

16. GENETIC AND BIOINFORMATIC INSIGHTS INTO IRON AND SULFUR OXIDATION MECHANISMS OF BIOLEACHING ORGANISMS

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16.1 INTRODUCTION.

Arguably, the most important role played by microorganisms in the solubilization of metals in bioleaching operations is their ability to oxidize iron- and sulfur-containing minerals. Most progress has been made in understanding these fundamental processes in the mesophilic bacterium *Acidithiobacillus ferrooxidans* because its role in bioleaching was recognized earliest and, therefore, it has had the longest history of investigation. In addition, the complete genome sequence of *At. ferrooxidans* was recently released by The Institute for Genome Research (TIGR) which has allowed the bioinformatic prediction of several important biochemical pathways and has provided insight into the biochemistry and physiology of iron and sulfur oxidation. This chapter will describe some of the recent progress in understanding iron and sulfur oxidation reactions in this microorganism using molecular genetics and bioinformatics tools.

In the last decade it has become increasingly clear that several other bacteria and archaea play crucial roles in mineral solubilization, especially at the elevated temperatures that occur in tank reactors for gold recovery and in the later stages of copper heap leaching when exothermic biooxidation reactions have driven temperatures up to the 45⁰C - 80⁰C range. Several of these bacteria and archaea have been identified and some initial information is available regarding their iron and sulfur metabolisms are described in this chapter.

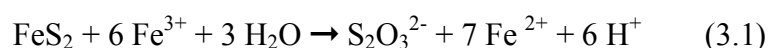
It has also become increasingly obvious that metal solubilization is promoted by the concerted effort of a consortium of microorganisms and that it is imperative to understand how these microorganisms cooperate in this endeavor. The identification of these microorganisms and the investigation of their interactions in bioleaching operations is a study in microbial ecology. To date, investigations into the microbial ecology of bioleaching operations has relied primarily on the use of standard techniques of

molecular genetics, especially in the description of the spatial and temporal distribution of microorganisms in heap bioleaching operations and these advances are discussed in chapters 12-14 of this book. However, the emerging fields of comparative genomics and metagenomics are beginning to impact on our understanding of the microbial ecology and metabolic processes of bioleaching operations and some of the recent advances in these areas related to our understanding of microbial iron and sulfur oxidation will be described.

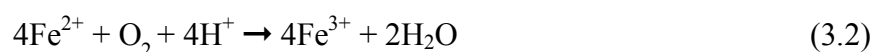
16.2. RELEVANT BIOCHEMICAL AND CHEMICAL REACTIONS.

The important biochemical and chemical reactions involved in iron and sulfur oxidation in bioleaching processes have been described elsewhere in this book (to be properly referenced by the editors of the book). However, for the purposes of this chapter it is important to highlight the following two points:

(i) The initial substrates for iron and sulfur oxidation enzymes in bioleaching microorganisms are insoluble metal sulfides. For example, pyrite (FeS_2) is the most common sulfide mineral present in sulfide ore deposits and its oxidation can be written:



The Fe^{2+} generated in equation (3.1) can be biologically oxidized to Fe^{3+} :



The Fe^{3+} product of equation (3.2) is a strong oxidant that can oxidize metals present in the ore aiding in their solubilization and regenerating Fe^{2+} in the process. However, in the absence of this regeneration, Fe^{3+} may subsequently hydrolyzed and precipitated as ferric oxyhydroxide:



The generation of a precipitate precludes the possibility that the oxidation of Fe^{2+} for energy generation occurs inside the cell and, therefore, it must be localized to the exterior of the outer membrane. This places an emphasis on where we must look for the key initial enzymes involved in iron oxidation.

(ii) The E'_0 of the redox couple $\text{Fe}^{2+}/\text{Fe}^{3+}$ is + 0.74 V at pH 2, whereas that of the $\text{NA(P)D}^+/\text{NAD(P)H}$ couple is -0.32 at pH 7. This means that the electrons biologically extracted during the oxidation of Fe^{2+} must be pushed “uphill” against a thermodynamically favorable gradient to reduce NAD(P)^+ to NAD(P)H . Therefore a source of energy must be found for the process and any proposed intermediate electron carriers must have values of E'_0 between those of $\text{Fe}^{2+}/\text{Fe}^{3+}$ and $\text{NA(P)D}^+/\text{NAD(P)H}$.

16.3. GENETICS OF BIOLEACHING MICROORGANISMS.

16.3.1 Introduction.

Molecular genetics refers to a collection of techniques and knowledge that can be used to investigate gene and protein function. For example, in the case of bioleaching organisms, it is relatively straightforward to isolate genes from bioleaching microorganisms and clone them into a well characterized surrogate host such as *Escherichia coli* where their function can be analyzed by complementing mutants or where sufficient gene product (protein) can be isolated to carry out standard biochemical assays to investigate function. One problem is that these strategies seem to work best for proteins that are expressed in the cytoplasm of bioleaching organisms where the pH is close to neutral, as is the cytoplasm of *E. coli*. Analyzing the function of periplasmic or outer membrane proteins of bioleaching microorganisms in *E. coli* results in proteins that may exhibit either no or poor function because they probably require an acid pH to fold and work correctly (Brown et al., 1994; Bengrine et al., 98; Appia-Ayme, 1998; Bruscella et al., 2005). This is unfortunate because many of the most relevant enzymes and electron carriers involved in iron and sulfur oxidation are outside the cytoplasm or embedded within the membrane with loops exposed to the acid pH of the periplasm.

A significant challenge has been the difficulty of returning genes to bioleaching microorganisms once they have been studied in a surrogate host. This means that their function cannot be proved directly in the bioleaching organism in question, nor can genes that have been modified by genetic engineering in surrogate hosts be re-introduced back into the microorganisms from which they were derived. This has seriously impeded progress in understanding the physiology of bioleaching organisms and has prevented the development of genetically modified strains. Some promising

progress has, however, been made in this direction recently and will be discussed in this chapter.

Genetic analysis of bioleaching microorganisms is also a challenge because, in general, they are strict or moderate acidophiles and obligatory or facultative chemoautolithotrophs. They grow slowly in laboratory culture with very low cell yields making them difficult to culture and posing severe problems for the isolation of sufficient quantities of enzymes for biochemical analysis.

A word of caution is in order regarding bioleaching microorganism strain classification and its implication for the identification of genes, enzymes and biochemical pathways. Even though two organisms may be classified as belonging to the same species, usually by rRNA spacer similarity, it does not necessarily mean that they share an identical repertoire of genes, meaning that biological interpretations, extrapolated from one strain to another, should be treated with caution. This potential problem is exacerbated when the identification of a particular strain is not adequately described. The literature regarding the genetics and biochemistry of *At. ferrooxidans* is particularly rampant with examples of the use of “private” strains, i.e. that are not deposited in public microbial banks and are often, lamentably, not adequately characterized. Such strains are often not publicly available and consequently results of investigations cannot be independently confirmed.

16.3.2 Gene cloning.

The majority of genes that encode proteins involved in the oxidation of sulfur (S^0), reduced inorganic sulfur compounds, or ferrous iron (FeII) that have been cloned and studied are from *At. ferrooxidans*, and are described below. However, advances are being made in the cloning and analysis of FeII- and S^0 -related genes in other microorganisms. For example, genes from *Leptospirillum ferrooxidans* (Delgado, et al., 1998) and *Thermoplasma acidophilum* have been investigated. Recently, a tetrathionate hydrolase gene from *Acidithiobacillus caldus* has been sequenced, and preliminary evidence suggests that it may belong to an operon that also contains genes encoding a terminal quinol oxidase (Rzhepishevskaya et al., 2005, personal communication).

16.3.3 Gene transfer systems.

The real challenge is to introduce DNA into bioleaching microorganisms. Without a reliable and efficient gene transfer system, expression of heterologous genes in these bacteria and the construction of mutants, precisely defined at the molecular level, are not possible. The classical approaches followed are either (i) transduction, that is transfer of genetic information via a bacteriophage (virus) particle, (ii) conjugation, that is the transfer of conjugative or mobilizable plasmids from one bacterium to another by cell-to-cell contact, or (iii) electrotransformation, by exposing the cells in the presence of free DNA to a pulsed electric field which destabilizes transiently the bacterial membrane and permits the entry of the DNA into the cell.

16.3.3.1 *Acidiphilium* spp. *Acidiphilium* spp. are acidophilic and facultative heterotrophic Gram-negative proteobacteria (Hiraishi et al., 1998). *Acidiphilium acidophilum* (formerly *Thiobacillus acidophilus*) can grow autotrophically with reduced inorganic sulfur and is capable of mixotrophic ferric iron (FeIII) reduction (Johnson and McGinness, 1991). A bacteriophage, ϕ AC1, that infects *Acidiphilium* and can integrate into its genome, has been described (Ward et al., 1993) but no transduction system with this phage has been reported so far. However, genetic transfer by conjugation between *E. coli* and different *Acidiphilium* species (Roberto et al., 1991; Glenn et al., 1992; Quentmeier and Friedrich, 1994; Bruhn and Roberto, 1993) and by electrotransformation (Glenn et al., 1992; Inagaki et al., 1993) has succeeded with frequencies from 10^{-2} to 10^{-9} transconjugants per recipient cell and 10^3 to 10^4 transformants mg^{-1} of plasmid DNA, respectively, depending on the plasmid and on the *Acidiphilium* species.

16.3.3.2 *Acidithiobacillus thiooxidans*. This Gram-negative proteobacterium is a strict acidophile and an obligate autotroph. However, in spite of these differences with the neutrophilic and heterotrophic *E. coli*, direct mating is possible, and self-transmissible broad-host-range plasmids were transferred between these two microorganisms. Transconjugants were obtained at a low but workable frequency (10^{-5} to 10^{-7} per recipient) (Jin et al., 1992). With this approach, the *E. coli* phosphofructokinase was cloned and expressed successfully in *At. thiooxidans* (Tian et al., 2003).

16.3.3.3 *Acidithiobacillus ferrooxidans*. For many years, considerable but unsuccessful efforts were made by several groups to find conditions

that would permit conjugation between *E. coli* and *At. ferrooxidans*. The main problem was to find a compatible medium in which both bacteria could survive and produce the energy for the conjugation process to take place. Nevertheless, Peng et al. (1994b and 1994c) succeeded in designing a mating medium with which plasmids from different incompatibility groups were conjugated in several private strains of *At. ferrooxidans*. Low but workable frequencies of transconjugants (10^{-5} to 10^{-7} transconjugants per recipient) were obtained, depending on the incompatibility group of the plasmid and on the strains (Peng et al., 1994c). In spite of this low frequency, the transposon Tn5 was successfully introduced with a mobilizable suicide vector and shown to be able to transpose into the chromosome of *At. ferrooxidans*. This result is encouraging and opens the way to random transposon insertion mutagenesis (Peng et al., 1994c). In addition, the arsenic resistance genes from a narrow host range non-mobilizable plasmid were cloned into a mobilizable plasmid and transferred by conjugation from *E. coli* to *At. ferrooxidans* and expressed (Peng et al., 1994a). The transfer of plasmids from different incompatibility groups from *E. coli* to different *At. ferrooxidans* strains, including strains ATCC33020 and ATCC19859, was later improved to get a frequency as high as $2.5 \cdot 10^{-3}$ transconjugants per recipient (Liu et al., 2000; Liu et al., 2001a). More recently, transconjugants with the type strain ATCC23270 were obtained with even higher frequencies (Ratouchniak and Bonnefoy, unpublished data).

Electroporation of native and exogenous plasmids into *At. ferrooxidans* has been reported (Kusano et al., 1992a). However, only one out of the 30 private strains tested was transformed and 50% of colonies obtained were false positives and not transformants. Furthermore, in spite of all these efforts, the frequency was very low (120 to 200 colonies mg^{-1} of DNA) (Kusano et al., 1992a; Rawlings and Kusano, 1994). By following the efflux of ATP out of the cells, strain ATCC33020 was shown to be electropermeabilizable (Guiliani et al., 1995). However, under these conditions, only false positives were obtained, even with a plasmid which has been purified from *At. ferrooxidans* ATCC33020 transconjugants, suggesting that the restriction barrier of this strain is very stringent (Liu, Ratouchniak and Bonnefoy, unpublished data).

16.3.4 Mutant construction.

Only two *At. ferrooxidans* mutants, affected in their FeII oxidation capacities,

have been described. The first was obtained by nitrosoguanidine mutagenesis and was reported to affect the rusticyanin gene, but no proof was provided (Cox and Boxer, 1986). The second was due to the insertion of ISAfe1 (an insertion sequence element) within the *resB* gene resulting in mutant cells that could grow on S^0 but not FeII medium (Zhao and Holmes, 1993; Cabrejos et al., 1999; Holmes et al., 2001). Interestingly, *resB* encodes a protein involved in the maturation of cytochrome *c* suggesting that this electron carrier is required for FeII oxidation but is not necessary for S^0 oxidation, an hypothesis which was later confirmed (Brasseur et al., 2004; Bruscella, 2004). Unfortunately, this mutant has been lost and the attempts to construct a new one by marker exchange mutagenesis have failed.

Marker exchange mutagenesis (MEM), in contrast to the techniques described above, permits the construction of specifically designed mutants and is therefore called a “targeted” mutagenic approach. In MEM, a “suicide plasmid”, carrying a selectable marker inserted inside the gene to be mutated, is introduced into *At. ferrooxidans* cells by conjugation. This plasmid is unable to replicate in this bacterium, and the only way to maintain the selection is by plasmid insertion into the chromosome (one cross-over), or by exchange between the “targeted” normal (wild-type) gene in the chromosome and the mutated gene in the plasmid (two cross-overs). A *recA* mutant was successfully constructed in this way (Liu et al., 2000; Liu et al., 2001b). The same strategy was later used to construct a *hip* mutant, the *hip* gene encoding a high redox potential iron-sulfur protein whose function remains controversial (Bruscella, 2004, and see below). Unfortunately, even after one year of subculturing in FeII medium without selection, only one cross-over could be detected, and it was demonstrated that both the mutated and the wild type alleles were present in the transconjugant (Bruscella, 2004). Since the wild type allele was still expressed, this strain behaves phenotypically as a wild type, suggesting that the *hip* gene is essential in the conditions tested and cannot be inactivated by MEM. In conclusion, the MEM approach, while feasible, is fastidious and can be only applied to non essential genes.

16.4 IRON AND SULFUR OXIDATION AND REDUCTION IN *AT*.

FERROOXIDANS.

16.4.1 Ferrous iron oxidation.

16.4.1.1 Introduction. The oxidation of FeII by *At. ferrooxidans* can be considered to proceed in a “downhill” and an “uphill” reactions, although both pathways are interconnected in nature. In the “downhill” pathway, electrons removed biologically from FeII proceed, through a series of electron carriers, from the outer membrane to the periplasm where they are used to reduce O₂ to water, consuming protons in the process. The majority of the protons consumed have entered the cell via the ATP synthetase complex embedded in the inner membrane. *At. ferrooxidans* generates ATP using this proton motive force (PMF) generated by the tremendous protein gradient across the inner membrane and the electrons from the oxidation of iron are used only to neutralize the incoming protons.

However, in addition, *At. ferrooxidans* has to regenerate the reduced pyridine nucleotides NADPH and NADH necessary for CO₂ and N₂ fixation and other anabolic processes. Because the standard reduction half-potential of the FeII/FeIII couple (+0.77 V at pH 2, the pH of the medium) is much more positive than that of the NAD(P)/NAD(P)H couple (-0.32 V at the cytoplasmic pH 7), the electrons have to be “pushed uphill” from FeII to NAD(P) against the redox potential gradient. This “uphill” electron transfer requires energy which is probably provided by the PMF and the “uphill” flow of electrons can considered somewhat similar to a mitochondrion working in reverse, an hypothesis originally proposed by Ingledew over 20 years ago (Ingledew and Cobley, 1980; Ingledew, 1982). In addition, ATP hydrolysis via the ATP synthetase working in reverse may be used to generate an electrochemical proton gradient that may provide some of the force to push electrons “uphill” (Elbehti et al., 2000). This invokes an interesting possible regulatory mechanism for adjusting the balance between the production of NAD(P)H and ATP. When ATP concentrations are high, the ATP synthetase functions like an ATPase and the ATP hydrolysis may provide additional PMF to push electrons “uphill” to reduce NAD(P) to NAD(P)H that can then be used to fix carbon. Conversely, when the concentration of fixed carbon is high, ATP synthetase, working in the normal respiratory direction, will produce ATP (Elbehti et al., 2000).

16.4.1.2 The “downhill” electron pathway. Because of the relatively high redox potential of the FeII/FeIII couple, a considerable amount of FeII has to be oxidized to sustain the growth of FeII-oxidizing acidophiles and, as a result, many electrons have to be transferred from this compound to oxygen. Based on the

assumption that the proteins involved in the “downhill” electron transfer chain have to be synthesized in large quantities, the redox proteins present in relative abundance in FeII-grown cells were proposed to be involved in this pathway and were characterized. From these studies, a number of models have been designed, which differ slightly on the proteins involved and on their order in the respiratory chain (Ingledew et al., 1977; Yamanaka et al., 1991; Yamanaka and Fukumori, 1995; Blake and Shute, 1994; Bruschi et al., 1996; Giudici-Orticoni et al., 2001). However, these models contained several questionable points, the main one being that the first electron acceptor proposed in all these models is located in the periplasm while it is known that (i) an important natural substrate is pyrite, an insoluble sulfide mineral, (ii) soluble FeII cannot enter the cell because of its rapid auto-oxidization and the highly insoluble nature of the ferric oxy-hydroxide product at the pH of the cell. Following a completely different approach, another model has been proposed based on the studies on genetic organization, gene regulation and sub-cellular localization of the different partners (Appia-Ayme, 1998; Appia-Ayme et al., 1999; Yarzabal et al., 2002b; Yarzabal et al., 2004).

The first gene encoding a redox protein was the *iro* gene from Fe-1 strain (Kusano et al., 1992b), encoding a high potential redox iron sulfur protein (HiPIP) which was proposed by several authors to be the first electron acceptor from FeII (Fukumori et al., 1988; Cavazza et al., 1995; Yamanaka and Fukumori, 1995; Bruschi et al., 1996; Yamanaka et al., 1991). This gene is located between the *purA* and a tRNA encoding genes but is transcribed independently (Kusano et al., 1992b). It should be noted that in three collection strains including the type strain (ATCC23270, ATCC33020 and ATCC19859), only one gene encoding a HiPIP was identified and this gene, referred to as the *hip* gene, encodes a protein presenting only about 51% similarity to Iro (Bruscella et al., 2005). This *hip* is in a completely different genetic context than *iro* and its expression is higher in S^0 - than in FeII-grown cells (Bruscella, 2004; Bruscella et al., 2005; Quatrini et al., 2005a). This data, and the periplasmic location of the corresponding Hip protein, raises doubts about its proposed role at least in these three strains (see below). For several years, a number of laboratories worldwide have tried to isolate the gene encoding rusticyanin, whose concentration is very high in FeII-grown cells (up to 5% of the cell protein (Cox and Boxer, 1978) but without any success. The sequence of a 260 bp internal fragment of the *rus* gene was determined by Pulgar et al. (1993), and a synthetic *rus* gene was constructed, as well as site-

specific mutants, and overexpressed in *E. coli* to study the biochemical and biophysical properties of the protein (Casimiro et al., 1995). Also, in 1995, the sequence of the entire *rus* gene and of the flanking regions from the ATCC33020 strain was determined (Guiliani et al., 1995; Bengrine et al., 1995; Bengrine et al., 1998). One year later, the DNA sequence encoding the mature rusticyanin from the type strain ATCC23270 was published (Hall et al., 1996). From the analysis of the genomic organization of the *rus* locus, a model for the iron respiratory chain was proposed (Appia-Ayme, 1998; Appia-Ayme et al., 1999) which was later confirmed by subcellular localization of the different components (Yarzabal et al., 2002a; Yarzabal et al., 2002b) and by genetic regulation (Yarzabal et al., 2003; Yarzabal et al., 2004; Quatrini et al., 2005a). The *rus* gene belongs to an operon which is more highly expressed in FeII- than in S⁰-grown cells supporting the involvement of the *rus* operon encoded products in the oxidation of FeII (Yarzabal et al., 2003; Yarzabal et al., 2004; Quatrini et al., 2005a). This operon encodes, in addition to rusticyanin, three other electron transfer proteins, two cytochromes *c* (Cyc1 and Cyc2) and an *aa*₃-type cytochrome oxidase (CoxABCD) (Appia-Ayme, 1998; Appia-Ayme et al., 1999). These redox proteins constitute likely a "respiratory supercomplex" involved in FeII respiration, as proposed by Appia-Ayme et al. (1999). This operon encodes also a protein (ORF) which presents some similarities (32%) to the *Halobacterium* sp. NRC-1 Pan1 protein of unknown function. ORF has been located in the outer membrane (Yarzabal and Bonnefoy, unpublished data) but its function remains unknown.

As discussed above, it was assumed that the electrons have to pass from the outside medium, where pyrite (FeS₂) is oxidized, to the cytoplasm where H₂O reduction takes place. The order of the different partners encoded by the *rus* operon in the respiratory chain is dependent on their subcellular localization. The cytochrome *c* Cyc2 has been shown to be located in the outer membrane, with a domain facing the external environment where it may interact with insoluble substrates (Yarzabal et al., 2002b) while the cytochrome *c* Cyc1 is bound to the inner membrane (Yarzabal et al., 2002a). Rusticyanin was confirmed to be periplasmic and the cytochrome oxidase to be an integral inner membrane complex (Yarzabal et al., 2002b). Therefore, the electron transporters appear to constitute an "electron wire spanning both the outer and the inner membranes to conduct electrons from pyrite to oxygen", as suggested by Yarzabal et al. (2002b), through Cyc2→ rusticyanin→Cyc1→CoxABCD (Figure

16.1).

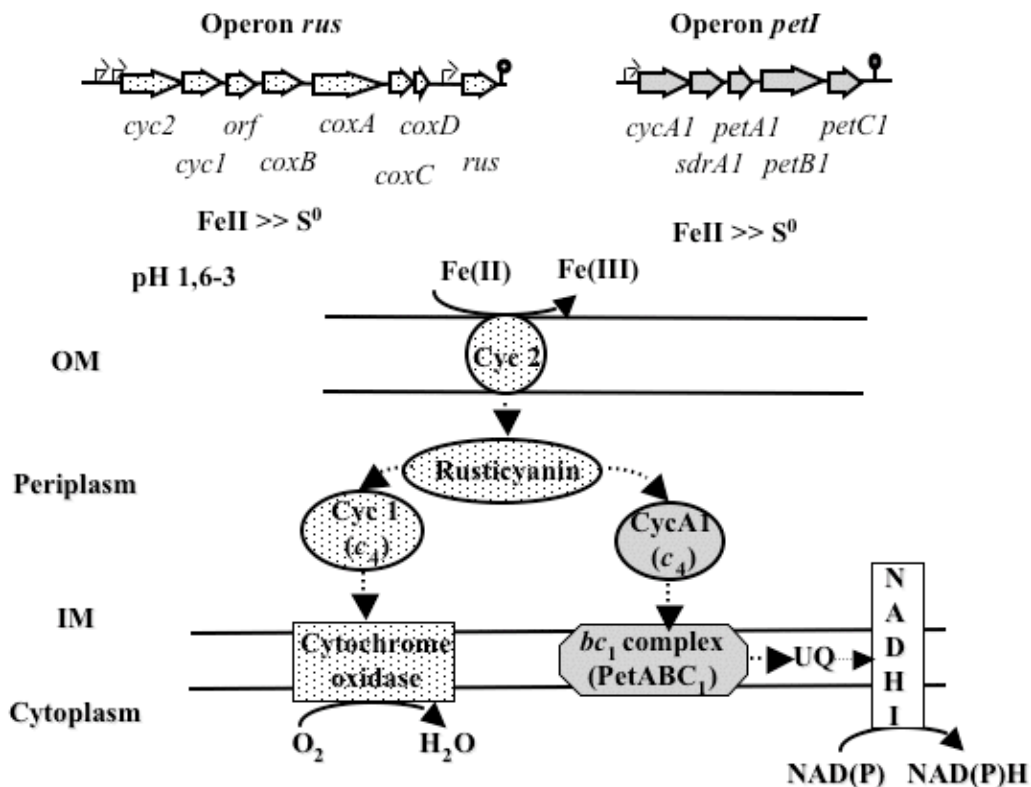


Figure 16.1. Proposed model for FeII energetic metabolism in *At. ferrooxidans*. The transcriptional units and the corresponding redox proteins are presented with the same background pattern. Dotted lines represent the electron transport. The small arrows and black dots in the transcriptional units represent predicted promoters and *rho* independent stop sites respectively.

16.4.1.3 The “uphill” electron pathway. Ingledew (1982) suggested a role for the *bc*₁ complex in the pathway to regenerate NAD(P)H in FeII-grown cells. The existence of a reverse electron flow from a cytochrome *c* through the cytochrome *bc*₁ complex to quinone and the NAD(P)H dehydrogenase was later clearly demonstrated in *At. ferrooxidans* (Elbehti et al., 2000; Basseur et al., 2002). An operon encoding a *bc*₁ complex has also been characterized in the *At. ferrooxidans* ATCC19859 (Levicán et al., 2002) and ATCC33020 (Bruscella, 2004) strains. This operon has been shown to be more highly expressed in FeII- than in S⁰-grown cells (Bruscella, 2004; Quatrini et al., 2005a) in agreement with its role proposed in the “uphill” pathway between FeII and NAD(P). This operon encodes, in addition to the three subunits of the *bc*₁ complex (PetA1B1C1), a cytochrome *c* (CycA1) and a

ribose/ glucose dehydrogenase (SdrA1), the latter of unknown function (Levican et al., 2002). The cytochrome c_4 encoded by the *cycA1* gene has been characterized and proposed to belong to the electron transfer chain between FeII and oxygen, and more precisely to receive the electrons directly from FeII and to transfer them to rusticyanin (Giudici-Orticoni et al., 2000). Because *cycA1* belongs to the *petI*, and not to the *rus* operon, this cytochrome is more likely to be involved in the same electron transfer chain than the bc_1 complex, that is the reverse electron pathway between FeII and NAD(P) (Bruscella, 2004; Quatrini et al., 2005a).

In the reverse electron pathway, the bc_1 complex receives electrons from a cytochrome c and transfers them to the quinol pool (Griesbeck et al., 2000). This cytochrome c has been suggested to be the cytochrome c_4 encoded by the *cycA1* gene (Bruscella, 2004; Quatrini et al., 2005a) (Figure 16.1). FeII oxidation and NAD(P) reduction have been proposed to be coupled to explain the balance of the reducing equivalent from FeII between the two pathways: the exergonic one, through the aa_3 type oxidase towards oxygen, and the endergonic one, through a bc_1 complex toward NAD(P) (Elbehti et al., 2000). As previously reported (Bruscella, 2004; Brasseur et al., 2004; Quatrini et al., 2005a), the bifurcation is likely at the level of rusticyanin which gives electrons to two different cytochromes c_4 : CycA1 encoded by the *petI* operon, or Cyc1 encoded by the *rus* operon. In the former case, electrons are transferred to the endergonic pathway while in the later case, they are transferred to the exergonic pathway (Figure 16.1). Recent evidence from a combination of inhibitors and electron uncouplers support this model (Chen and Suzuki, 2005).

16.4.2. Sulfur oxidation.

Unraveling the biochemistry of sulfur oxidation has proved particularly challenging. Sulfur can exist in various oxidation states from -2 to $+6$, complicating the resolution of the enzymatic steps involved. Also, some steps can proceed spontaneously exacerbating the search for reactions catalyzed by enzymes. Sulfur oxidation is widespread in prokaryotes and can proceed by a number of distinct routes. Recent genomic and biochemical data suggest that the sulfur oxidation pathways in archaea and bacteria differ substantially (Friedrich et al., 2005). Furthermore, acidophilic bacteria, including *At. ferrooxidans* and *At. thiooxidans*, oxidize sulfur by a system that is different from the majority of other bacteria (Friedrich et al., 2005). For example, neither the *sox* genes that encode the sulfur-oxidizing system widely distributed in a number of bacteria, nor the *sor* gene encoding the archaeal-type sulfur oxygenase reductase, were identified in the partial

genome sequence of the type strain ATCC23270 (Urich et al., 2004; Friedrich et al., 2005); an observation that has now been confirmed by inspection of the complete genome sequence of this bacterium (Appia-Ayme, Bonnefoy, Quatrini and Holmes unpublished data). It is clear that the sulfur oxidation pathways of *At. ferrooxidans* are different from those of most archaea and bacteria, which makes their study an exciting challenge.

Several enzymes of *At. ferrooxidans* have been suggested to be involved in the oxidation of sulfur, sulfide and reduced inorganic sulfur compounds (Figure 16.2): a thiol-bearing outer membrane protein, which mobilizes elemental sulfur and transports it into the periplasmic space as persulfide sulfur (Rohwerder et al., 2003), a sulfur dioxygenase which oxidizes persulfide sulfur to sulfite (Silver and Lundgren, 1968a; Rohwerder and Sand, 2003), a sulfite: oxidoreductase which oxidizes sulfite to sulfate (Vestal and Lundgren, 1971), a sulfide:quinone oxidoreductase which oxidizes sulfide to sulfur (Wakai et al., 2004), a thiosulfate oxidase which catalyses the oxidation of thiosulfate to tetrathionate (Silver and Lundgren, 1968 b), a rhodanase, which splits the thiosulfate to sulfur and sulfite (Tabita et al., 1969) and a tetrathionate hydrolase which hydrolyses tetrathionate to thiosulfate, sulfur and sulfate (De Jong et al., 1997). While the enzymatic steps to oxidize polythionates are still not very clear, it is now generally agreed that S^0 is transported into the periplasmic space as persulfide-sulfur where it is oxidized by sulfur dioxygenase to sulfite, which is further oxidized to sulfate by a sulfite oxidoreductase (Pronk et al., 1990; Kuenen et al., 1993; Lorbach et al., 1993; Rohwerder and Sand, 2003).

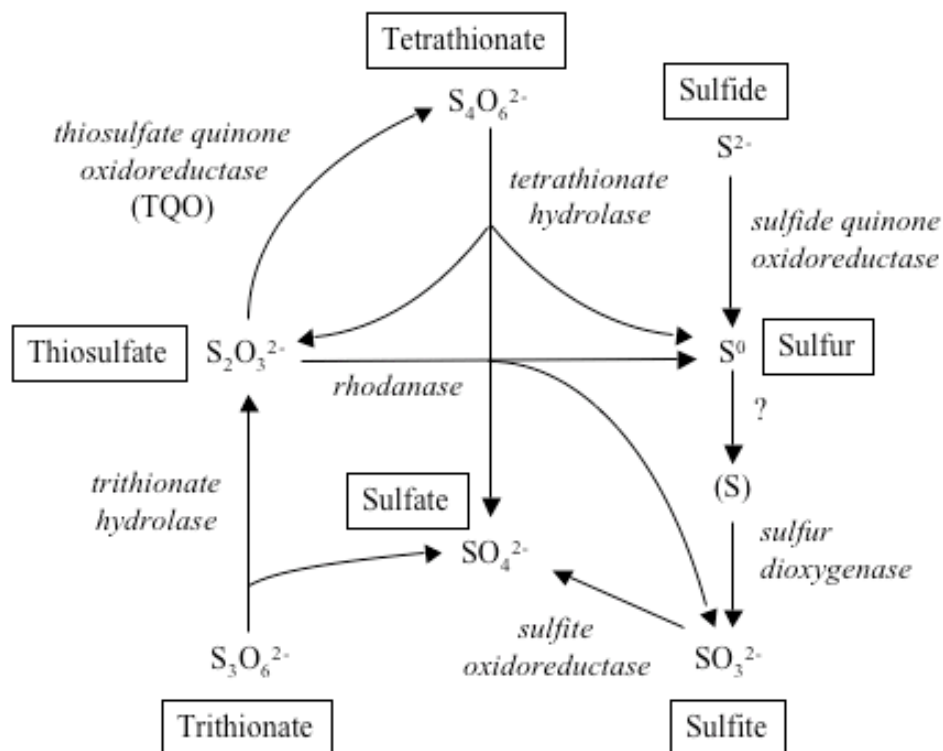


Figure 16.2. Scheme representing the proposed enzymatic steps involved in the oxidation of inorganic sulfur compounds by *At. ferrooxidans* (after Kuenen et al., 1993, and Rohwerder and Sand, 2003).

Some of the electrons that are transferred in the reactions described above can be fed into the respiratory chain for energy conservation. Corbett and Ingledew (1987) proposed that the electrons from S^0 oxidation are passed to oxygen through a bc_1 complex and a terminal oxidase. However, lower levels of total cytochromes *c* were observed in S^0 - compared with FeII-grown cells, suggesting that these electron transporters are not absolutely required for S^0 oxidation (Yarzabal et al., 2002a). The same conclusion was inferred from the analysis of a *resB* mutant in which cytochromes *c* cannot be matured but in which growth on S^0 is not affected (Cabrejos et al., 1999). In addition, oxygen reduction in FeII and S^0 was suggested to occur via two separate terminal oxidases (Pronk et al., 1991; Harahuc et al., 2000). All together, these data suggested that electrons from S^0 are transferred to oxygen by at least two respiratory chains, one of which is devoid of cytochrome *c*. Indeed, a branching point at the level of the quinol pool has been demonstrated and the electrons can either be transferred to a *bd* type oxidase, or to a ba_3 (or bo_3 depending on the strain) type oxidase via a bc_1 complex (Brasseur et al., 2004). This bc_1 complex has been shown to function in direct (downhill) mode (Brasseur et al., 2004), contrary to the one detected in FeII-grown cells which can function only in reverse (“uphill”) mode (Brasseur et al., 2002). The most likely explanation is therefore that *At. ferrooxidans* has two bc_1 complexes, one functioning in direct mode in S^0 -grown cells, and one functioning in reverse in FeII-grown cells. Indeed, two operons encoding bc_1 complexes have been detected in the genome sequence of the ATCC23270 strain (Brasseur et al., 2002; Bruscella, 2004). These two operons are also present in strains ATCC33020 and ATCC19859, indicating that the presence of two bc_1 complexes is a general property of bacteria categorized as “*At. ferrooxidans*”, a feature which is unique so far to this species (Bruscella, 2004). Surprisingly, not only *petA*, *petB* and *petC* genes are duplicated but also the cytochrome *c* gene, *cycA*, and the ribitol/ glucose dehydrogenase gene, *sdrA*. In addition, in the second operon, referred to as *petII*, a gene encoding a high potential iron sulfur protein (HiPIP), *hip*, is located downstream from *petC2*. All these genes, including the *hip* gene, have been shown to be co-transcribed (Bruscella, 2004). Furthermore, they are expressed in S^0 -grown cells

(Bruscella, 2004; Quatrini, et al., 2005a) suggesting that the proteins encoded by this operon, including the HiPIP, are involved in S^0 oxidation. Therefore, the bc_1 complex encoded by *petI* is the one functioning in reverse and transfers the electrons from FeII to NAD(P), while the bc_1 complex encoded by *petII* is the one functioning in direct mode and transfers electrons from S^0 to oxygen. When functioning in direct mode, the bc_1 complex receives electrons from the quinol pool and transfers them either to a membrane-bound cytochrome *c*, and/or to a soluble redox protein such a cytochrome *c*, or a high potential iron-sulfur protein (HiPIP) which then transfers the electron to the terminal oxidase where oxygen reduction takes place (Trumpower, 1990; Bonora et al., 1999; Pereira et al., 1999). Since a membrane-bound cytochrome *c* (CycA2) and a periplasmic HiPIP are encoded by the *petII* operon, these two redox proteins are good candidates for transferring electrons between the two integral membrane complexes: the bc_1 and the terminal oxidase (Bruscella, 2004; Quatrini et al., 2005a) (Figure 16.3).

The model is far from being complete. For example, how are the electrons transferred to the quinol pool? Which is the terminal oxidase involved, since it is known from the genome sequence that there are at least three oxidases, an aa_3 type, encoded by the *rus* operon and involved in the downhill pathway between FeII and oxygen, and *bd* and bo_3 types (Brasseur et al., 2004). Furthermore, *At. ferrooxidans* is able to oxidize not only S^0 , but also sulfide and reduced inorganic sulfur compounds, such as thiosulfate, tetrathionate and sulfite, which are oxidized to sulfate. Where are the electrons produced by the oxidation reactions fed in the respiratory chain? To answer all these questions, a global approach using a genome-wide microarray transcript profiling analysis was undertaken to facilitate an overall view of the genes involved in S^0 or FeII oxidation. Oligonucleotides corresponding to each genes of the *At. ferrooxidans* type strain ATCC23270 were spotted onto glass-slides and hybridized with cDNA retrotranscribed from RNA extracted from FeII- and S^0 -grown cells (Quatrini et al., 2005a). The expression of the redox protein encoding genes which are preferentially transcribed in FeII- or S^0 -conditions has been validated by real-time PCR, Northern blot hybridization and/or immunodetection analysis (Yarzabal et al., 2004; Bruscella, 2004; Quatrini et al., 2005a). These transcriptome analyses have shown that the operons encoding the *bd* and the bo_3 terminal oxidases, the operon encoding thiosulfate quinone reductase and the gene encoding sulfide quinone oxidoreductase are more expressed in S^0 - than in FeII-conditions (Quatrini et

al., 2005a), in agreement with previous results obtained on the ATCC19859 strain (Brasseur et al., 2004; Ramirez et al., 2004; Acosta et al., 2005). These data suggest that the electrons from S^0 and reduced sulfur compounds enter the respiratory chain through the sulfide quinone oxidoreductase (SQR) or the thiosulfate quinone reductase (TQR) at the level of the quinol pool and through the sulfite oxidoreductase at the level of the cytochrome c_4 or of the HiPIP. These electrons can then either be transferred to a bd terminal oxidase or, through a bc_1 complex to a bo_3 terminal oxidase (Figure 16.3).

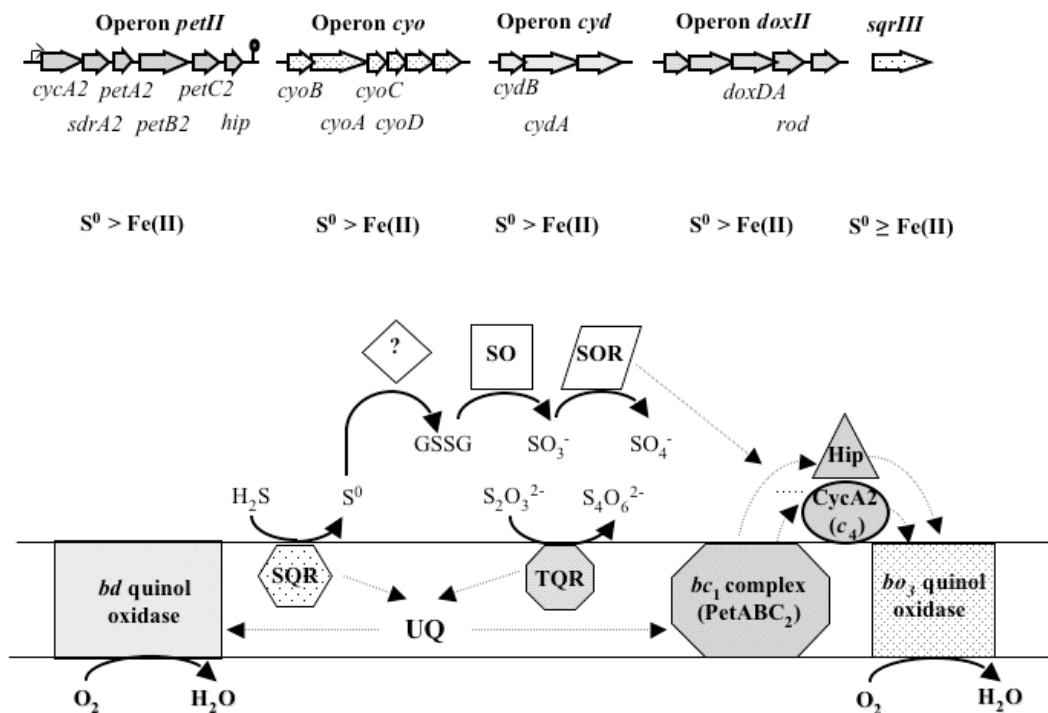


Figure 16.3. Model proposed for sulfur energetic metabolism in *At. ferrooxidans*. The transcriptional units and the corresponding redox proteins are presented with the same background pattern. Dotted lines represent the electron transport. TQR: thiosulfate quinone reductase; SQR: sulfide quinone reductase; SO: sulfur oxygenase; SOR: sulfite oxidoreductase; Hip: High Potential Iron-sulfur protein.

An alternative mechanism has been proposed in which S^0 oxidation is coupled to the reduction of Fe^{III} (Figure 16.4; Sugio et al., 1985; Sugio et al., 1987; Sugio et al., 1988a; Sugio et al., 1988b; Sugio et al., 1989; Sugio et al., 1992a; Sugio et al., 1992b) but this model has been contested mainly based on bioenergetic considerations (Corbett and Ingledew, 1987; Pronk et al., 1991, Kuenen et al., 1993).

16.4.3 Ferric iron and sulfur reduction in *At. ferrooxidans*.

In large scale leaching operations, where dissolved oxygen concentrations are low and FeIII concentrations high, anaerobic FeIII reduction may be an important process. It has been known for a number of years that *At. ferrooxidans* is able to reduce FeIII under anaerobic conditions coupled to the oxidation of S⁰ (Brock and Gustafson, 1976; Kino and Usami, 1982; Sugio et al., 1985; Das et al., 1992; Pronk et al., 1992) or formate (Pronk et al., 1991).

Anaerobic S⁰ oxidation coupled to FeIII reduction appears to be catalyzed by a series of enzymes apparently different from those involved in the aerobic oxidation of S⁰ (Sugio et al., 1985): after chemical reduction of S⁰ by glutathione to hydrogen sulfide (Sugio et al., 1989), a periplasmic hydrogen sulfide:FeIII oxidoreductase reduces hydrogen sulfide to sulfite (Sugio et al., 1987), and a membrane bound sulfite:FeIII oxidoreductase reduces sulfite to sulfate (Sugio et al., 1988a; Sugio et al., 1992a) (Figure 16.4).

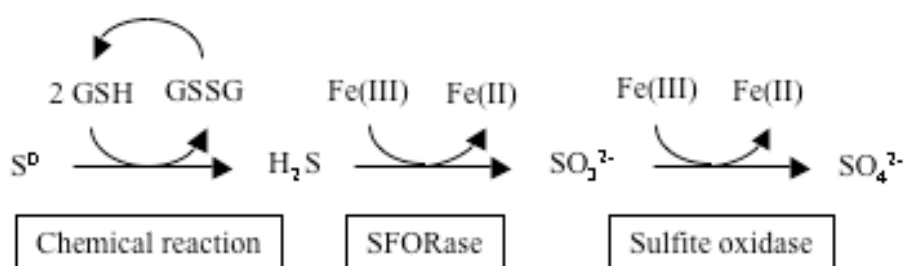


Figure 16.4. Model proposed by Sugio et al. (1989) for the oxidation of S⁰ coupled to the reduction of FeIII. SFORase: hydrogen sulfide:FeIII oxidoreductase; GSH: glutathione; GSSG: oxidized glutathione.

Anaerobic S⁰ oxidation coupled to FeIII reduction was shown to be an energy-transducing process (Pronk et al., 1991; Pronk et al., 1992). The pathway of electrons in S⁰ oxidation seems to be the same whether FeIII (anaerobiosis) or oxygen (aerobiosis) are the terminal electron acceptors (Corbett and Ingledew, 1987). A pathway for aerobic and anaerobic oxidation of formate and S⁰ was proposed (Figure 16.5) (Corbett and Ingledew, 1987; Pronk et al., 1991). In this model, the electrons from formate and S⁰ are transported to oxygen, or FeIII, via a *bc*₁ complex. The oxidoreductase involved in the reduction of FeIII is proposed to be that which catalyzes the aerobic oxidation of FeII. Finally, different terminal oxidases are

proposed to be involved in the reduction of oxygen to water.

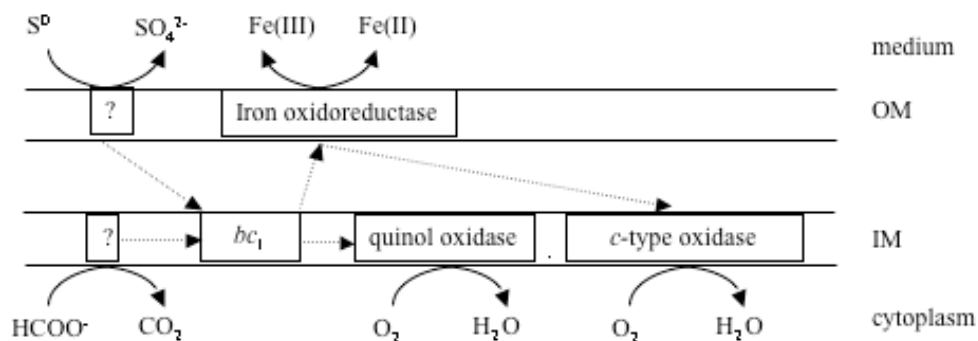


Figure 16.5. Proposed model for the aerobic and anaerobic electron pathway in S^0 and formate oxidation. Dotted lines represent the electron transport. Adapted from Pronk et al. (1991).

Ohmura et al. (2002) have shown that some *At. ferrooxidans* strains are able to grow under anaerobic conditions using FeIII or S^0 as electron acceptor and hydrogen as electron donor. Furthermore, a 28 kDa cytochrome *c* has been shown to be synthesized in the cells growing by respiring FeIII, but not S^0 , with either hydrogen or S^0 as electron donor. However, the type strain ATCC23270 was shown to be unable to grow on hydrogen by the anaerobic reduction of FeIII.

16.5 IRON OXIDATION IN OTHER BIOLEACHING MICROORGANISMS.

16.5.1 Introduction.

Although *At. ferrooxidans* remains the paradigm for understanding acidophilic FeII and S^0 oxidation in acidic conditions, evidence for electron transport proteins involved in these two processes is beginning to be accumulated for other microorganisms involved in bioleaching or close relatives of these microorganisms (Table 16.1).

FeII	→	FeIII	S ⁰	→	SO ₄
<i>Acidithiobacillus ferrooxidans</i>			<i>Acidithiobacillus. ferrooxidans</i>		
<i>Leptospirillum spp.</i>			<i>Acidithiobacillus thiooxidans</i>		
<i>Ferroplasma acidarmanus</i>			<i>Acidithiobacillus caldus</i>		
<i>Acidimicrobium ferrooxidans</i>			<i>Alicydobacillus tolerans</i>		
<i>Ferromicrobium acidophilus</i>			<i>Sulfolobus sp.</i>		
<i>Alicydobacillus tolerans</i>			<i>Thermoplasma acidophilum</i>		
<i>Sulfolobus metallicus</i>			<i>Picrophilus oshimae</i>		
<i>Metallosphaera sp.</i>			<i>Sulfobacillus acidophilus</i>		
<i>Acidianus sp.</i>			<i>Metallosphaera sp.</i>		
			<i>Acidianus sp.</i>		

Table 16.1. Some of the microorganisms involved in bioleaching that can oxidize iron and/or sulfur.

More than a decade ago the acidophilic bacteria *At. ferrooxidans*, *L. ferrooxidans*, an unidentified iron-oxidizing bacterium (m1) and two archaea (*Sulfobacillus metallicus* and *Metallosphaera sedula*) were shown to contain spectrally-distinct redox active proteins during autotrophic growth on soluble FeII (Barr et al., 1990; Blake et al., 1992; Blake et al., 1993). This suggested that different pathways or variations of pathways for FeII oxidation have evolved, an idea that is largely supported by recent bioinformatic analysis of sequenced or partially sequenced genomes of several acidophilic bacteria and archaea, coupled with experimental evidence in a few cases.

16.5.2 *Ferroplasma* spp.

An electron transport chain involved in iron oxidation has been proposed for *Ferroplasma* Type II and “*F. acidarmanus*” Fer1 based on an analysis of sequence information derived from an environmental genome shotgun library (Tyson et al., 2004; and reviewed in Golyshina and Timmis, 2005). Genes coding for putative haem-copper terminal oxidases, cytochrome *b*, an associated Rieske iron sulfur proteins and blue copper proteins were predicted. The blue copper protein had sequence similarity with rusticyanin of *At. ferrooxidans* and sulfocyanin (SoxE) of *Sulfolobus acidocaldarius*. It was suggested that in *Ferroplasma* Type II, these proteins formed a terminal oxidase supercomplex similar to the SoxM supercomplex of *Sulfolobus acidocaldarius*. However,

this view is not entirely supported from spectral studies of membrane proteins and proteomic analysis of *Ferroplasma* strains (Dopson et al., 2005). In addition, some genes of a SoxM complex are missing from the nearly complete genome of *F. acidarmanus*' Fer1 and the complete genome of *Ferroplasma* Type II.

A preliminary model for electron transport for "*F. acidarmanus*" Fer1 has been proposed by Dopson et al. (2005; Figure 16.6). This model was based on inhibitor studies, spectral information and differential protein expression. Several key differences between this scheme and the one proposed for *At. ferrooxidans* (Figure 16.1) can be detected. Firstly, the absence in *Ferroplasma* of an "uphill" electron pathway from FeII to the NADH ubiquinone oxidoreductase complex. Presumably, this pathway is not required because low potential electrons can be derived from the oxidation of organic compounds in *Ferroplasma* and fed directly into the NADH ubiquinone oxidoreductase complex to generate reducing power. Secondly, *Ferroplasma* lacks the outer membrane cytochrome Cyc2, proposed to be the primary catalyst of FeII oxidation in *At. ferrooxidans*. The role of Cyc2 is suggested to be assumed by the blue copper protein sulfocyanin. *Ferroplasma* is an archaeon and lacks the outer membrane found in Gram-negative bacteria such as *At. ferrooxidans*. Therefore, direct contact with insoluble substrates such as pyrite or soluble FeII could presumably be made by the blue copper protein embedded in the cell envelope. Also, in *At. ferrooxidans* the cytochrome c_4 (Cyc1) is proposed to connect rusticyanin with the terminal oxidase whereas it is absent in *Ferroplasma* and the blue copper protein connects directly with the terminal oxidase. Thus, the "downhill" electron components connecting FeII oxidation to the terminal oxidase are fewer in *Ferroplasma* (one protein) than in *At. ferrooxidans* (three proteins). Finally, the cytochrome bc_1 complex of *At. ferrooxidans* postulated to be involved in "downhill" electron flow of electrons during S^0 oxidation has been replaced by a cytochrome b /FeS complex with similarity to the cytochrome b and Rieske protein of *At. ferrooxidans* but lacking the cytochrome c_1 of the complex.

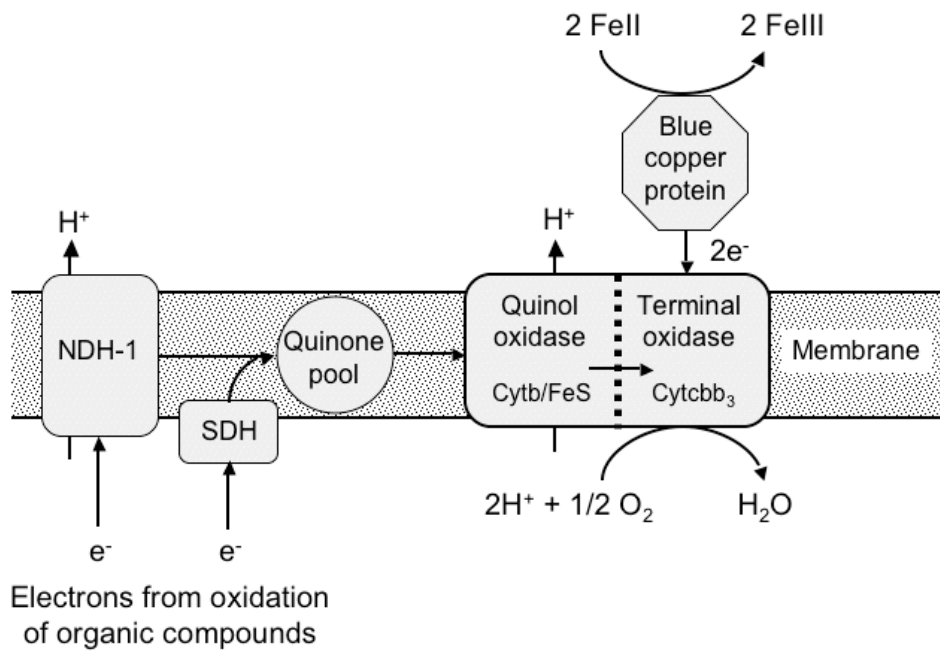


Figure 16.6. Model for electron transport during chemotrophic or chemomixotrophic growth of “*F. acidarmanus*” Fer1 (redrawn after Dopson et al., 2005). NDH-1, NADH ubiquinone oxidoreductase; SDH, succinate dehydrogenase.

16.5.3 *Leptospirillum* spp.

Leptospirillum spp. belong to the deep-branching *Nitrospirae* phylum, and consist of three main groups represented by: *L. ferrooxidans* (Group I), *L. ferriphilum* (Group II) and *L. ferrodiazotrophum* (Group III) (Tyson et al., 2005). Based on the almost complete genome sequence of a *Leptospirillum* Group II-like bacterium derived from an environmental genome shotgun library, Tyson et al. (2004) reported the discovery of putative genes potentially encoding a red cytochrome, a cytochrome *cbb*₃-type haem-copper terminal oxidase, a cytochrome *b* and an associated FeS-containing protein and a cytochrome *bd*-type quinol oxidase. A preliminary electron transport chain for *Leptospirillum* group II was proposed for FeII oxidation including both a “downhill” respiration and an “uphill” NADH synthesis electron flow (Figure 16.6). Most notably there is an absence of a blue copper protein corresponding to rusticyanin of *At. ferrooxidans* or sulfocyanin of *Ferroplasma*. The initial oxidation of FeII is suggested to occur via the red cytochrome which is positioned, according to the scheme of Tyson et al. (2004), within the periplasmic space. This is an unlikely location for the initial oxidation of FeII because of the insolubility of iron substrates such as pyrite, possible problems with

spontaneous oxidation of soluble FeII within the cell and the potential problems that would be caused by precipitation of FeIII within the periplasm. Interestingly, a metaproteomic analysis of the community revealed the presence of copious quantities of this red cytochrome in the biofilm and it was suggested to play an important role in FeII oxidation in the community (Ram et al., 2005)

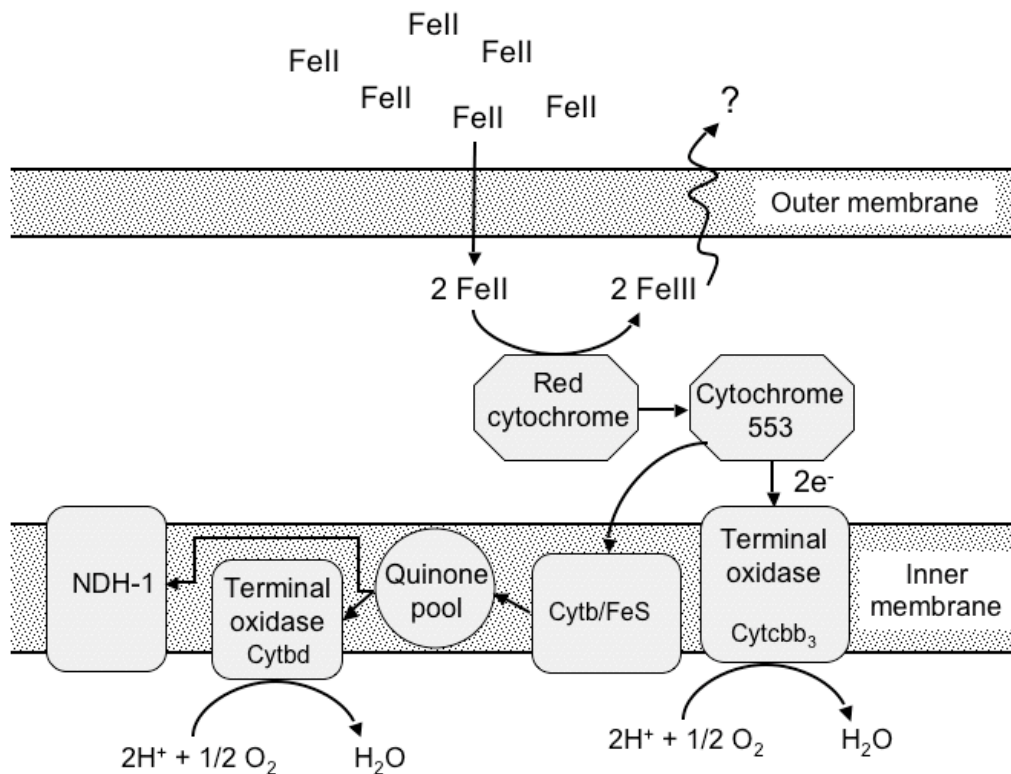


Figure 16.7. Preliminary model of iron oxidation in *Leptospirillum* Group II, redrawn according to Tyson et al. (2004). NDH-1, NADH ubiquinone oxidoreductase.

Uphill electron flow is postulated to occur via a cytochrome *b*/FeS complex, similar to the *bc*₁ complex of *At. ferrooxidans* but lacking the cytochrome *c*₁ component. This then feeds electrons to a quinone pool and subsequently to a NADH ubiquinone oxidoreductase as has been proposed for *At. ferrooxidans*. Although not discussed by Tyson et al. (2004), implicit in this model is that the energy for “uphill” electron flow comes from the PMF as has been postulated for *At. ferrooxidans*. Clearly, additional bioinformatic analysis of potential genes and pathways involved in FeII oxidation coupled with experimental validation is now required.

The dissimilarity of the components of the FeII oxidation electron transfer pathways between *At. ferrooxidans* and *Leptospirillum* Group II could account for the observed

differences in their FeII oxidation capabilities in bioleaching operations. Optimum bioleaching efficiency was obtained at lower substrate concentrations with *L. ferrooxidans* than with *At. ferrooxidans* (Sand et al., 1992). This may be explained by the greater affinity for FeII of *L. ferrooxidans* (K_m 0.25 mM) compared to *At. ferrooxidans* (K_m 1.34 mM), implying that its FeII-oxidizing system needs less substrate for saturation than the system of *At. ferrooxidans* (Norris et al., 1988). Also, the tolerance of *L. ferrooxidans* to FeIII is significantly greater than that of *At. ferrooxidans* (Norris et al., 1988).

Furthermore, while oxidation of Fe^{+2} by *At. ferrooxidans* was possible only at redox potentials of up to +850 mV, FeII oxidation by *Leptospirillum* was able to occur at redox potentials of up to +950 mV (Boon et al., 1999) This accounts for the observation that *At. ferrooxidans* can outgrow *L. ferrooxidans* at high ratios of FeII to FeIII which occurs during the earlier stages of FeII oxidation in bioleaching, but that *L. ferrooxidans* outcompetes *At. ferrooxidans* once the FeIII concentration becomes high (Rawlings et al., 1999). An additional explanation that could play a role in accounting for the reduced FeII oxidation capabilities of *At. ferrooxidans* at high FeIII concentrations is that this microorganism possesses more predicted FeII and FeIII uptake complexes than *Leptospirillum* spp., perhaps rendering it more susceptible to higher FeIII concentrations (Quatrini et al., 2005b).

16.5.4 Metallosphaera sedula.

A recent paper provides an initial glimpse at the proteins involved in electron transport in the thermoacidophilic Crenarchaeon *Metallosphaera sedula* (Kappler et al., 2005). Respiratory complexes were investigated when grown heterotrophically or chemolithotrophically on either S^0 or pyrite. Gene clusters, encoding two terminal oxidase complexes, a quinol oxidase SoxABCD and a SoxM oxidase supercomplex, were detected; the former is upregulated in cells grown on S^0 and the latter is upregulated when cells are grown on yeast extract. Both terminal oxidase complexes are downregulated when the cells are grown on pyrite but there appear to be oxidase-associated haems in these conditions, suggesting the presence of additional, as yet uncharacterized, genes encoding terminal oxidases perhaps involved in FeII oxidation. A gene cluster encoding a high redox-potential membrane-bound cytochrome *b* and components of a bc_1 complex system were also detected. The cytochrome *b* is strongly upregulated when cells are grown on pyrite compared to yeast extract, suggesting a role for this protein in FeII oxidation. This cytochrome *b* is not co-transcribed with the bc_1 complex genes and it was

suggested that made it unlikely to be part of the bc_1 complex. No mention of the possible presence of a blue copper protein was made in the report. Further work is required to firmly establish its presence or absence.

16.5.5 Sulfur oxidation in other bioleaching microorganisms.

Substantial progress has been made in understanding sulfur oxidation in a wide range of bacteria and archaea including some known to be involved in bioleaching such as “*F. acidarmanus*” or close relatives of known bioleaching microorganisms such as *Acidianus ambivalens* and the reader is directed towards an excellent recent review that covers current knowledge of prokaryotic sulfur oxidation (Friedrich et al., 2005).

16.6 OUTSTANDING QUESTIONS AND FUTURE DIRECTIONS.

Of all the major energetic pathways in nature, FeII oxidation is perhaps the least understood. One of the reasons for this lacuna in our knowledge is the apparent diversity of proteins that can extract electrons from iron and the multiplicity of ways to subsequently feed them into energy-yielding pathways. This would suggest that biological FeII oxidation has evolved separately many times. However, future work might reveal common underlying mechanisms such as the use of multiheme cytochromes and small copper proteins that could be homologous members of multifamily proteins. If this proves to be the case, then FeII oxidation might have evolved just a few times and the apparent diversity of pathways results from variations on a limited number of themes. This issue will become clearer as more genomes are sequenced.

More research is needed to understand FeIII reduction processes and how these might impact dump and heap bioleaching where at times, or in specific locations, there might be an inadequate supply of air to support biooxidation. Nor is enough known about S^0 oxidation and S^0 reduction to suggest ways that might prevent passivation of mineral surfaces by S^0 deposits resulting from mesophilic bacterial activity.

Cross-species genome analysis is already beginning to impact our understanding of bioleaching as shown, for example, by the discovery of possible reasons why *Leptospirillum* is outcompeted by *At. ferrooxidans* in early stages of a bioleaching operation but how, at later stages, it is able to continue biooxidation at higher FeIII loads that inactivate *At. ferrooxidans*.

An exciting potential of metagenomics is to provide community-wide assessment of metabolic and biogeochemical function. Analysis of specific functions across all members of a community can generate integrated models about how organisms share the workload of maintaining the nutrient and energy budgets of the community. The models can then be tested with genetic and biochemical approaches. The best example of such an analysis is the nearly complete sequencing of the metagenome of a community in acid drainage of the Richmond Iron Mountain mine (Tyson et al., 2005). The metagenomic sequence challenged a number of significant hypotheses. First, it appears that *Leptospirillum* group III contains genes with similarity to those known to be involved in nitrogen fixation, suggesting that it provides the community with fixed nitrogen. This was a surprise because the previous supposition was that a numerically dominant member of the community, such as *Leptospirillum* group II, would be responsible for nitrogen fixation. However, no genes for nitrogen fixation were found in the *Leptospirillum* group II genome, leading the authors to suggest that the group III organism is a keystone species that has a low numerical representation but provides a service that is essential to community function.

Furthermore, the prevailing idea that *Ferroplasma* strains, including those found at Iron Mountain, can fix CO₂ has been challenged (Dopson et al., 2005). If it turns out that they are organomixotrophs incapable of fixing CO₂ then some other member such as *Leptospirillum* must be providing them with fixed carbon.

Lessons learnt from the Iron Mountain metagenomic project can be applied to further our understanding of bioleaching. For example, it is already known that tank biooxidation (reviewed in Rawlings, 2005) and heap bioleaching (Demergasso et al., 2005) proceed in three stages, resulting from temperature increases due to exothermic biological oxidation of FeII and S⁰: an early stage favoring mesophilic microorganisms (30-40⁰C) such as *At. ferrooxidans*, *At. thiooxidans*-like bacteria and *Sulfurisphaera*-like archaea; a second stage when the temperature begins to rise (40-55⁰C) when *At. caldus*, *Leptospirillum* and *Ferroplasma* groups become dominant and a final stage (55-65⁰C or higher) where *Sulfobacillus*- and *Alicyclobacillus*-like bacteria (Karavaiko et al., 2005) became dominant and archaea such as *Ferroplasma* thrive. This means that the development and interaction of each of these microbial communities, including possible community biofilm formation in the case of heap bioleaching, must be considered in order to comprehend bioleaching processes and suggest ways by which bioleaching can be improved.

16.7 ACKNOWLEDGEMENTS.

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