

MINI-REVIEW

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Sulfur chemistry, biofilm, and the (in)direct attack mechanism – a critical evaluation of bacterial leaching

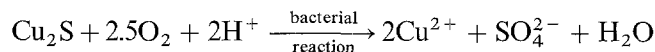
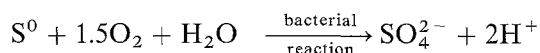
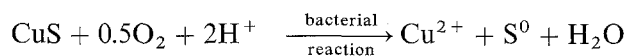
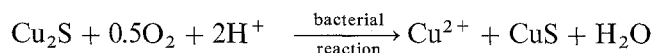
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Abstract It has been shown (a) that bacterial leaching of metal sulfides apparently requires the attachment of leach bacteria to metal sulfides, (b) that exopolymer-bound iron compounds are responsible for or at least considerably increase the rate of the biological attack over the chemical rate, (c) that the primary attacking agent in leaching environments is the ferric iron hexahydrate ion, (c) that thiosulfate is the first intermediate sulfur compound, giving rise to a variety of other compounds including polythionate-containing periplasmic granula, and (d) that we have no idea about the actual concentrations of protons, ferrous/ferric and/or other cations, and sulfur compounds in the reaction space between the bacterium and the sulfide surface.

for bacterial leaching, because they are unable to metabolize metal sulfides. The biological oxidation requires an acidic pH (Colmer et al. 1950; Hallmann et al. 1993), the presence of oxygen and carbon dioxide, and humidity (Hallmann et al. 1993). Since the discovery of bacterial leaching in 1950 (Colmer et al. 1950), two hypotheses for the chemical reactions have been discussed: the direct and the indirect attack mechanisms (Rossi 1990).

The direct attack mechanism is generally believed to proceed via a simultaneous enzymatic oxidation of the metal and the sulfide moiety of the mineral. As an example consider the following equations of chalcocite (Cu_2S) oxidation.

Direct:



Iron ions, either ferric or ferrous, seem not to be involved in this reaction. Owing to the insolubility of metal sulfides, a direct contact between the bacterium and the sulfide surface is required.

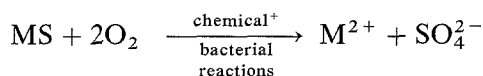
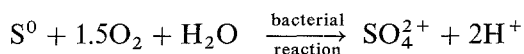
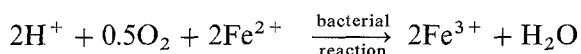
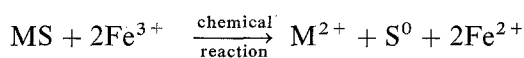
The indirect attack mechanism is based upon the chemical oxidation of metal sulfides (MS) by ferric ions. The reduced form, the ferrous ion, in turn serves as the substrate for *T. ferrooxidans*, for example, which will reoxidize it to ferric ion. Because of this shuttle mechanism, a direct bacterium/sulfide contact is not necessary. The following equations demonstrate the catalytic effect of the ferrous/ferric couple and bacteria.

The extent of our knowledge

Bacterial leaching is performed by the oxidation of insoluble metal sulfides by specialized lithoautotrophic bacteria to sulfuric acid containing dissolved heavy-metal ions (Ehrlich 1990; Tuovinen 1990). The most important bacteria are *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* (Colmer et al 1950; Sand et al. 1992). *T. thiooxidans* is often involved also. Besides these, microorganisms like the mixotrophic *T. acidophilus* and the heterotrophic *Acidiphilum* sp. (which are closely related; Lane et al. 1992) occur in these habitats. The latter especially was shown to interact with *L. ferrooxidans* causing an increased metal solubilization (Hallmann et al. 1993). The other sulfur oxidizers like *T. neapolitanus*, *T. intermedius*, or *T. thioparus* are believed to be of little or no importance

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Indirect:



Up to now it has been a subject of controversy whether the direct attack mechanism exists and, if it does, to what extent this mechanism contributes to leaching. Generally it is claimed that results in favor of direct leaching have been achieved from experiments with pure metal sulfides, some of which may even be of synthetic origin like zinc sulfide, in an iron-ion free nutrient solution with an inoculum of repeatedly washed, iron-ion-free cells (Duncan et al. 1967; Rickard and Vanselow 1978). If natural sulfides were used, the amount of iron compounds was so low that it seemed to be negligible.

Doubts

Over the years of our working in this field, several inconsistencies arose that shed serious doubt on whether the direct attack mechanism really existed.

The first doubts resulted from an experiment with *T. ferrooxidans*. Subculturing the organism in an iron-free salt solution (Mackintosh 1978) for analyses of sulfur metabolism resulted in a complete loss of any measurable substrate degradation (Sand and Schröter, unpublished results). Thus, the cells were totally dependent on the availability of iron ions.

During the study of *L. ferrooxidans* (Sand et al. 1992) experiments were carried out comparing the ferrous-iron-oxidizing activities of ferrous-iron-grown cells with those of sulfide-ore-grown cells. In addition, the activities of strains of *T. ferrooxidans* were compared with those of *L. ferrooxidans*. After growth on a sulfidic ore the cells of the latter had a ferrous-iron-oxidizing activity comparable with that of *T. ferrooxidans*, although their activity after growth on ferrous sulfate was quite different (half that of *T. ferrooxidans*). Consequently, the metal output in leaching experiments with pure cultures was similar for those metals that require an oxidative attack, such as copper ores (Sand et al. 1992, 1993). If the direct and the indirect mechanisms were to act jointly, metal solubilization by *T. ferrooxidans* would be more prominent than that by *Leptospirillum*.

A study of microbial diversity in uranium mine waste heaps, which has been conducted only recently (Schipers et al. 1995), yielded an astonishing result: irrespective of the sample depth, moderately acidophilic thiobacilli like *T. neapolitanus*, *T. novellus*, or *T. intermedius* were at least as abundant as *T. ferrooxidans*. In samples from 3–4 m below the surface, these thiobacilli were a hundred times more frequent than the former. The source of substrate remained unclear. The uranium ore contained only pyrite as a nutrient source, which is known to be non-degradable by these bacteria. Even the intermediary sulfur cannot account for the substrate source since a few leach bacteria cannot oxidize enough pyrite to support the majority of commensals.

Last but not least, concern about the direct attack mechanism results from biochemical work. For 2 years studies have been under way investigating the importance of the adhesion of leach bacteria to mineral sulfides. Chemical analysis demonstrated that isolated extracellular polymeric substances of *T.* and *L. ferrooxidans* contained between 0.5% and 5% of tightly bound ferric iron (on a dry-weight basis; Sand, Gehrke, and Flemming, unpublished results). The iron compounds were not removable by any washing procedure. Thus, the cells of the two leaching bacteria carry a considerable supply of these compounds, even if the mineral is devoid of them.

This may, however, be of purely academic interest, because only synthetic sulfides are free from iron compounds. In all other cases, these compounds are associated, although in varying amounts, with sulfide ores. A limiting value for the concentration of iron compounds in the leach solution has so far not been determined (besides the experiment cited above with sulfur). It may, thus, be concluded that iron compounds in any given natural ore are not limiting the microbial leaching activity.

Sulfur chemistry

The chemical reactions occurring at the interface between mineral sulfide and leaching solution have received considerable attention during the last one or two decades, although the overall reactions have been known for quite a while. The interest of inorganic chemists has focussed on the mechanism of attack by either oxygen or ferric iron or both (Lowson 1982; Luther 1987; Moses et al. 1987). In 1987 two research groups came up with a similar mechanism. Both were analyzing the chemistry of the attack on pyrite. Their first result was the finding that, at neutral or alkaline pH, oxygen was the important oxidant for pyrite whereas at acid pH ferric iron became mainly responsible. The dominance of oxygen is a result of the low solubility of ferric iron above pH 3.5. The proposed reaction mechanism for the acidic pH range, which

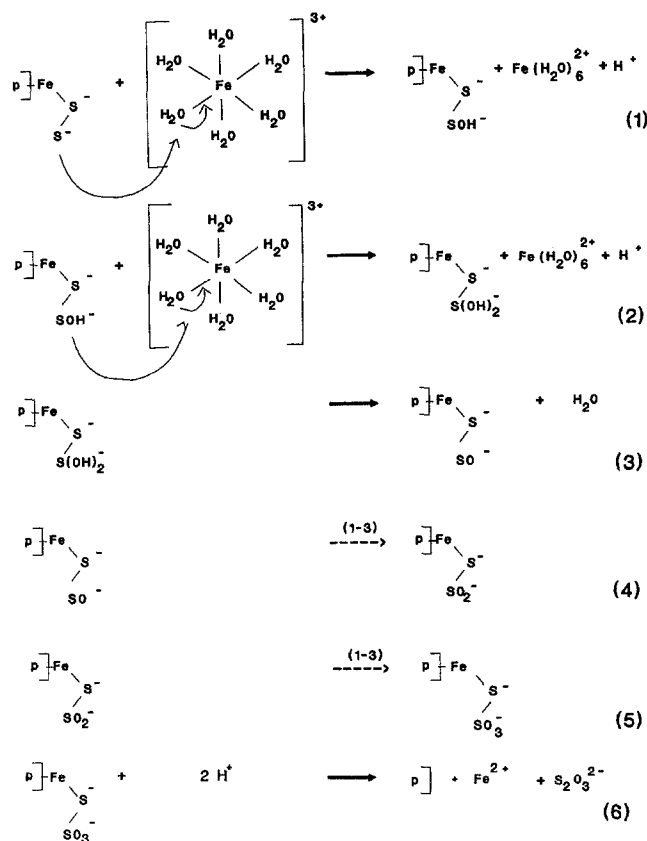
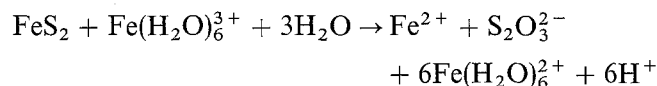


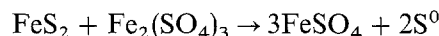
Fig. 1 Reaction mechanism of ferric iron hexahydrate attack on pyrite. Reactions 1, 2, and 3 are repeated three times to produce, via reaction 4, the sulfonic acid in equation 5, which hydrolyzes to ferrous iron and thiosulfate (based on Moses et al. 1987). The arrow in reactions 4 and 5 indicates that reactions 1–3 are repeated. *p* pyrite

finally is the relevant range for bacterial leaching, is based on an attack of the pyrite surface by ferric iron hexahydrate, as shown in Fig. 1.

The overall equation runs as follows:



In contrast, the “classical” equation has a different stoichiometry and sulfur as an intermediate product:



Sulfur and ferrous iron will be oxidized with oxygen to sulfate and ferric iron. The basic difference lies in the primary reaction with the pyrite molecule/surface, which in an aqueous environment is highly likely to be the reaction with the hexahydrate. Thus, thiosulfate instead of elemental sulfur becomes the first intermediate sulfur compound of the indirect attack mechanism.

Thiosulfate is not stable at acidic pH. A series of reactions follows resulting in the formation of polythionates, elemental sulfur, and possibly sulfide. The environmental conditions giving rise to the prevalence of one of these products are not known precisely. It is generally accepted that sulfane monosulfonic acids are

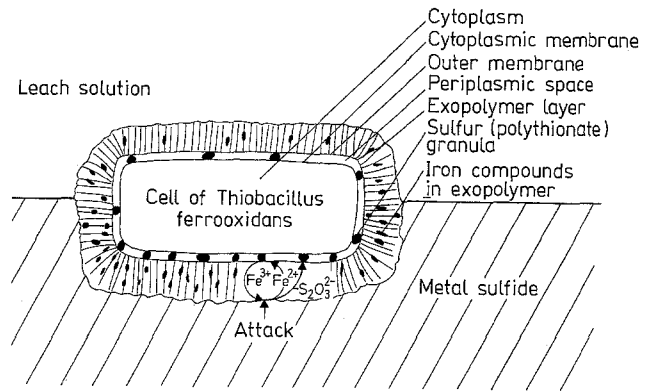
involved (Steudel et al. 1987; Wentzien et al. 1994). Thiosulfate is oxidized chemically or enzymatically to tetrathionate. The latter has been proven for *T. intermedius*, *T. ferrooxidans*, *T. thiooxidans*, and *T. acidophilus* (Wentzien et al. 1994; Sinha and Walden 1966; Okuzumi and Kita 1965; Pronk et al. 1990b respectively), whereas the former has been shown to occur under sterile conditions in an acidified ferric iron solution in the laboratory (Schippers and Sand, unpublished results). Tetrathionate is hydrolyzed to sulfane monosulfonic acid, as has been observed with *T. ferrooxidans* (Steudel et al. 1987). The anion of this acid, $\text{S}_3\text{O}_3^{2-}$, is highly reactive and may be oxidized, giving rise to various polythionates like tri-, penta-, or hexathionate (Wentzien et al. 1994). It is also possible that the anion may be decomposed in a complex multistep reaction to elemental sulfur and sulfite. Even a second hydrolysis of the anion is possible, yielding thiosulfate, sulfate, and two protons.

As a consequence, all sulfur compounds that have been detected in leaching operations may arise from reactions starting from thiosulfate. It is not surprising that elemental sulfur was believed to be the main intermediate compound. Because of its low solubility in water ($5 \mu\text{l/l}$ at 20°C ; Steudel and Holdt 1988), sulfur remains in the system and is easily, sometimes even visually detectable. Polythionates as well as thiosulfate are highly soluble and need special equipment for an unequivocal detection (Steudel et al. 1987). Nevertheless, several reports have described their occurrence during leaching (Steudel et al. 1987; Pronk et al. 1990a) Pichtel and Dick 1991a, b; Schippers and Sand, unpublished results). In addition, preliminary results from experiments with *T. ferrooxidans* and *L. ferrooxidans* oxidizing pyrite indicated the occurrence of thiosulfate and tetrathionate in the culture solution (Schippers and Sand, unpublished results). Summarizing the preceding paragraph: it has been shown that considerable evidence has been accumulated indicating that ferric iron in the form of a hexahydrate ion is the first agent to attack pyrite. The first resulting sulfur component is thiosulfate.

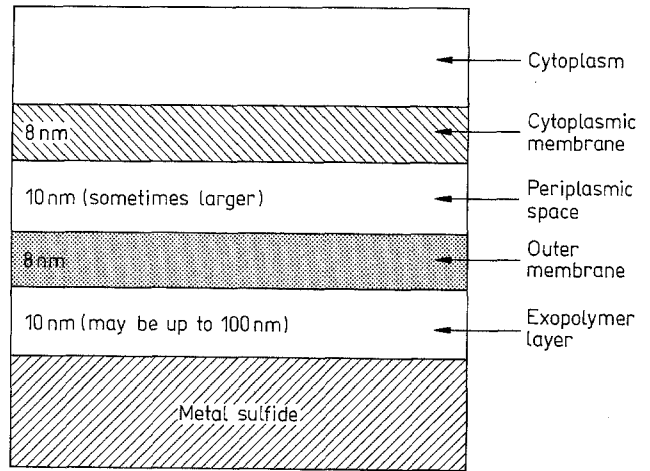
Ecological and biochemical considerations

As mentioned above, leaching bacteria attach to ore particles during growth. This type of growth is generally called biofilm, and its thickness does not exceed one or two cell layers. We have never observed an extensive, multilayered growth in contrast to other biotopes with a biofilm development. Obviously the active leaching bacteria need to be directly attached to the mineral surface in order to be able to leach and grow (Arredondo et al. 1994). The morphology of leaching patterns supports this assumption. The leach pits demonstrate unambiguously that a single bacterial

cell is responsible for the traces of degradation (Rodriguez-Leiva and Tributsch 1988; Mustin et al. 1992). *T. ferrooxidans* and *L. ferrooxidans* exhibit considerable differences in the amount of attached cells compared to planktonic cells. In our field studies *L. ferrooxidans* was usually abundant only in samples containing mud, not however, in clear effluents or temporarily dry ones (Sand et al. 1992; Hallmann et al. 1993; Sand unpublished results). Percolator leach studies demonstrated that *Leptospirillum* attached more rapidly to the ore than *T. ferrooxidans*. The attachment could be furthered by using mixed cultures of *Leptospirillum* and *Acidiphilium* (Hallmann et al. 1993). Attachment to the mineral surface is mediated by exopolymeric compounds. The nature of these compounds and their interaction with the mineral sulfide surface is unknown. The work published by Rodriguez-Leiva and Tributsch (1988) indicated that a layer of organic substances filled the void between a bacterial cell and the solid sulfide. The distance between these is known to amount to 3–7 nm (van Loosdrecht et al. 1990). The organic layer seems to be a kind of reaction space where all degradative reactions take place. The actual concentrations of the reactants in this space are unknown. It is quite likely that the real concentrations are considerably higher than those reported in the literature for culture solutions. This is due to the method of sampling, which involves the homogenisation of the sample. At this point it needs to be recalled that the extracellular polymers of *T. ferrooxidans* and *L. ferrooxidans* bear a considerable load of ferric iron. These iron compounds are thus available in direct proximity with the surface for an attack on the metal sulfide. In addition, they may be a prerequisite for the attachment of the cells to the insoluble nutrient. In an elegant method, using zeta potential measurements, Blake et al. (1994) very recently demonstrated that metal ions are necessary to overcome the repellent effect between negatively charged sulfide minerals or sulfur and microbial cells. The inclusion of metal cations in the exopolymers of the cells caused a shift of the zeta potential from negative to slightly positive values. From the electrochemical point of view the attachment now becomes possible. Our own work, although using a different system, yielded comparable results. Cells of *T. ferrooxidans*, from which the extracellular polymers had been removed by centrifugation, needed several hours before attachment to pyrite became detectable, whereas untreated cells started to adhere within the first hour (Gehrke and Sand, unpublished results). The isolated extracellular polymers exhibited a similar stickiness to pyrite. Within 1 h after mixing they were no longer detectable in the supernatant. These findings enabled us to explain the problem of the iron-deficient cells of *T. ferrooxidans* being unable to degrade sulfur. Owing to the lack of iron compounds in the exopolymers the cells were not able to attach to the sulfur particles.



a



b

Fig. 2a, b A model for the indirect leaching attack mechanism as catalyzed by a metal-sulfide-attached cell of *Thiobacillus ferrooxidans*. **a** A general description of the bacterial leaching attack exaggerating the exopolymer layer in order to show its importance. **b** The correct dimensions of the cellular structures where leaching biochemistry takes place. Note: the attack takes place in the exopolymer layer between the cell and the metal/sulfide and is mediated by the iron compounds in the exopolymer layer (at least primarily). Thiosulfate is an intermediate compound; for clarity the ensuing reactions were omitted

The model

Combining all the evidence that has been outlined in the previous sections, a topical model for the bacterial leaching of metal surfaces has emerged. The scheme is presented in Fig. 2.

In this model *T. ferrooxidans* may be replaced by *L. ferrooxidans* only with respect to iron ion metabolism, because *L. ferrooxidans* only oxidizes ferrous to ferric iron but not sulfur or its compounds. It is evident from Fig. 2, that leach bacteria grow and attach to the surface of a mineral sulfide. The mechanism for recognition of suitable sites for attachment remains to be elucidated. It has been shown that *T. ferrooxidans* and

L. ferrooxidans do not attach randomly. Jerez demonstrated convincingly that a chemotactic mechanism is involved, with Ni^{2+} , Fe^{2+} and Cu^{2+} ions being positive attractants for cells of *Leptospirillum* (Acuna et al. 1992). The genes responsible were detectable in *T. ferrooxidans* as well. In addition, thiosulfate was shown to be a positive attractant for *T. ferrooxidans* (Chakraborty and Roy 1992). Thus, both leach bacteria are able to recognize sites on the mineral sulfide surface where probably electrochemically controlled processes cause a dissolution (generation of an anode and cathode due to charge imbalances, faults, electron gaps etc. in semiconductors, to which metal sulfides like pyrite belong; Tributsch and Bennett 1981a, b) and liberate ferrous ions and thiosulfate. It may be speculated that the mechanism for attachment is mainly a result of the amount of complexed iron ions (or possibly other metal ions; Mittelman and Geesey 1985; Ferris et al. 1989) in the exopolymers. Wherever free ferrous or ferric ions are available, it is highly likely that the amount of iron ions complexed in the extracellular polymeric substances starts to increase. If the zeta potential of the cells has increased to sufficiently positive values because of this process (Blake et al. 1994), adhesion may occur. This mechanistic model also makes sense from another point of view. The cells attach to those sites where dissolution processes make substrates available.

In any event, the attachment is mediated by the ferric-iron-containing exopolymers. After attachment, the ferric ions start to attack the mineral sulfide (or increase the attack?). As a result, ferrous iron hexahydrate and thiosulfate are generated. In the case of *T. ferrooxidans* both compounds are used by the cells. Ferrous iron is reoxidized to ferric iron. Thiosulfate may either be oxidized as well, giving rise to sulfate, or be partially included in the periplasmic polythionate granula, which have been described by Steudel (1989). Only the finding of thiosulfate as an intermediate satisfactorily allows an explanation of the presence of these granula. In the case of sulfur it would have been necessary to assume that a part of the sulfur became oxidized to sulfite, which could react with the remaining sulfur, finally giving rise to thiosulfate – a complicated process. In addition, sulfite is quite unstable in strongly acidic environments containing a considerable amount of dissolved heavy-metal cations.

The presence of thiosulfate also provides a convincing explanation of the abundance of moderately acidophilic thiobacilli in uranium mine waste heaps, described above, since this compound is the preferred substrate for these bacteria. Thiosulfate may well have been the reason why *T. neapolitanus* was supposed to be involved in the leaching process of metal sulfides (Goroll 1976).

Further support for the leach model results from the work of Sugio on the enzyme sulfur: ferric iron oxidoreductase in cells of *T. ferrooxidans* (Sugio et al.

1987). Thiosulfate (Sugio used sulfur) is rapidly chemically oxidized to tetrathionate, as explained above, concomitantly reducing ferric to ferrous iron. Most of the tetrathionate is incorporated into the periplasmic polythionate granula, the rest is oxidized to sulfate. The often hypothesized elemental sulfur will be a degradation product of polythionate chemistry (see above). The ferrous iron is directly oxidized.

The implications of the model are somewhat different for *Leptospirillum* because of its inability to use sulfur compounds (Sand et al. 1992). The degradation of thiosulfate will progress by purely chemical reactions – an oxidation by ferric iron ions and the effect of high proton concentrations in these acidic environments. Consequently, polythionates should be produced by chemical oxidation, causing a reduction of ferric iron. Recent results proved the hypothesis. Tetra- and pentathionate were detected in cultures of *L. ferrooxidans* oxidizing pyrite (besides the end-product sulfate; Schippers and Sand, unpublished results).

The fact that ferrous iron is the sole (known) substrate for *Leptospirillum* (Sand et al. 1992) might explain why these bacteria attach more tightly to mineral sulfides and are less abundant in leachates. The highest concentration of ferrous ions will exist in close proximity to the mineral surface at leach pits. Support for this hypothesis may be derived from our ecological research. At several sites *L. ferrooxidans* could not be enumerated when ferrous sulfate was used as substrate (Schippers et al. 1995; Sand unpublished results). Only the use of pyrite allowed a detection and a quantification. It is highly likely that the K_m value for ferrous iron in these organisms is so different from that of strains traditionally isolated that the usual nutrient solution is inadequate for them. The competing organism, *T. ferrooxidans*, does not exclusively need to rely on ferrous iron. Thus, these cells do not need to be as tightly attached as those of *L. ferrooxidans* because of the increased availability of substrates. In addition, sulfur compounds provide considerably more energy for metabolism than ferrous iron. In any case, attachment seems to be advantageous for these bacteria and is a requirement for the attack on the metal sulfide, which may be caused or at least be enhanced by the exopolymer-bound iron compounds. Returning to the question about the direct or the indirect attack mechanism it seems quite obvious that, according to the definition given above, bacterial leaching proceeds only via the indirect attack.

We are fully aware that many of the details presented above may be explained by other mechanisms. However, taking into consideration the recent research on biofilm growth as well as the sulfur chemistry, these data fit perfectly into the leach model outlined in Fig. 2. A considerable amount of further work is necessary for the verification of this hypothesis, some of which is under way and will be presented soon.

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