Progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation—part A

Mario Vera · Axel Schippers · Wolfgang Sand

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Abstract Bioleaching of metal sulfides is performed by a diverse group of microorganisms. The dissolution chemistry of metal sulfides follows two pathways, which are determined by the mineralogy and the acid solubility of the metal sulfides: the thiosulfate and the polysulfide pathways. Bacterial cells can effect this metal sulfide dissolution via iron(II) ion and sulfur compound oxidation. Thereby, iron(III) ions and protons, the metal sulfide-attacking agents, are available. Cells can be active either in planktonic state or in forming biofilms on the mineral surface; however, the latter is much more efficient in terms of bioleaching kinetics. In the case of Acidithiobacillus ferrooxidans, bacterial exopolymers contain iron(III) ions, each complexed by two uronic acid residues. The resulting positive charge allows an electrostatic attachment to the negatively charged pyrite. Thus, the first function of complexed iron(III) ions is the mediation of cell attachment, while their second function is oxidative dissolution of the metal sulfide, similar to the role of free iron(III) ions in non-contact leaching. In both cases, the electrons extracted from the metal sulfide reduce molecular oxygen via a redox chain forming a supercomplex spanning the periplasmic space and connecting both outer and inner membranes. In this review, we summarize some recent discoveries relevant to leaching bacteria which contribute to a better understanding of these fascinating microorganisms. These include surface science, biochemistry of iron and sulfur metabolism, anaerobic metabolism, and biofilm formation. The study of microbial interactions among multispecies leaching consortia, including cell-to-cell communication mechanisms, must be considered in order to reveal more insights into the biology of bioleaching microorganisms and their potential biotechnological use.

Keywords Bioleaching · Acidithiobacillus · Metal sulfides · Extracellular polymeric substances

Introduction

Understanding and application of bacterial leaching of metal sulfides has developed rapidly in the course of the last two decades. The mobilization of metal cations from often almost insoluble ores by biological oxidation and complexation processes is referred to as bioleaching. The recovery of heavy metals by an application of microorganisms is now a worldwide established biotechnological process. Metals for which this technique is mainly employed include copper, cobalt, nickel, zinc, and uranium. These are extracted either from insoluble sulfides or—in the case of uranium—from oxides. However, for the recovery of gold and silver, the activity of leaching bacteria is applied only to dissolve interfering metal sulfides from ores bearing the precious metals prior to cyanidation treatment. Here, the term biooxidation is used preferentially because the bioleached solubilized metals, in most cases iron and arsenic, are not intended to be recovered. A general term to cover both bioleaching and biooxidation techniques is “biomining” (Bosecker 1997; Rawlings 1997; Ehrlich 2009; Olson et al. 2003; Rohwerder et al. 2003). Emerging possibilities from developments in the fields of molecular biology, “omics” techniques, chemical analysis, and surface science (nanobiotechnology) have contributed to an improved understanding of this bioprocess. Nevertheless, which processes are actually occurring at the molecular scale at bacterial–mineral interfaces is still unknown.
Especially in the case of bioleaching, the use of thermophiles has been shown to have considerable advantages since industrial processes like tank leaching suffer from costs caused by cooling (in processes using mesophilic bacteria). Also, the problem of acid mine/rock drainage (ARD/AMD), mainly caused by psychrophilic and mesophilic leaching bacteria, must be considered since in many countries environmental legislation forces companies to apply effective countermeasures or at least to reduce its impact on the environment (Johnson and Hallberg 2005). However, improved AMD countermeasures can be developed only if the basis of microbial interactions is thoroughly understood. The (bio)chemical fundamentals of the leaching reactions have been the subject of intensive research in the last 30 years. In this context, the sulfur chemistry behind the leaching mechanisms has been solved (Schippers et al. 1995; Schippers and Sand 1999; Rohwerder and Sand 2003). Furthermore, it is now generally accepted that the originally discussed “direct mechanism” of biological metal sulfide oxidation, i.e., the direct enzymatic oxidation of the sulfur moiety of heavy metal sulfides (Sand et al. 1995; Ehrlich 2009), does not exist. The “indirect mechanism”, i.e., the non-enzymatic metal sulfide oxidation by iron(III) ions combined with an enzymatic (re)oxidation of the resulting iron(II) ions, remains. There are two leaching modes: “contact” and “non-contact” leaching (Sand et al. 2001; Rawlings 2002). Non-contact leaching is basically exerted by planktonic bacteria, which oxidize iron(II) ions in solution. The resulting iron(III) ions (somehow) come into contact with a mineral surface, where they are reduced and the sulfide moiety is oxidized. Thus, iron(II) ions enter the cycle again. In a strict sense, this is in effect the previously designated indirect mechanism (Sand et al. 1995). Contact leaching takes into account that most cells attach to the surface of sulfide minerals. This means that the electrochemical processes resulting in the dissolution of sulfide minerals take place at the interface between the bacterial cell and the mineral sulfide surface. This space is filled with extracellular polymeric substances (EPS). However, even after several years of research, many open questions remain. In both contact and non-contact leaching, the bacteria contribute to mineral dissolution by the generation of the oxidizing agent, the iron(III) ions, and by a subsequent oxidation of the released sulfur compounds arising from the metal sulfide to sulfuric acid. What is still missing is the analysis of interactions at the strain and also at the species/genus level in bioleaching communities. Cell-to-cell communication systems of “quorum sensing” (QS) have been described to be present in some leaching bacteria (Farah et al. 2005; Ruiz et al. 2008; González et al. 2012), but their importance for the whole process becomes obvious only in fragments. A detailed knowledge of the interactions among the microorganisms in leaching environments, including elucidation of the role of the known bacterial communication mechanisms and identification of still unknown potential cell–cell communication signals, may be a future option for further process optimization. As a consequence, this review, which is based on three previous ones (Sand et al. 1995, 2001; Rohwerder et al. 2003), needed considerable revision.

Diversity among leaching microorganisms

The predominant metal sulfide-dissolving microorganisms are extremely acidophilic bacteria and archaea (meaning organisms thriving at pH values below 3) that are able to oxidize either inorganic sulfur compounds and/or iron(II) ions. Several recent reviews have given a broad view of the microbial diversity within mining biotopes (Schippers et al. 2010; Hedrich et al. 2011; Johnson 2011; Dopson and Johnson 2012). Leaching bacteria are distributed among the Proteobacteria (Acidithiobacillus, Acidiphilium, Acidiferrobacter, Ferrovum); Nitrospirae (Leptospirillum); Firmicutes (Alicyclobacillus, Sulfolabillus); and Actinobacteria (Ferrimicrobium, Acidimicrobium, Ferriithrix). Within all groups, mesophilic as well as moderately thermophilic microorganisms can be found (Clark and Norris 1996; Norris et al. 2000). Leaching archaea mostly belong to the Sulfolobales, a group of extremely thermophilic, sulfur and iron(II) oxidizers including genera such as Sulfolobus, Acidianus, Metallosphaera, and Sulfrusphaera (Norris et al. 2000). Also, within the Thermoplasmales, two iron(II)-oxidizing species, Ferroplasma acidiphilum (Golyshina et al. 2000) and Ferroplasma acidarmanus (Edwards et al. 2000), are known.

The first isolated and best-studied iron and/or sulfur-oxidizing bacterium is Acidithiobacillus ferrooxidans, formerly Thiobacillus ferrooxidans (Kelly and Wood 2000). Physiological and genetic data pointed out the diversity of T. ferrooxidans, for which 23 strains were classified in seven subgroups based on DNA–DNA hybridization patterns (Harrison 1982). Recently, 21 At. ferrooxidans strains have been analyzed using molecular techniques such as multilocus sequence analysis. This study revealed the existence of four subgroups that could correspond to different species (Amouric et al. 2010). One of these subgroups includes the Acidithiobacillus ferrivorans strains (Hallberg et al. 2009), whose psychrophilic growth abilities and motility may cause them to be the predominant microorganisms in low-temperature leaching environments (Liljeqvist et al. 2011, 2012). Such analyses will allow the elucidation of several new species in the future, contributing to a better understanding of the microbial diversity within leaching environments. Recently, strain m-1, for a long time classified as At. ferrooxidans, has been proposed to belong to the
new genus *Acidiferrobacter* among the proteobacterial division and closely related to the alkaliphilic *Ectothiorhodospira* spp. It was shown that this strain is a “moderate osmophile” (Hallberg et al. 2011b).

A large diversity has been found with respect to carbon assimilation pathways among leaching bacteria. *Acidithiobacillus* spp. and *Leptospirillum* spp. can grow only chemolithoautotrophically. In contrast, *Acidiphilium acidophilum* and *Acidimicrobium ferrooxidans* are able to grow autotrophically with reduced sulfur compounds and iron(II) ions, heterotrophically with glucose or yeast extract, and mixotrophically with all of these substrates (Clark and Norris 1996; Hiraishi et al. 1998). In addition, several *Acidiphilium* spp. and *Acidisphaera rubrifaciens* possess pigments that may confer the ability for some photosynthetic activity (Hiraishi et al. 2000; Hiraishi and Shimada 2001). Inorganic phosphate (Pi) is essential for all living cells. Leaching bacteria must be able to deal with the problem of Pi scarcity due to precipitation in environments, where Fe(III) ions are present. It has been shown that *At. ferrooxidans* possesses a “Pho” genetic response system, probably involved in scavenging traces of Pi from the environment (Vera et al. 2003). This bacterium is also able to use alternative Pi sources such as phosphonates, which might confer it a selective growth advantage by providing access to a unique Pi pool (Vera et al. 2008). *At. ferrooxidans* as well as several other bioleaching microorganisms accumulate high levels of inorganic polyphosphate, which, among its several functions, can serve as Pi reservoir, modulate stress responses, as well as being a contributing factor to the high copper resistance shown by these microorganisms (Alvarez and Jerez 2004; Remonsellez et al. 2006; Orell et al. 2010, 2012).

*At. ferrooxidans* is also endowed with a remarkably broad metabolic capacity. This species lives on the oxidation of iron(II) ions and/or reduced inorganic sulfur compounds and, in addition, is able to oxidize molecular hydrogen, formic acid, and other metal ions. Anaerobic growth is possible by oxidation of sulfur compounds or hydrogen coupled with iron(III) reduction (Das et al. 1992; Pronk et al. 1992; Johnson et al. 2012; Osorio et al. 2013). Also, one strain showed a capacity to reduce elemental sulfur in the course of anaerobic hydrogen oxidation (Ohmura et al. 2002). In *At. ferrooxidans*, the utilization of electron acceptors other than oxygen is reflected by the presence of various electron transport components. For example, at least 11 different cytochromes of the c type have been identified in the genome of *At. ferrooxidans* (Yarzabal et al. 2002a). Recently, a transcriptomic and proteomic study suggested that iron reduction in anaerobic growth of *At. ferrooxidans*, when using sulfur as the electron donor, can be explained partially due to an indirect mechanism in which sulfur was disproportionated, forming H2S and sulfate. Consequently, the generated H2S could be responsible for the reduction of iron(III) ions under acidic conditions. The presence of an alternative mechanism, probably involving the transfer of electrons from sulfur to iron(III) ions via a respiratory chain, which would explain their reduction when using H2 as an electron donor, was also suggested (Osorio et al. 2013).

The fact that many leaching bacteria control the complete aerobic and anaerobic components of the sulfur and iron cycles could be especially of great importance for AMD treatment (Schippers et al. 2001; Johnson and Hallberg 2005; Sand et al. 2007). If natural bioleaching in waste heaps and tailings is stopped by flooding or with organic covers (both common AMD countermeasures that create an anoxic environment), leaching bacteria could remain active due to their anaerobic capacities. Several other acidophiles have been reported to grow via dissimilatory reduction of iron(III) ions by using inorganic electron donors, as in the case of *At. ferrooxidans*, or organic electron donors in the case of *Acidiphilium* and *Ferrophilum* (Johnson et al. 2012). A big diversity of Gram-positive strains related to *Acidimicrobium* and *Ferrimicrobium* has been found in enrichment cultures from sulfide mine dumps, which partially represent novel species (Schippers et al. 2010). The “anaerobic biomining” of metal sulfides at low pH has been demonstrated to be feasible with *At. ferrooxidans* for bioleaching of nickel limonites, in which the metal is associated to ferric oxohydroxides such as goethite (FeOOH; Hallberg et al. 2011a). This concept of bioreductive leaching may be applicable for oxide ores and has already been used for a process called “Ferreodox” (du Plessis et al. 2011). Most of the known acidophilic iron oxidizers are inhibited by high chloride concentrations, which is a problem relevant for mining operations performed in desert areas such as Chile and Australia, where freshwater resources are scarce. Attempts to obtain leaching at high salt concentrations as well as studies of the response of leaching organisms to increased amounts of chloride ions have been reported (Zammit et al. 2011). Recently, a microbiological survey in sulfidic mine tailings in the north of Chile has revealed the existence of halotolerant, acidophilic iron oxidizers active at concentrations up to 1 M NaCl. This finding may open a possibility for developing biomining processes with these halotolerant species (Korehi et al. 2013).

**Bioleaching mechanisms**

The mechanisms of bioleaching have been intensively discussed in the past. In the older literature, “direct” vs. “indirect” bioleaching is described (Rossi 1990; Bosecker 1997; Ehrlich 2009). Direct leaching means a direct electron transfer from the metal sulfide to the cell attached to the mineral surface. Indirect leaching proceeds via the metal sulfide-oxidizing agent, iron(III) ions, which are generated.
by iron(II)-oxidizing bacteria either planktonic or attached to the mineral surface. Since a direct electron transfer via enzymes, nanowires, etc., between the metal sulfide and the attached cell has not been demonstrated, a direct mechanism does not seem to exist. Instead, attached cells provide an efficient EPS-filled reaction compartment for indirect leaching with iron(III) ions (Sand et al. 1995, 2001). Thus, to improve the understanding of these processes, the terms “contact leaching” and “non-contact leaching” have been proposed for bioleaching by attached and planktonic cells, respectively. A third term, “cooperative leaching,” describes the dissolution of sulfur colloids, sulfur intermediates, and mineral fragments by planktonic cells (Tributsch 2001; Rawlings 2002). These new terms may be useful for a description of the physical status of cells involved in bioleaching, but they do not tell us anything about the underlying chemical mechanisms of biological metal sulfide dissolution. Metal sulfide oxidation can be described by two different pathways, namely, the thiosulfate mechanism and the polysulfide mechanism (Fig. 1), which are in fact chemical pathways (Schippers et al. 1996, 1999; Schippers and Sand 1999; Sand et al. 2001). The formation of the intermediate sulfur compounds in the two reaction pathways depends on the mineralogy of the metal sulfide and the geochemical conditions in the environment, mainly the pH and the presence of different oxidants (Schippers 2004). Microorganisms play a crucial role in the oxidation of intermediate sulfur compounds, which are formed by the chemical dissolution of the metal sulfides. Under oxic and acidic conditions relevant for bioleaching, microorganisms oxidize Fe(II) to Fe(III) ions, which serve as oxidants for the metal sulfides and for most of the intermediate sulfur compounds. Additionally, microorganisms may catalyze the oxidation of intermediate sulfur compounds to sulfuric acid.

Metal sulfides are conductors, semiconductors, or insulators, and their metal and sulfur atoms are bound in the crystal lattice (Vaughan and Craig 1978; Xu and Schoonen 2000). According to the molecular orbital and valence band theory, the orbitals of single atoms or molecules form electron bands with different energy levels. The metal sulfides FeS2 (pyrite), MoS2 (molybdenite), and WS2 (tungstenite) consist of pairs of sulfur atoms (Vaughan and Craig 1978) which form nonbonding orbitals. Consequently, the valence bands of these metal sulfides are only derived from orbitals of metal atoms, whereas the valence bands of all other metal sulfides are derived from both metal and sulfur orbitals (Borg and Dienes 1992). Thus, the valence bands of FeS2, MoS2, and WS2 do not contribute to the bonding between the metal and the sulfur moiety of the metal sulfide. This fact explains the resistance of these metal sulfides against a proton attack. The bonds can only be broken via multistep electron transfers with an oxidant like the iron(III) ion. For the other metal sulfides, in addition to an oxidant like iron(III) ions, protons can remove electrons from the valence band, causing a cleavage of the bonds between the metal and the sulfur moiety of the metal sulfide. Consequently, these metal sulfides are relatively soluble in acid, whereas FeS2, MoS2, and WS2 are insoluble (Singer and Stumm 1970; Tributsch and Bennett 1981a, b; Crundwell 1988; Rossi 1993; Sand et al. 2001).

Based on the existence of two different groups of metal sulfides, two different metal sulfide oxidation mechanisms have been proposed (Schippers et al. 1996, 1999; Schippers and Sand 1999; Sand et al. 2001). These mechanisms are able to explain the occurrence of all inorganic sulfur compounds, which have been documented for bioleaching environments.

Pyrite and other acid-non-soluble metal sulfides: the thiosulfate pathway

Among metal sulfides, FeS2 and its oxidation is the most studied. For reviews, see (Dutrizac and MacDonald 1974; Lowson 1982; Nordstrom 1982; Evangelou 1995; Rimstidt and Vaughan 2003; Schippers 2004; Druschel and Borda 2006). It will be used as a representative for the three metal sulfides—FeS2, MoS2, and WS2—here. After initial attack of the oxidant Fe(III) ion, the sulfur moiety of pyrite is oxidized to soluble sulfur intermediates. Moses et al. (1987) and Luther (1987) presented a detailed reaction mechanism for FeS2 dissolution by iron(III) ions, in which thiosulfate is the first soluble sulfur intermediate. According to this mechanism, hydrated iron(III) ions oxidize the disulfide moiety of FeS2 to a sulfonic acid group by several electron extractions. Due to this transformation, the bonds between iron and the two sulfur atoms are cleaved and hydrated iron(II) ions and thiosulfate are formed. Thiosulfate, as the first soluble sulfur compound intermediate, is then almost quantitatively oxidized to tetrathionate (Williamson and Rimstidt 1994; Schippers et al. 1996). Tetrathionate is further degraded to various sulfur compounds, i.e. trithionate, pentathionate, elemental sulfur, and sulfite (Schippers et al. 1996; Druschel 2002; Schippers 2004; Druschel and Borda 2006). These sulfur compounds are finally oxidized to sulfate in chemical and/or biological reactions. Overall, the thiosulfate pathway can be summarized by the following equations:

\[
\text{FeS}_2 + 6 \text{Fe}^{3+} + 3 \text{H}_2\text{O} \rightarrow \text{S}_2\text{O}_3^{2-} + 7 \text{Fe}^{2+} + 6 \text{H}^+ \quad (1)
\]

\[
\text{S}_2\text{O}_3^{2-} + 8 \text{Fe}^{3+} + 5 \text{H}_2\text{O} \rightarrow 2 \text{SO}_4^{2-} + 8 \text{Fe}^{2+} + 10 \text{H}^+ \\
(2)
\]

The stoichiometry of the thiosulfate pathway has been confirmed in bioleaching experiments with \textit{At. ferrooxidans}, in
which the stable isotopes of oxygen and sulfur were determined in the pyrite oxidation reaction products (Balci et al. 2007).

Most metal sulfides: the polysulfide pathway

In contrast to FeS$_2$ oxidation, the metal–sulfur bonds in the acid-soluble metal sulfides can be cleaved before the sulfidic sulfur is oxidized. These metal sulfides like As$_2$S$_3$ (orpiment), As$_4$S$_6$ (realgar), CuFeS$_2$ (chalcopyrite), FeS (troilite), Fe$_7$S$_8$ (pyrrhotite), MnS$_2$ (hauerite), PbS (galena), and ZnS ( sphalerite) can thus be dissolved by protons. At low pH, the sulfur moiety of these metal sulfides is oxidized mainly to elemental sulfur (Dutrizac and MacDonald 1974; Schippers and Sand 1999; McGuire et al. 2001). A series of reactions for acid-soluble metal sulfides inherently explain the formation of elemental sulfur via polysulfides (Schippers and Sand 1999), which have been detected during the dissolution of, e.g., Fe$_7$S$_8$ (Thomas et al. 1998, 2001), PbS (Smart et al. 2000), and CuFeS$_2$ (Hackl et al. 1995). Consequently, the oxidation mechanism for acid-soluble metal sulfides has been named polysulfide mechanism (Schippers and Sand 1999).

Although elemental sulfur is chemically inert in natural environments, it can be biologically oxidized to sulfuric acid. Overall, the polysulfide mechanism can be described by the following equations (Schippers and Sand 1999):

$$MS + Fe^{3+} + H^+ \rightarrow M^{2+} + 0.5 H_2S_n + Fe^{2+}(n \geq 2)$$ (3)

$$0.5 H_2S_n + Fe^{3+} \rightarrow 0.125 S_8 + Fe^{2+} + H^+$$ (4)

$$0.125 S_8 + 1.5 O_2 + H_2O \rightarrow SO_4^{2-} + 2 H^+$$ (5)

The polysulfide pathway is in agreement with the results of bioleaching experiments with *At. ferrooxidans*, in which the stable isotopes of oxygen and sulfur were determined in the products of chalcopyrite and sphalerite oxidation (Thurston et al. 2010; Balci et al. 2012).

Sulfur chemistry—implications for bioleaching kinetics

In both pathways, the main role of leaching bacteria consists of the regeneration of iron(III) ions—the most important oxidants in acidic biotopes (Fig. 1). Thus, the acidophilic iron(II) oxidizers control the redox potential in their environment, which is determined mainly by the iron(II)/iron(II) ratio in leaching solutions. Besides this, acidophilic sulfur oxidizers contribute to the transformation of the intermediary sulfur compounds to sulfuric acid (Schippers et al. 1999; Schippers and Sand 1999). In the case of elemental sulfur, oxidation is exclusively carried out by microorganisms because this sulfur species is inert to abiotic oxidation in acidic environments (Fig. 1b). Consequently, elemental sulfur may accumulate in the course of metal sulfide dissolution if sulfur-oxidizing microorganisms are absent or inhibited. In general, the production of sulfuric acid from reduced sulfur compounds is needed to regenerate protons consumed by the initial leaching processes via the polysulfide pathway (Fig. 1b). In addition, sulfur oxidizers can influence leaching kinetics in a particular manner. Elemental sulfur may occur suspended as free aggregates and crystals or can form a layer on the metal sulfide surface (Mustin et al. 1993; Fowler et al. 1999). In the latter case, the electrochemical properties of the metal sulfide surface might change and/or a barrier may be formed, reducing the diffusion rates for ions and oxygen. Both phenomena negatively influence leaching kinetics. Leaching rate decreasing sulfur layers were observed on acid-soluble sphalerite at low redox potentials in the absence of sulfur oxidizers (Fowler et al. 1999). Similar problems are known for chalcopyrite (Bevilaqua et al. 2002). In contrast, at high redox potentials [about 750 mV vs. standard hydrogen electrode (SHE)], no inhibiting sulfur layers were observed, either with acid-soluble or with the acid-insoluble metal sulfide sphalerite (Fowler and Crundwell 1998; Fowler et al. 1999). Although elemental sulfur was also formed in the latter two cases, it probably occurred only as free aggregate, which does not decrease leaching rates under pH-controlled conditions.

**Biochemistry of iron(II) oxidation**

Within the last years, more information has been obtained by analysis of the redox chains of aerobic iron(II)-oxidizing bacteria such as *At. ferrooxidans* and *Leptospirillum ferrooxidans* (Castelle et al. 2008, 2010; Blake and Griff 2012). Although most biochemical details are best known for *At. ferrooxidans*, on the basis of spectroscopic biochemical and “omics” analyses, it can be stated that the iron(II)-oxidizing systems of the other acidophilic iron-oxidizing bacteria are different with respect to the redox components used. There are at least 14 genera able to oxidize iron(II) ions with molecular oxygen as the electron acceptor. Within this diversity of microbial groups, it is not astonishing that different mechanisms exist (Bonnefoy and Holmes 2011; Blake and Griff 2012). It has been postulated that these biochemical differences in the respiratory chains could determine whether *At. ferrooxidans* or *L. ferrooxidans* are the dominant bacteria in various mining habitats such as leach dumps (underground), ore bodies, or bioreactors (Sand et al. 1992; Schrenk et al. 1998; Rawlings et al. 1999; Bond et al. 2000; Rawlings 2002). Consequently, clarification of these oxidizing systems is of industrial importance as it will affect
possible improvements in the use of microbes and/or the design of bioleaching plants. Boon and Hansford with co-workers (Hansford 1997; Boon et al. 1998) described the phenomenon that, in the case of experiments with *At. ferrooxidans*, iron(II) oxidation was possible only at redox potentials of up to +850 mV (vs. SHE, here and for all following redox values), whereas with *L. ferrooxidans*, iron(II) oxidation occurred at redox potentials of up to +950 mV. This finding must be related to the fact, as already described (Norris et al. 1988), that the inhibitory concentration of iron(III) ions is much lower for *At. ferrooxidans* (3.1 mM) than for *L. ferrooxidans* (42.8 mM). The reason must lie in the energy-conserving electron transport chain from iron(II) ions to molecular oxygen. It has been shown that *Leptospirillum* spp. possess two cytochromes, Cyt572 and Cyt579, which are proposed to be key proteins in aerobic iron(II) oxidation. In biofilms, both showed posttranslational modifications dependent on the maturation state of the biofilm, and Cyt579 showed sequence variants with decreased redox potentials (Singer et al. 2010). The aerobic iron respiratory chain of *L. ferrooxidans* was dominated by the redox status of an abundant cellular cytochrome that had an absorbance peak at 579 nm in the reduced state (Blake and Griff 2012). This helps explain the increased redox potential at which *L. ferrooxidans* can still oxidize iron (II) ions. Consequently, *L. ferrooxidans* has considerably lower oxidation and growth rates on iron(II) ions than *At. ferrooxidans* (Sand et al. 1992; Hallmann et al. 1993). Although this adaptation to high redox potentials is rather inefficient with respect to energy conservation, it has been proposed to explain the dominance of *Leptospirillum* strains found in bioleaching operations (Rawlings et al. 1999).

In the case of *At. ferrooxidans*, the functionality of the respiratory chain coupling iron(II) oxidation with oxygen reduction has been shown. The respiratory system is flexible and gene expression can be modulated according to the main energy source and the growth condition (Quatrini et al. 2009). Electrons from iron(II) ions can either be transported along a “downhill” or an “uphill” pathway (Bonnefoy and Holmes 2011). The first pathway allows for ATP synthesis, whereas the second one allows for the production of reducing power for biosynthetic reactions. The rus operon encodes the proteins involved in the “downhill” pathway, which includes two c-type cytochromes.

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**Fig. 1** Schematic comparison of the thiosulfate (a) and polysulfide (b) mechanisms in the (bio)leaching of metal sulfides (from Schippers and Sand 1999, modified). Iron(III) ions attack metal sulfides (MS) by electron extraction and are thereby reduced to the iron(II) ion form. As a result, the metal sulfide crystal releases metal cations (M²⁺) and water-soluble intermediary sulfur compounds. Iron(II)-oxidizing bacteria such as *At. ferrooxidans* (Af) and *L. ferrooxidans* (Lf) catalyze the recycling of iron(III) ions in acidic solutions. In the case of acid-soluble metal sulfides (b), an additional attack is performed by protons, which can bind the valence band electrons of these metal sulfides. The liberated sulfur compounds are oxidized abiotically and by sulfur-compound-oxidizing bacteria such as *At. ferrooxidans* and *At. thiooxidans* (At). In case of mainly abiotic reactions, the contribution of sulfur compound oxidizers is indicated in brackets. The main electron acceptors of oxidation reactions other than the initial iron(III) ion attack on the metal sulfide are given to the right of the arrows. The main reaction products that accumulate in the absence of sulfur compound oxidizers are boxed, i.e., sulfuric acid in (a) and elemental sulfur in (b). The equations given are not stoichiometric. For details, see text and Pronk et al. (1992), Schippers and Sand (1999), Rohwerder and Sand (2003), and Schippers (2004).
(Cyc1 and Cyc2), the blue copper protein rusticyanin (Rus), and an aa3-type cytochrome oxidase (CoxABCD; Appia-Ayme et al. 1999). The 46-kDa Cyc2, with a midpoint potential of +560 mV, is located in the outer membrane and functions as the primary electron acceptor in iron(II) oxidation (Yarzabal et al. 2002b, 2004). The electron is then transferred to the periplasmic cupredoxin rusticyanin and afterwards to the Cyc1 (a dihemic c4-type cytochrome), which hands over the electron to the aa3-type cytochrome oxidase. It has been shown that these proteins are organized in a supramolecular structure spanning the outer and inner membranes. This supercomplex has been proven to be functional since, after its purification in mild conditions, iron(II) oxidation as well as oxygen reduction activities were present (Castelle et al. 2008). Also, the proteins belonging to the bc1 complex (uphill pathway) as well as the cytochrome Cyc42 (c4-type) have been found in this supercomplex, suggesting a strong physical association of the “uphill and downhill” respiratory chains, where, as stated earlier, Rus could modulate the delivery of electrons to both chains. Rus is an essential component of the electron transport chain in *At. ferrooxidans*. An organism that survives on a substrate with such little energy, such as iron(II) ions, may contain up to 5 % of its total cell protein in the form of Rus (Cox and Boxer 1978). With a $\Delta G$ of only about $-30 \text{kJ/mol}$ available from iron(II) oxidation (with oxygen as electron acceptor at pH 2), *At. ferrooxidans* could not afford to produce several percent of its biomass as Rus, if it did not have an important function. It is accepted that Rus functions as an electron reservoir in *At. ferrooxidans*. Furthermore, this assumption also explains the redox dependence discussed above. As determined by Ingledew and Cobley (1980), Rus has a midpoint redox potential of $+680 \text{mV}$. As a consequence, it may take up electrons to become reduced up to potentials of around $+800 \text{mV}$. This agrees well with data presented by Boon et al. (1998), which are also supported by other studies (Meruane et al. 2002). Rus, due to its large concentration, could efficiently take up every electron that becomes available at the outer membrane and channel it into the downhill oxidation pathway. The primary electron acceptor (probably Cyc2) remains oxidized. Consequently, the driving force for iron(II) oxidation is at maximum (i.e., for a certain iron(II)/iron(III) ratio), the $\Delta G$ value of iron(II) oxidation is highest because the other redox partner, the electron acceptor Cyc2, is fully oxidized. This has the advantage that most electrons available from iron(II) ions can be collected, however, only in the redox range of Rus. This seems to be highly beneficial, especially when using such a low-energy substrate. It must be noted that among the Fe(II)-oxidizing acidithiobacilli, at least two different Fe(II) oxidation pathways exist. One is via RusA, working in *At. ferrooxidans*. The second pathway, present in some *At. ferrooxidans* and *At. ferrivorans* strains, includes a high potential iron–sulfur protein (HiPIP; Bruscella et al. 2005) and the rusticyanin isoenzyme RusB (Amouric et al. 2010; Bonnefoy and Holmes 2011).

Surface science—extracellular polymeric substances, attachment, and biofilm formation

In general, the majority of leaching bacteria grow attached to the surfaces of mineral sulfides. In case of a non-limiting surface space, more than 80 % of an inoculum can disappear from the solution within 24 h (Dispirito et al. 1983; Baghdijian and Myerson 1986; Gehrke et al. 1998; Harneit et al. 2006). Nevertheless, some cells remain in the planktonic state, even though the surface area (coverage below 5 %; own data) for attachment is not limiting. The reason for this is unknown. It is known that the attachment process is predominantly mediated by the EPS surrounding the cells. Attachment/surface contact stimulates EPS production (Vandevivere and Kirchman 1993; Bellenberg et al. 2012; Vera et al. 2013). In the case of *At. ferrooxidans* strain R1 and FeS$_2$, it was demonstrated that these EPS consist of the sugars glucose, rhamnose, fucose, xylose, mannose, C12–C20 saturated fatty acids, glucuronic acid, and iron(III) ions (Gehrke et al. 1998–2001). Primary attachment occurs mainly by electrostatic interactions between positively charged cells (actually, the EPS surrounding the cells, in which likely 2 mol negatively charged glucuronic acid residues complex 1 mol positively charged iron(III) ions, resulting in a net positive charge) with the negatively charged pyrite surface (at pH 2 in sulfuric acid solution; Solari et al. 1992; Blake et al. 1994). Also, hydrophobic interactions contribute somewhat to the attachment to metal sulfide surfaces (Gehrke et al. 1998; Sampson et al. 2000), although this applies especially to very hydrophobic surfaces, e.g. of elemental sulfur. Hydrophobic interactions as well as covalent bonds seem to mediate the secondary (tight) surface attachment. Cells grown on elemental sulfur do not attach to FeS$_2$ due to a considerably modified EPS composition in comparison to pyrite-grown cells. These EPS contain considerably less sugars and uronic acids, but much more fatty acids than EPS from pyrite-grown cells. The most important difference, however, is the total lack of complexed iron(III) ions or other positively charged ions. Consequently, exclusively hydrophobic interactions are relevant for the attachment of cells of *At. ferrooxidans* to sulfur (Gehrke et al. 1998). The molecular mechanisms used by leaching bacteria to adapt the composition and amount of their EPS according to the growth substrate (planktonic cells grown with soluble substrates, e.g., iron(II) sulfate, produce almost no EPS) remain to be elucidated in detail.

The site of attachment and the detection/sensing of this site by the cells are still open questions. There are indications from the literature (Andrews 1988; Ohmura et al.
1993; Shrihari et al. 1995; Dziurla et al. 1998; Sanhueza et al. 1999; Edwards et al. 2001) and our own work (Gehrke et al. 1998, 2001; Telegdi et al., unpublished) that attachment to metal sulfides does not occur randomly (Fig. 2). For example, atomic force microscopy (AFM) as well as confocal laser microscopy (CLSM) images demonstrate that cells of *At. ferrooxidans* preferentially (>80 %) attach to sites with visible surface imperfections (scratches, etc.). Furthermore, attachment to areas with a low degree of crystallization is favored, and the sessile cells seem to orient themselves along crystallographic axes, in whose direction oxidation fronts propagate. Whereas adhesion to scratches might be explained by mere contact area enhancement, areas with low crystallization and crystallographic axis are often not related to changes in surface topography. Therefore, attachment to specific sites on the mineral surface is principally related to different attractants, most likely caused by charge imbalances on the surface as caused by, e.g., oxidation processes. Several strains of *At. ferrooxidans* and *L. ferrooxidans* have been shown to possess a chemosensory system—chemotaxis—reacting positively to gradients of iron(II)/(III) ions, thiosulfate, etc. (Acuña et al. 1992; Meyer et al. 2002). These compounds occur compulsorily in the course of metal sulfide dissolution (Fig. 1). Dissolution occurs (in an electrochemical sense) at local anodes, bringing iron(II) ions and thiosulfate in solution in the case of FeS$_2$; a review on the anodic and cathodic reactions is given by Rimstidt and Vaughan (2003). It may be speculated that these local anodes are the sites toward which the cells are chemotactically attracted. These anodes and cathodes may have resulted from imperfections in the crystal lattice where the iron-to-sulfur ratio is not exactly 1:2, inclusion of other metal atoms during the process of crystallization (from saturated solutions), and/or from variations of temperature during crystallization (causing amorphous up to highly crystalline structures).

Experiments with a Kelvin probe to detect local anodes and cathodes on a FeS$_2$ surface were unsuccessful due to a lateral resolution of the instrument of 10 μm (Gehrke et al. 1998). Obviously, an increased resolution is necessary. This may be achieved by the development of a combination of AFM with a Kelvin probe. Recent work using an AFM equipped for Kelvin probe force mapping (Kuklinski and Sand, unpublished) indicates that the cells of *L. ferrooxidans* attached to a pyrite surface are more negatively charged (about 100–200 mV) than the surrounding surface (i.e., extracting electrons from the pyrite; see Fig 2). In a similar case, Little et

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**Fig. 2** Atomic force microscopy (left) and AFM–Kelvin probe scans (right) of cells of *At. ferrooxidans* (a) and *L. ferrooxidans* (b–d) specifically attached to dislocation areas (surface defects) on the pyrite surface. Arrows in (a) indicate cell-free EPS. In (b), a single cell of *L. ferrooxidans* appears embedded in EPS. The topographical image of a colony of *L. ferrooxidans* (scanned in intermittent contact mode) is shown in (c). The same colony is shown in (d), imaged using Kelvin probe force microscopy, where a negative potential difference among the cells and the pyrite surface is shown. The blue line represents the zone, for which the potential differences are shown in (e)
al. (2000), testing the attachment sites of sulfate-reducing bacteria on steel surfaces, detected that the bacteria were attached in the immediate vicinity (nanometer range) of the anode. The latter is negatively charged until a release of iron(II) ions occurs. As a consequence of bacterial attachment, the anode and the cathode become permanent (manifest) and steel dissolution commences. This observation also seems to be relevant for the bioleaching of metal sulfides. To summarize, cells are attracted to transiently (electrically charged) dissolution sites by their chemotactic sensory systems and cause the anodes and cathodes on the metal sulfide surface to become permanent. The dissolution process occurs in the EPS layer (Fig. 3). This layer fills the void volume between the outer membrane (of the cells) and the surface layer (of the metal sulfide) and can, thus, be considered as a reaction space. The pioneering work of Rodriguez-Leiva and Tributsch (1988) demonstrated that this distance is 10–100 nm wide. The At. ferrooxidans EPS thickness was estimated for iron(II)-grown cells by in vivo AFM to be 28.7 nm (±13.5). The polymer density calculations estimated that this bacterium has 51,000–105,000 exopolymer molecules on its outer surface, a value which is 20–30 times lower than Escherichia coli (Taylor and Lower 2008). The EPS thickness values for sulfur- or pyrite-grown cells remain to be elucidated. Presumably, these will be much higher than the aforementioned ones since it is already known that EPS levels increase when the bacteria are grown with these substrates (Gehrke et al. 1998). Bacterial biofilm formation is linked to an increase in the production of EPS (Flemming and Wingender 2010). We have shown that induction of capsular polysaccharide (CPS) biosynthesis in pyrite cultures occurs simultaneously with the formation of microcolonies on pyrite grains. Interestingly, the biosynthesis of CPS seems to be regulated at several levels. We have recently shown by CLSM that it can be enhanced by the addition of reduced sulfur species to iron(II)-grown cells. CPS levels were also increased by the addition of glucose or galactose in the medium, and as part of the Pi starvation response (Bellenberg et al. 2012). Also, At. ferrooxidans possess a canonic QS system and synthesizes N-acylhomoserine lactones (AHL; Farah et al. 2005; Ruiz et al. 2008, 2011). Recently, it has been shown that the addition of a synthetic C-14 AHL and a C14-AHL mixture resulted in an enhancement of biofilm formation on sulfur and FeS₂ surfaces and that this enhancement was likely a consequence of an enhanced EPS biosynthesis (Gonzalez et al. 2012). By high-throughput proteomics, the estimated differences among planktonic and FeS₂-attached cell subpopulations after 24 h of biofilm formation account for around 10 % of the detected proteins. Functions such as glutathione metabolism, stress responses, and EPS biosynthesis seem to be pivotal (Vera et al. 2013).

In the case of metal sulfides such as FeS₂, which need an oxidizing attack by iron(III) ions for dissolution, the EPS-complexed iron(III) ions must fulfill this function (Fig. 3b). However, this very process is not at all understood. Currently, the most likely explanation is based on two plausible assumptions. In order that the iron(III) ions are reduced, the first assumption considers the electron tunneling effect. It is known that electrons can bridge distances of up to 2 nm by tunneling from one electron hole to another (Medvedev and Stuchebrukhov 2001). Consequently, the iron(III) ions have to be exposed to the pyrite surface within this distance (to be reducible by tunneling electrons). Considering the 2-nm distance between the cell membrane and the substrate surface, this hypothesis seems to be reasonably sound and would explain the reduction of the iron(III) ions. The second assumption is that iron(II) ion–glucuronic acid complexes are less stable than the corresponding iron(III) ion complexes. This has been demonstrated for various iron–carboxylic acid complexes (NIST 2004). Consequently, iron(II) ions produced by the cathodic electron transfer are released from their EPS chelators. The

**Fig. 3** a Model for contact leaching catalyzed by a cell of At. ferrooxidans (from Sand et al. 1995, modified). Overview showing the bacterial cell embedded in its EPS attached to pyrite via electrostatic interactions. CM cytoplasmic membrane, PS periplasmic space, OM outer membrane. b CLSM image showing a 3D projection of a pyrite grain (50–100 mesh) colonized with cells of At. ferrooxidans ATCC 23270 after 1 week of incubation. Cells were double stained with Syto9 (green) for nucleic acids and TRITC-labeled concanavalin A (ConA) lectin for capsular polysaccharides as described (Bellenberg et al. 2012). Signals from both fluorescent channels plus light reflection (to image the pyrite grain surface) were recorded; the merged image from all three channels is shown. The bacterial colonization pattern strongly correlates with surface imperfections.
remaining uronic acid residue will bind a new iron(III) ion at the FeS$_2$ surface out of solution as it stands in equilibrium with the dissolved as well as other complexed iron(III) ions. If mobile iron(II) ions diffuse toward the outer membrane, they will be (re)oxidized by the enzymatic system of the cells. These two assumptions currently underlie the most likely explanation of the electrochemical mechanism of the (bio)leaching of metal sulfides. The chemical reactions occur outside the cells, in fact outside the outer membrane, but still within the EPS-generated microenvironment (Fig. 3b). Similar observations have been recorded for *Geobacter* species by Lovley and coworkers, in which the genetic deletion of several genes encoding outer membrane c-type cytochromes in *Geobacter metallireducens* impaired its capacity for Fe(III) reduction (Smith et al. 2012).

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