.*, 1999). Most of the iron-oxidizing phototrophs that have been described are affiliated to the class Alphaproteobacteria, with the notable exception of *Thiodictyon* strain L7, which is a gammaproteobacterium (Fig. 2).

Ferrous iron is used by this group of bacteria as a source of reductant for carbon dioxide (equation [4]; CH₂O indicates fixed biomass carbon):

However, while most photosynthetic bacteria that oxidize ferrous iron use this reaction for carbon assimilation, it can also be used as a detoxification mechanism, as described below.

As with other neutrophilic iron oxidizers, ferric iron precipitates are generated as waste products. They represent a potential hazard to iron-oxidizing phototrophs, as the bacteria risk being enshrouded by these ferrihydrite-like minerals, which would restrict their access to light (Heising & Schink, 1998). However, this phenomenon has only, so far, been noted for cultures of *Rhodomicrobium vannielii* (*Rm. vannielii*), in which encrustation of cells has been reported to result in incomplete oxidation of ferrous iron due to restricted light access (Heising & Schink, 1998).

The mid-point potential of the photosystem I in purple bacteria is about +450 mV, and is therefore more positive than that of the ferrous carbonate/ferric hydroxide couple (about +200 mV at pH 7; Fig. 1), though that ferrous iron is a less favourable electron donor in energetic terms than sulfide, which is more widely used by anaerobic photosynthetic bacteria (the redox potential of the sulfide/sulfur couple is –180 mV). Table 1 lists the alternative electron donors used by phototrophic iron-oxidizing bacteria. Phototrophic iron oxidizers can use soluble ferrous iron and minerals such as FeS or FeCO₃ as sources of reductant, but are not able to access ferrous iron in more crystalline minerals such as magnetite (Fe₃O₄) or pyrite (FeS₂; Kappler & Newman, 2004).

Table 1. Alternative electron donors of phototrophic iron-oxidizing proteobacteria (Duchow & Douglas, 1949; Heising & Schink, 1998; Imhoff, 2005; Jiao *et al.*, 2005; Straub *et al.*, 1999)

Organism	Alternative electron donor
<i>Rhodovulum iodosum</i>	S ₂ O ₃ ²⁻ , HS ⁻ , S ⁰
<i>Rhodovulum robiginosum</i>	S ₂ O ₃ ²⁻ , HS ⁻ , S ⁰
<i>Rm. vannielii</i>	H ₂ , HS ⁻ , organic compounds
<i>Rhodobacter</i> sp. SW2	H ₂ , organic compounds
<i>Rp. palustris</i> TIE-1	H ₂ , S ₂ O ₃ ²⁻
<i>Thiodictyon</i> strain L7	H ₂ , organic compounds

Most of the currently known phototrophic iron oxidizers belong to the *Rhodobacteraceae*, a highly diverse family within the class Alphaproteobacteria that includes photoheterotrophs that can also grow photoautotrophically under appropriate environmental conditions, aerobic and facultatively anaerobic heterotrophs, fermentative bacteria and facultative methylotrophs (Imhoff, 2005). The nitrate-reducing iron oxidizer '*P. ferrooxidans*' is also affiliated to the *Rhodobacteraceae*. The first iron-oxidizing phototroph to be characterized was *Rhodobacter* sp. strain SW2, which oxidizes ferrous iron only when provided with an organic carbon source, and also utilizes hydrogen and organic compounds (Ehrenreich & Widdel, 1994). In contrast, Poulain & Newman (2009) obtained data that suggested that ferrous iron oxidation by a related *Rhodobacter* sp. (*Rhodobacter capsulatus*, formerly classified as a species of *Rhodopseudomonas* and first described in 1907) served as a defence mechanism. This phototroph is highly sensitive to ferrous iron (5 µM can inhibit its growth) and this toxicity is relieved when the ferrous iron is oxidized to the highly insoluble ferric form. Other iron-oxidizing photosynthetic purple bacteria of the family *Rhodobacteraceae* include species of *Rhodovulum* (*Rhodovulum robiginosum* and *Rhodovulum iodosum*). Both species are marine bacteria and oxidize ferrous iron and sulfide when provided with an organic co-substrate, such as acetate (Straub *et al.*, 1999).

A phototrophic isolate, identified as a strain (BS-1) of *Rm. vannielii*, a heterotrophic non-sulfur purple bacterium of the family *Hyphomicrobiaceae*, was shown by Heising & Schink (1998) to oxidize ferrous iron; this trait was subsequently confirmed in the type strain of this species. Interestingly, *Rm. vannielii* had been tentatively identified by Widdel *et al.* (1993) as one of the iron-oxidizing phototrophic isolates that they obtained from freshwaters. Growth of strain BS-1 in the presence of ferrous iron was stimulated by adding acetate or succinate as co-substrates. Heising & Schink (1998) concluded that the oxidation of ferrous iron is only a peripheral activity for *Rm. vannielii* strain BS-1. Another member of the *Hyphomicrobiaceae*, *Rhodopseudomonas palustris* (*Rp. palustris*) strain TIE-1, was isolated from an iron-rich mat by Jiao *et al.* (2005) and used subsequently as a model organism for genetic studies.

Currently, only two photosynthetic iron-oxidizing gamma-proteobacteria have been described, and both are strains of *Thiodictyon*. One of these, strain L7, was isolated from the same source as *Rhodobacter* sp. SW2 (Ehrenreich & Widdel, 1994), while *Thiodictyon* sp. strain f4 was isolated from a marsh by Croal *et al.* (2004a). *Thiodictyon* sp. strain f4 displays the fastest rates of iron oxidation of all phototrophic iron-oxidizing bacteria that have been isolated (Hegler *et al.*, 2008). Interestingly, Widdel *et al.* (1993) reported that none of the authenticated *Thiodictyon* spp. that they tested was able to oxidize ferrous iron, suggesting that this trait is not widespread among this genus.

Photosynthetic iron oxidizers only possess photosystem I, and therefore do not evolve oxygen. The fact that these bacteria can promote iron oxidation in the absence of both molecular oxygen and an oxidized alternative electron donor (such as nitrate) has major implications for the perceived early development of planet earth. Large-scale oxidation of ferrous iron, originating from the weathering of ferro-magnesium and other reduced minerals associated with the extensive volcanism that is thought to have characterized the young planet, could have been mediated by phototrophic bacteria in Pre-Cambrian times while the planet was still essentially anoxic. This would help explain the occurrence of vast deposits of oxidized BIFs, which are thought to pre-date the development of an oxygen-enriched atmosphere (Ehrenreich & Widdel, 1994).

Oxido-reduction of iron by proteobacteria

Some species of proteobacteria that oxidize ferrous iron are also able to catalyse the dissimilatory reduction of ferric iron, where the latter acts as the sole or major electron acceptor in anaerobic respiration (Lovley, 1997; Lovley *et al.*, 2004; Nealson & Saffarini, 1994; Pronk & Johnson, 1992). Both organic and inorganic materials can be used as electron donors for iron reduction. While in most cases proteobacteria that reduce or oxidize iron are distinct species, some, described below, can both oxidize and reduce iron, depending on the prevailing environmental conditions. Cycling of iron mediated by microbiological oxido-reduction of iron is an important process in the environment, both on the micro and the global scales (Fig. 4). Iron-oxidizing proteobacteria have an important role in facilitating ferric iron reduction in the environment, as their solid-phase end products (e.g. schwertmannite and ferrihydrite) are much more reactive than other ferric iron minerals such as goethite and haematite, which are often more abundant in the environment (Emerson *et al.*, 2010). In the case of extreme acidophiles, the end product of iron oxidation is often soluble ferric iron, which is much more readily reduced than amorphous or crystalline forms (Bridge & Johnson, 1998), and ferric iron respiration appears to be widespread among acidophilic proteobacteria (Coupland & Johnson, 2008; Johnson & Hallberg, 2008). Since the redox potential of the soluble ferric/ferrous couple is not much less positive than that of the oxygen/

water couple, facultative anaerobic acidophiles that can use ferric iron as an electron acceptor do not suffer such a thermodynamic 'penalty' for switching electron acceptors as their neutrophilic counterparts.

The only iron-oxidizing proteobacteria known to be also capable of dissimilatory ferric iron reduction are the neutrophile *Geobacter metallireducens* (Lovley *et al.*, 1993) and three acidophilic species, *At. ferrooxidans* (Pronk *et al.*, 1992), *At. ferrivorans* (Hallberg *et al.*, 2010) and *Af. thiooxydans* (Hallberg *et al.*, 2011), though this trait appears to be more widespread among Gram-positive acidophilic bacteria (Johnson & Hallberg, 2008). *Geobacter metallireducens* has been shown to reduce amorphous iron oxides readily (Lovley & Phillips, 1986), but to reduce crystalline iron oxides only after the removal of surface ferrous iron from the mineral (Roden & Urrutia, 1999). Various organic compounds, such as acetate, ethanol, butyrate and propionate, can be used by *Geobacter metallireducens* to reduce ferric iron (Ehrlich & Newman, 2009). *At. ferrooxidans*, *At. ferrivorans* and *Af. thiooxydans* can all couple the oxidation of elemental sulfur to the reduction of ferric iron, while *At. ferrooxidans* can also reduce ferric iron under anaerobic conditions using hydrogen as electron donor (Ohmura *et al.*, 2002).

Genomic and molecular biology

New insights into the physiologies of iron-oxidizing proteobacteria are emerging as increasing numbers of their genomes are sequenced and annotated (Cárdenas *et al.*, 2010). A summary of the genomes of iron-oxidizing proteobacteria completed or known to be under way at the time of writing is shown in Table 2. As with other bacteria, advances in genomics, transcriptomics and proteomics technologies have helped to understand the mechanisms involved in the metabolisms of iron-oxidizing proteobacteria. Bioinformatic analysis can predict previously unrecognized potential gene functions and help to construct metabolic pathways, including those involved in iron oxidation (Bonnefoy, 2010).

Acidovorax ebreus TSY was the first nitrate-dependent iron oxidizer to have its genome sequenced, which in this case preceded the full physiological description of the bacterium (Byrne-Bailey *et al.*, 2010). The annotated genome of *Dechloromonas aromatica* RSB intimated that this proteobacterium is a nitrate-dependent iron oxidizer, and that it can also use chlorate or perchlorate as an electron acceptor (Weber *et al.*, 2006). The *pio* operon in *Rp. palustris* strain TIE-1, which is necessary for phototrophic iron oxidation (deletion results in loss of ability to oxidize iron), has been shown to contain three genes (Jiao & Newman, 2007) encoding a cytochrome *c*, a putative outer membrane β -barrel protein, and a high-potential iron-sulfur protein which is similar to the Iro protein found in *At. ferrivorans*, and which is thought to be involved in iron oxidation in that betaproteobacterium (Fig. 3). Another three-gene operon, the *fox* operon, has

Fig. 4. Microbially mediated cycling of iron in neutral and acidic environments.

been identified in *Rhodobacter* sp. strain SW2 and shown to confer enhanced phototrophic iron oxidation reactivity upon the genetically tractable strain *Rhodobacter capsulatus* SB1003 (Croal *et al.*, 2007).

Annotation of the genome sequence of *At. ferrooxidans*^T has confirmed the known physiological capabilities of this

acidophilic iron-oxidizing and iron-reducing proteobacterium and provided new insights into the metabolic pathways involved (Bonney, 2010; Cárdenas *et al.*, 2010; Levican *et al.*, 2008; Quatrini *et al.*, 2007; Valdés *et al.*, 2008). These include carbon metabolism, sulfur uptake and assimilation, hydrogen metabolism, biofilm formation, nitrogen fixation and anaerobic respiration. The enzymo-

Table 2. Currently completed or draft in-progress genomes of iron-oxidizing proteobacteria (Cárdenas *et al.*, 2010)

Organism	Class	Accession number	Reference
<i>At. ferrivorans</i>	Gammaproteobacteria	In progress	Unpublished
<i>At. ferrooxidans</i> ATCC 23270 ^T	Gammaproteobacteria	NC_011761	Valdés <i>et al.</i> (2008)
<i>At. ferrooxidans</i> ATCC 53993	Gammaproteobacteria	NC_011206	Unpublished
<i>T. denitrificans</i> ATCC 25259	Betaproteobacteria	NC_007404	Beller <i>et al.</i> (2006)
' <i>T. prosperus</i> ' M7	Gammaproteobacteria	In progress	Unpublished
<i>Gallionella</i> sp. strain ES-2	Betaproteobacteria	NC_014394	Unpublished
<i>Dechloromonas aromatica</i>	Betaproteobacteria	NC_007298	Unpublished
<i>M. ferrooxydans</i> PV1	'Zetaproteobacteria'	(Shotgun sequencing)	Unpublished
<i>Rp. palustris</i> TIE-1	Alphaproteobacteria	NC_011004	Unpublished
<i>Rp. palustris</i> BisB18	Alphaproteobacteria	NC_007925	Oda <i>et al.</i> (2008)
<i>Rp. palustris</i> BisA53	Alphaproteobacteria	NC_008435	Oda <i>et al.</i> (2008)
<i>Rp. palustris</i> BisB5	Alphaproteobacteria	NC_007958	Oda <i>et al.</i> (2008)
<i>Rp. palustris</i> HaA2	Alphaproteobacteria	NC_007778	Oda <i>et al.</i> (2008)
<i>Rp. palustris</i> CGA009	Alphaproteobacteria	BX571963	Larimer <i>et al.</i> (2004)
' <i>Sideroxydans</i> ' strain ES-1	Betaproteobacteria	NC_013959	Unpublished
<i>Acidovorax ebreus</i> strain TPSY	Betaproteobacteria	NC_011992	Byrne-Bailey <i>et al.</i> (2010)
<i>Rhodobacter</i> sp. SW2	Alphaproteobacteria	(Shotgun sequencing)	Unpublished
<i>R. capsulatus</i> SB 1003	Alphaproteobacteria	CP001312	Strnad <i>et al.</i> (2010)
<i>Rm. vannielii</i>	Alphaproteobacteria	CP002292	Unpublished

logy of iron oxidation has also been far more thoroughly studied in *At. ferrooxidans* than in other proteobacteria. Most of these studies have been carried out with strain ATCC 33020, which has been recently identified as a group II iron-oxidizing *Acidithiobacillus* sp. (Amouric *et al.*, 2011), although the same mechanism has been confirmed with the type strain (group I) (ATCC 23270) of *At. ferrooxidans* (Appia-Ayme *et al.*, 1999; Quatrini *et al.*, 2007; Yarzabal *et al.*, 2004). Genes encoding proteins involved in ferrous iron oxidation in these strains are located on the *rus* operon (Fig. 3). Four electron transport proteins are encoded on this operon: two cytochromes *c* (Cyc1 and Cyc2), an *aa₃*-type cytochrome oxidase, and the low-molecular-mass copper protein rusticyanin. Hallberg *et al.* (2010) reported that the rusticyanin in group I and group II iron-oxidizing acidithiobacilli is type A, whereas a variant of this (type B) is found in group III (*At. ferrivorans*) and group IV iron-oxidizing acidithiobacilli. Groups III and IV do not appear to possess the *rus* operon, and iron oxidation is thought to proceed by another, as yet not fully elucidated, mechanism (Fig. 3). Interestingly, although rusticyanin has long been postulated to play a central role in ferrous iron oxidation in *At. ferrooxidans*-like bacteria, the absence of both isozymes (A and B) in one strain (CF27) of *At. ferrivorans* infers that it is not essential to this function in all iron-oxidizing acidithiobacilli (Hallberg *et al.*, 2010).

Metagenomic approaches make it possible to study the metabolisms of entire microbial communities and to predict which organisms carry out essential community functions, without cultivating these bacteria (Hugenholtz & Tyson, 2008). The metagenome of a biofilm community in acid mine drainage is one example of how the method has been used to investigate communities of iron-oxidizing proteobacteria and other prokaryotes (Lo *et al.*, 2007; Tyson *et al.*, 2004).

Environmental and applied aspects

Iron-oxidizing bacteria have had a major influence on the geochemical evolution of our planet and continue to have a significant impact in terrestrial and aquatic environments. More recently, mankind has begun to learn how to harness their activities in biotechnological processes. Microbial (phototrophic) iron oxidation is thought to have been pivotal to the formation of oxidized BIFs in the Pre-Cambrian era (about 3.85 billion years ago) at a time when the atmosphere was anoxic or only partly oxygenated (Koehler *et al.*, 2010). BIFs are the major primary iron ore used by modern society, while bog iron ores (goethite-rich iron deposits found in bogs and swamps, and associated with gradient neutrophilic iron oxidizers) were an important source of iron in earlier human history.

Spoilage of well waters, blockages in water pipes and other water quality issues associated with ferric iron precipitates have led to neutrophilic iron oxidizers sometimes being considered as nuisance organisms (Taylor *et al.*, 1997; Tuhela *et al.*, 1997). They have also been implicated

in microbial-enhanced corrosion of steel by consuming oxygen and generating microaerobic/anaerobic niches, which are favoured conditions for the sulfate-reducing bacteria actively involved in metal-surface corrosion (Hamilton, 2003). The role of acidophilic species of proteobacteria and other bacteria in the formation of acid mine drainage, a pernicious form of water pollution, has been widely documented (e.g. Hallberg, 2010). Increasingly though, iron-oxidizing proteobacteria and other bacteria are being seen as potentially useful micro-organisms. For example, bacterial ferric iron oxyhydroxides (Supplementary Fig. S2a, b) can be used to adsorb anions such as phosphate, arsenate (Kappler & Straub, 2005) and humic colloids (Cornell & Schwertmann, 2003) in polluted and contaminated waters. Some metals can also co-precipitate with biogenic ferric iron minerals and thereby aid remediation of polluted waters (Richmond *et al.*, 2004). Currently, the major biotechnological application of iron-oxidizing prokaryotes, however, is the use of acidophilic species (proteobacteria and other bacteria, as well as some archaeal species) to solubilize metals from mineral ores or, in the case of gold, to make it accessible to chemical extraction. Over the past 50 years, biomining has developed into a global technology, responsible for ~20% of current copper production, as well as lesser amounts of nickel, cobalt, uranium, zinc and gold (Rawlings & Johnson, 2007).

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