# A REVIEW OF BIOENERGETICS AND ENZYMOLOGY OF

# SULFUR COMPOUND OXIDATION BY ACIDOPHILIC THIOBACILLI

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Abstract

For many years the basic research on acidophilic thiobacilli and especially Thiobacillus ferrooxidans has been focussed mainly on the ferrous iron oxidizing system and metal tolerance. Now it is generally accepted that for an effective solubilization of sulfidic minerals the microbial system should possess both a ferrous iron- and a sulfur-oxidizing capacity. Thus for a full understanding of the performance of acidophilic thiobacilli their sulfur-oxidizing system should also be studied. The direct oxidation of sulfur compounds by these organisms might also represent also a bioenergetic advantage as the oxidation of sulfur compounds has a much higher Gibbs (free) energy change than of iron. Yield data obtained in our laboratory support this hypothesis. A recent publication, however, suggests that there is a strong interdependence between the iron- and sulfur-oxidizing system in T. ferrooxidans and related organisms. It is proposed that the sulfur oxidation is catalyzed by a ferric-iron dependent periplasmic protein (sulfur-ferric iron respiration). As a consequence, electrons produced by the oxidation of sulfur compounds should enter the respiratory chain at the same site as electrons from ferrous iron oxidation. This would imply that the yields per electron should be similar for iron and sulfur compounds. Data from recent studies dealing with the bioenergetics of anaerobic growth of T. ferrooxidans on elemental sulfur and with ferric iron demonstrate that the electrons from the sulfur compound oxidation enter the respiratory chain at a higher level than those from ferrous iron. Further studies in Delft focussed on the enzymology of inorganic sulfur oxidation in both T. ferrooxidans and T. acidophilus. Three enzyme activities, thiosulfate oxidoreductase, trithionate hydrolase, and an enzyme catalyzing the cleavage of tetrathionate to thiosulfate, sulfur, and sulfate, were purified. All three activities exhibited very low pH optima (2-4), suggesting that inorganic sulfur oxidation by these acidophiles predominantly occurs in the periplasm. Evidence was obtained that sulfite oxidation also occurs in the periplasm of T. ferrooxidans.

## Introduction

Although acidophilic thiobacilli have been exploited since ancient times, awareness that organisms such as Thiobacillus ferrooxidans are responsible for the solubilization of metal sulfides dates from the end of the fourties of this century. Since then this organism has been used extensively in mission oriented research on microbial metal leaching and in basic physiological, enzymological and molecular genetical studies aimed at improvement and better control of metal recovery processes from low grade ores and heavy metal containing waste materials, and of desulfurization of coal.

Progress in the basic research on thiobacilli has always been comparatively slow because of the fastidious character of these organisms and the low cell yields obtained on inorganic energy substrates such as ferrous iron and reduced sulfur compounds. This type of research has been focussed mainly on the ferrous iron

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oxidizing system and heavy metal tolerance. The long-existing interest in the iron-oxidizing capacity is important in view of the role that T. ferrooxidans plays in the reoxidation of ferrous to ferric iron in leaching and "remote leaching" operations. For a number of years it has been assumed that sulfide minerals were oxidized not directly by T. ferrooxidans, but indirectly by a chemical reaction with ferric iron produced by the organisms. In this chemical reaction ferrous iron would be formed and the role of T. ferrooxidans would thus be the recycling of ferrous iron.

However, it is now generally accepted that for an effective solubilization of sulfidic minerals the microbial system should preferably possess both a ferrous iron and a sulfur-oxidizing capacity. Studies by Arkesteyn (1) have demonstrated that, as far as pyrite oxidation is concerned, the biological sulfur oxidation must be involved. If sulfur oxidation is specifically inhibited by a thiol inhibitor (NEM), pyrite oxidation by T. ferrooxidans stops. Another argument which supports the important role of sulfur oxidation in pyrite oxidation can be found in the cell yield data obtained by Hazeu et al. (2) They found that the cell yield on pyrite expressed as g/mol electrons was far higher than the yield obtained in cultures growing on ferrous iron. This suggests that part of the electrons delivered by the oxidation of this mineral enters the respiratory chain at another site than electrons originating from ferrous iron. Continuous culture studies in our laboratory have demonstrated that cell yields expressed in g/mol electron on reduced sulfur compounds,such as thiosulfate and tetrathionate are also higher than those obtained on ferrous iron (Table 1). From these observations one can conclude that for a full understanding of the physiology of T. ferrooxidans and its performance in leaching operations not only its ferrous iron oxidizing, but also its sulfur-oxidizing system should be studied.

Table 1: Yields of Thiobacillus ferrooxidans grown in the chemostat under various conditions (2).

| Limiting substrate(s) | Conc. (mM) | Dilution rate ($h^{-1}$) | pH | Yield (g dry weight/mol electron) |
|---|---|---|---|---|
| $Fe^{2+}$ | 160 | 0.034 | 1.6 | 0.23 |
| $S_4O_6^{2+}$ | 10 | 0.030 | 3.0 | 0.92 |
| $Fe^{2+}/S_4O_6^{2+}$ | 15\10 | 0.030 | 1.6 | 0.76 |
| $Fe^{2+}/S_4O_6^{2+}$ | 70\15 | 0.032 | 1.6 | 0.55 |
| pyrite | (1\14) | batch | 1.8 | 0.35-0.5 |

For a review of the older literature on sulfur oxidation by acidophilic thiobacilli the reader is referred to the review of Pronk et al. (3) Recently the group of Sugio (4) published data which suggest that there is a strong interdependence between the iron- and sulfur-oxidizing system in T. ferrooxidans and related organisms. They proposed a ferric-iron dependent sulfur oxidation by T. ferrooxidans which is catalysed by a single periplasmic protein (sulfur-ferric iron respiration), whereby the ferrous iron would be the first product. In this redox reaction the electron transport chain would not be involved. As a consequence of their model, electrons produced by the oxidation of reduced sulfur compounds, including elemental sulfur, should enter the respiratory chain at the same site as electrons from ferrous iron oxidation. This would imply that the yields per mol electrons should be similar for iron and sulfur compounds. Clearly, this would contradict the cell yield data of Hazeu et al. (2) mentioned earlier in this introduction.

In the following, we will first present data on the bioenergetics of acidophilic thiobacilli, with special reference to work performed in Delft. Our data reinforce the contention that the electrons from the sulfur compound oxidation enter the respiratory chain at a higher level than those from ferrous iron. Secondly, we will also present some of our data on the enzymology of sulfur oxidation by these organisms. Again, the results strongly suggest that the sulfur oxidation pathway does not involve iron as an intermediate. In our studies we used two model organisms: the obligate chemolithoautotroph T. ferrooxidans and the facultative chemolithoautotroph T. acidophilus. As will be discussed later, the latter organism has a sulfur oxidizing system that shows a high similarity with that of T. ferrooxidans, but it has the experimental advantage with respect to enzymological studies that it can be grown at high cell densities on a mixture of organics and reduced S-compounds. The overall results obtained thus far will be used to present a

hypothetical pathway for sulfur compound oxidation in these organisms. Finally, we will discuss its implications for our understanding of the role of acidophilic thiobacilli in technology and environment.

## Bioenergetics of acidophilic thiobacilli

There is now conclusive evidence from the literature that energy metabolism in acidophiles involves 'classic' chemiosmotic coupling. The proton motive force is composed of a pH gradient ($\Delta$pH, which contributes the bulk of the proton motive force) and an electrical charge component ($\Delta\psi$). At very low pH values, $\Delta\psi$ may become inside positive, thereby compensating part of the excessively high $\Delta$pH (5). This mechanism ensures that the total proton motive force is not higher than in neutrophilic bacteria and that, bioenergetically, the maintenance of a large trans-membrane proton gradient is not more expensive (in energetical / thermodynamical terms) than the maintenance of a proton motive force in neutrophilic bacteria. Indeed, the maintenance energy requirement of T. acidophilus was not higher than that of neutrophilic bacteria and was not significantly affected by the medium pH (6).

The large $\Delta$pH across the cytoplasmic membrane of the acidophilic thiobacilli makes these organisms very vulnerable to compounds that dissipate this essential gradient. Many weak organic acids can relatively easily diffuse across the cytoplasmic membrane in their non-dissociated form. Upon entering the near-neutral cytoplasm, these acids dissociate and cause a decrease of the cytoplasmic pH (7). In batch cultures, low concentrations ($10^{-4}$ M) (8) of these organic acids are sufficient to completely inhibit growth of the acidophilic thiobacilli. However, when the supply to the cultures is growth-limiting, some organic acids can be used as sources of energy. This is for example the case with pyruvate in T. acidophilus (6) and with formate in T. acidophilus (9) and as found recently in T. ferrooxidans (10). The ability to actively metabolize and thus to 'scavenge' low concentrations of organic acids may be a vital defense mechanism during growth in acidic environments.

Since no fermentative acidophilic thiobacilli have been described to date, generation of a proton motive force has to occur by respiration-driven proton translocation. The facultatively autotrophic species T. acidophilus can use both organic and inorganic electron donors. The obligate autotroph T. ferrooxidans can use, besides ferrous iron, inorganic sulfur compounds (including sulfidic minerals), molecular hydrogen (11) and formic acid as electron donors for respiration. It has long been assumed that the acidophilic thiobacilli are strictly aerobic (12). However, T. ferrooxidans can use ferric iron as an alternative electron acceptor for oxidation of elemental sulfur in the absence of oxygen (13). It has recently been demonstrated that anaerobic ferric iron respiration with sulfur or formic acid can be used by the bacterium to provide metabolic energy (14). Moreover, it has been shown independently by Das et al. (15) and Pronk et al. (16) that T. ferrooxidans is in fact capable of anaerobic, autotrophic growth on elemental sulfur and ferric iron.

The observation that the anaerobic ferric iron respiration by T. ferrooxidans is an energy-transducing process implies that electron transfer from elemental sulfur to ferric iron involves at least part of the respiratory chain. This conclusion is in agreement with the sensitivity of ferric iron respiration to the inhibitor HOQNO, a specific inhibitor of the bc1 complex of the respiratory chain (14,17). The involvement of the respiratory chain in the transfer of electrons to ferric iron is difficult to reconciliate with the direct coupling of the sulfur oxidizing system to the iron oxidizing system as suggested by Sugio et al. (4).

Current models for the oxidation of ferrous iron by T. ferrooxidans state this process has an effective $H^+/O$ ratio of two due to the different locations of the ferrous/ferric iron couple (extracytoplasmic) and the $O_2/H_2O$ couple (cytoplasmic) (18). The higher yields on inorganic sulfur compounds as observed by Hazeu et al. (2) suggest the involvement of at least one additional proton translocating site. This is also compatible with crude estimates of anaerobic growth yields, derived from cell counts in batch cultures grown on elemental sulfur and ferric iron (16). These estimated yields are lower than aerobic growth yields on elemental sulfur, in agreement with a model proposing that reduction of ferric iron during sulfur compound oxidation under anaerobic conditions occurs at the same site of the respiratory chain as the oxidation of ferrous iron (14).

## Enzymology of acidophilic sulfur compound oxidation by thiobacilli

Attempts to unravel the pathways involved in acidophilic sulfur oxidation are hindered by the high chemical reactivity of many of the compounds involved (19). Therefore, knowledge of the enzymology of inorganic sulfur oxidation in acid environments is incomplete (for a review see Pronk et al. (3)).

In our group T. ferrooxidans has been used as a model organism to study growth and metabolism of sulfur compounds at low pH. Until recently it was, however, considered as an obligately autotrophic organism with low biomass yields, due to the low energy content per mol of inorganic substrate and due to the fact that substrate concentration must be kept relatively low because of osmotic stress and/or solubility. This was in particular a disadvantage for enzyme purification studies, where often substantial amounts of biomass are needed. To overcome this problem we introduced the facultative autotroph T. acidophilus into our studies. Meanwhile we now know how to grow T. ferrooxidans in high cell densities in formate-limited chemostat cultures (10). T. acidophilus is an acidophilic sulfur oxidizer and was initially isolated as an contaminant of a ferrous iron-grown culture of T. ferrooxidans (20). It can be grown heterotrophically and mixotrophically with relatively high yields. Carbon sources for heterotrophic growth include a number of monosaccharides, tricarboxylic-acid-cycle intermediates and some amino acids (6,20). Growth substrates for autotrophic growth include formate (9), elemental sulfur (20), tetrathionate (21), trithionate and thiosulfate (22).

Aim of our studies is the investigation of the kinetics and mechanism of sulfur compound oxidation in cell suspensions of both T. ferrooxidans and T. acidophilus and the purification and characterization of the enzymes involved in these oxidation reactions. These studies were restricted to the use of sulfide, thiosulfate, trithionate, tetrathionate, sulfite and elemental sulfur. T. ferrooxidans and T. acidophilus were grown in substrate-limited chemostat cultures on thiosulfate and on mixtures of glucose and thiosulfate, respectively.

Table 2: Characteristics of sulfur compound oxidation by cell suspensions of Thiobacillus ferrooxidans and Thiobacillus acidophilus. Experiments were carried out at pH 3 and 30°C.

| | Thiobacillus ferrooxidans | | | Thiobacillus acidophilus | | |
|---|---|---|---|---|---|---|
| sulfur compound | pH range (-) | pH optimum (-) | $K_s$ (μM) | pH range (-) | pH optimum (-) | $K_s$ (μM) |
| sulfide | 1->6 | 4 | 4-5 | 1-8 | 2 | 5 |
| sulfur | 1->6 | 2-4 | 6 | 1-8 | 4 | 2 |
| thiosulfate | 1-5 | 2 | 10-20 | 1-6 | 2 | 133 (8)[b] |
| tetrathionate | 1-5 | 3 | 4-15 | 1-6 | 4 | 1 |
| trithionate | ND[a] | ND[a] | ND[a] | 1-6 | 3 | 72 (9)[b] |
| sulfite | 1.5-5 | 3 | 30-60 | 2-8 | 6 | 13 |

[a]: ND = not determined.

[b]: Cell suspensions of Thiobacillus acidophilus exhibit a biphasic pattern of thiosulfate- and trithionate-dependent oxygen uptake. Due to the small amount of oxygen consumed during the first phase of thiosulfate and trithionate oxidation (oxidation of thiosulfate to tetrathionate), initial rates of oxygen uptake could only be determined accurately at substrate concentrations above 50 μM. This resulted in higher $K_s$ values than found for oxidation of thiosulfate and trithionate by heterotrophically grown cells (Table 2: between brackets), which exhibited a monophasic oxidation pattern with thiosulfate and trithionate.

Aerobic cell suspensions of T. ferrooxidans and T. acidophilus were able to oxidize the above-mentioned sulfur compounds roughly between pH 1 and 5, but the range for oxidation of sulfide and elemental sulfur was extended to pH values above 6 (Table 2). The pH optimum for sulfite oxidation by cell suspensions of T. acidophilus was 6. The affinity constants for the different sulfur compounds were all in the micromolar range (Table 2). Uncoupler studies and pH optima for oxidation suggested a periplasmic oxidation of trithionate, thiosulfate and tetrathionate and a cytoplasmic oxidation of elemental sulfur and

sulfide. Sulfite was oxidized in the periplasm by T. ferrooxidans whilst T. acidophilus appears to oxidize sulfite in the cytoplasm.

Sulfide was initially oxidized to some form of intermediary sulfur by both organisms. This sulfur was subsequently further oxidized to sulfate. Cell suspensions of T. ferrooxidans and T. acidophilus oxidized trithionate to sulfate. However, as was observed in anaerobic cell suspensions, the first step in the metabolism of trithionate was the hydrolysis to thiosulfate and sulfate. During thiosulfate oxidation tetrathionate is an intermediate in both organisms. Tetrathionate was subsequently further oxidized to sulfate.

When anaerobic cell suspensions of T. acidophilus were incubated with tetrathionate, a stoichiometric conversion of tetrathionate to thiosulfate, sulfur and sulfate was observed. During tetrathionate oxidation by T. ferrooxidans transient accumulation of insoluble sulfur was also observed.

At pH values above 5, T. ferrooxidans oxidized elemental sulfur completely to sulfite, which was excreted into the medium. Whether or not sulfite is an intermediate during sulfur compound oxidation in T. acidophilus remains unclear.

In Figure 1 a scheme is presented for sulfur compound oxidation in T. ferrooxidans and T. acidophilus. It is important to notice that the mechanism and the enzymes involved in the oxidation of intermediary sulfur to sulfate in T. acidophilus remain unknown at present, while in T. ferrooxidans sulphite is an intermediate during the oxidation of elemental sulfur to sulfate (Figure 1).

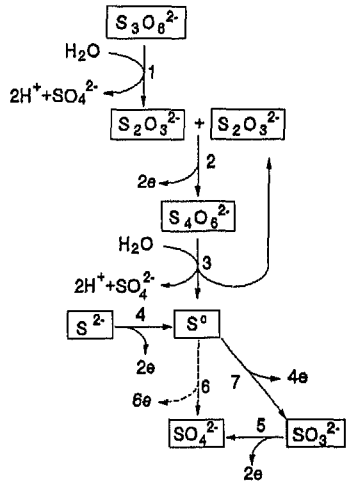


Figure 1: *Tentative scheme for sulfur compound oxidation by T. ferrooxidans and T. acidophilus.* 1: trithionate hydrolase, 2. thiosulfate dehydrogenase, 3: tetrathionate hydrolase, 4: not yet characterized, 5: sulfite oxidizing enzyme, 6: not yet characterized and 7: not yet characterized.

Cell-free extracts of T. ferrooxidans and T. acidophilus stoichiometrically hydrolysed trithionate to thiosulfate. The enzyme activity was stimulated by sulfate. In cell-free extracts of T. ferrooxidans optimum activity was found between pH 2 and 4, at a sulfate concentration of 50 mM. The activity was thermostable: after 10 min at 80°C, still 90% of the initial activity was retained.

Trithionate hydrolase was purified to near homogeneity from cell-free extracts of T. acidophilus. The molecular masses of the native enzyme and its subunit were 99 kDa (gel filtration) and 34 kDa

(SDS/PAGE), respectively. The purified enzyme showed optimum activity between pH 3.5-4.5 and the temperature optimum was 70°C. Enzyme activity was stimulated by sulfate up to a concentration of 2 M. This stimulation was half maximal at a sulfate concentration of 0.23 M. The $K_m$ for trithionate was 70 µM at 30°C and 270 µM at 70°C. Enzyme activity was lost after 36 days at 0°C, 27 days at 70°C, but after 97 days at 30°C, 40% of the initial activity was still present. The enzyme activity was inhibited by mercury chloride, N-ethylmaleimide, thiosulfate and tetrathionate.

Thiosulfate was oxidized to tetrathionate by cell-free extracts of T. ferrooxidans and T. acidophilus. This oxidation was catalysed by a ferricyanide-dependent thiosulfate dehydrogenase.

Thiosulfate dehydrogenase was purified to homogeneity from cell-free extracts of T. acidophilus. With ferricyanide, the pH optimum for enzyme activity was 3. At pH 7, also TMPD, horse heart cytochrome c and cytochrome $c_{550}$ from T. versutus could be used as an electron acceptor, but the specific activity with ferricyanide was the highest. The molecular mass of the native enzyme was 103 kDa (gel filtration). The enzyme contained two different subunits with molecular masses of 29 and 23 kDa (SDS/PAGE), respectively. Both subunits contained a $c_{553}$-type haem, with absorption bands at 553, 524 and 416 nm. The $K_m$ for thiosulfate was 0.54 mM at pH 7 and could not be determined at pH 3 due to the chemical reactivity of thiosulfate at low pH values. Sulfite was a very potent inhibitor of enzyme activity.

Cell-free extracts of T. ferrooxidans and T. acidophilus stoichiometrically converted tetrathionate to thiosulfate and sulfur. This tetrathionate-hydrolysing enzyme in cell-free extracts of T. ferrooxidans was stimulated by sulfate or selenate concentrations up to 50 mM. In cell-free extracts of T. acidophilus optimum specific activity was found when the extract was prepared in 2 M ammonium sulfate at pH 3. Due to the high background sulfate concentrations in the enzyme assays, we have not yet been able to accurately measure the expected formation of equimolar amounts of sulfate in this reaction.

Cell-free extracts of T. ferrooxidans were able to oxidize sulfite at pH 3, but not at pH 6. Tests for a periplasmic ferricyanide- or cytochrome c-dependent sulfite-oxidizing enzyme were all negative. In addition, no cytoplasmic AMP-dependent activity could be found.

## Concluding remarks

1. From the foregoing discussion of our data on the bioenergetics and the enzymology of acidophilic thiobacilli one can conclude that in contrast with the model suggested by Sugio et al.(4) the sulfur and iron oxidation are not interlinked. Some other observations, not yet mentioned in this paper, support this conclusion. T. ferrooxidans growing in a tetrathionate-limited chemostat culture looses its iron-oxidizing capacity. T. acidophilus with a sulfur-oxidizing system that shows a high similarity with that of T. ferrooxidans doesn't oxidize ferrous iron. The sulfur oxidizing activities that we have observed in cell free extracts compare well with the in vivo activities and are 2 to even 250-fold higher than required for the observed rate of growth. In contrast the activities of the sulfur compound:ferric iron oxidoreductase (SFORase) studied by Sugio et al. (4) represent only 1% of the rate of ferrous iron production required to explain the observed oxygen uptake rate of 44 nmol $O_2$/min/mg protein. This suggests that the quantitative role of SFORase with sulfur compound oxidation may be only of minor importance.

2. The facultatively anaerobic nature of T. ferrooxidans might be of importance in the environment and in technical applications. This finding suggest that T. ferrooxidans might also play an important role in underground remote leaching processes and in anaerobic zones of mining waste dumps. The question whether this organism can grow anaerobically on mineral sulfides with ferric iron as electron acceptor still has to be answered.

3. It should be noted that in the enzymological studies sofar only water soluble sulfur species are included and that even sulfide is missing. We did, however, observe a very high sulfide oxidation activity of intact cells, but failed until now to detect the corresponding activity in cell free extracts, because an adequate enzyme test is not yet available. With respect to microbial metal leaching, the enzymology of sulfide oxidation, or even the oxidation of the sulfur moiety as present in the mineral is far more interesting. Recently, a sulfur oxygenase-reductase, catalysing the conversion of elemental sulfur to sulfide and sulfite has been found in the extremely thermophilic, facultatively anaerobic archaebacterium Desulfurolobus ambivalens (23). Whether this enzyme activity plays a role in the pathways of sulfur oxidation in T.

ferrooxidans and T. acidophilus is under investigation at the moment. If this is indeed the case, it would produce an elegant sulfur compound oxidation scheme (Figure 2). Oxidation of sulfide, sulfite and elemental sulfur is then short-circuited and oxidation of soluble sulfur compounds in T. ferrooxidans and T. acidophilus can be described by only 6 enzyme activities (Figure 2). However, this would still leave the gap in our knowledge with respect to mineral bound sulfur species oxidation.

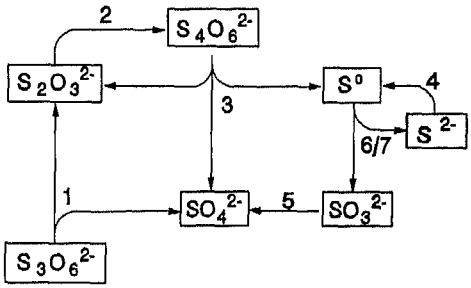


Figure 2: Hypothetic scheme for sulfur-compound oxidation in only 6 steps in acidophilic thiobacilli, assuming the presence of a sulfur oxygenase reductase (23). 1: trithionate hydrolase, 2: thiosulfate dehydrogenase, 3: tetrathionate hydrolase, 4: not yet characterized, 5: sulfite oxidizing enzyme, 6/7: sulfur oxygenase reductase.

References


1. G.J.M.W. Arkesteyn, "Contribution of microorganisms to the oxidation of pyrite" (Ph.D. thesis Agricultural University Wageningen, The Netherlands, 1980), 80 pp.

2. W. Hazeu, D.J. Schmedding, O. Goddijn, P. Bos and J.G. Kuenen, "The importance of the sulphur-oxidizing capacity of Thiobacillus ferrooxidans during leaching of pyrite," Proceedings 4th European Congress on Biotechnology 1987, Volume 3, ed. O.M. Neijssel, R.R. van der Meer, and K.Ch.A.M. Luyben (Amsterdam, Elsevier, 1987), 497-499

3. J.T. Pronk, R. Meulenberg, W. Hazeu, P. Bos and J.G. Kuenen, "Oxidation of reduced inorganic sulphur compounds by acidophilic thiobacilli," FEMS Microbiol. Rev. 75 (1990), 293-306.

4. T. Sugio, K.J. White, E. Shute, D. Choate, and R.C. Blake II, "Existence of a hydrogen sulfide:ferric iron oxidoreductase in iron-oxidizing bacteria," Applied and Environmental Microbiology 58 (1992), 431-433

5. A. Matin, "Keeping a neutral cytoplasm; the bioenergetics of obligate acidophiles," FEMS Microbiology Reviews, 75 (1990), 307-318

6. J.T. Pronk, P.J.W. Meesters, J.P. van Dijken, P. Bos, and J.G.Kuenen, "Heterotrophic growth of Thiobacillus acidophilus in batch and chemostat cultures," Archives of Microbiology, 153 (1990), 392-398

7. B. Alexander, S. Leach and W.J. Ingledew, "The relationship between chemiosmotic parameters and sensitivity to anions and organic acids in the acidophile Thiobacillus ferrooxidans," Journal of General Microbiology, 133, 1987, pp. 1171-1179.

8. G.S. Rao and L.R. Berger, "Basis of pyruvate inhibition in Thiobacillus thiooxidans," Journal of Bacteriology, 102 (1970), 462-466

9. J.T. Pronk, P. de Bruijn, J.P. van Dijken, P. Bos, and J.G. Kuenen, "Energetics of mixotrophic and autotrophic $C_1$-metabolism in Thiobacillus acidophilus," Archives of Microbiology, 154 (1990), 576-583

10. J.T. Pronk, W.M. Meijer, W. Hazeu, J.P. van Dijken, P. Bos and J.G. Kuenen, "Growth of Thiobacillus ferrooxidans on formic acid." Applied and Environmental Microbiology, 57 (1991), 2057-2062

11. E. Drobner, E. Huber and K.O. Stetter, "Thiobacillus ferrooxidans, a facultative hydrogen oxidizer," Applied and Environmental Microbiology, 56 (1990), 2922-2923

12. D.P. Kelly and A.P. Harrison, "Thiobacillus," Bergey's manual of systematic bacteriology, Vol.3, ed. J.T. Staley, M.P. Bryant, N. Pfennig and J.G. Holt (Baltimore, Williams & Wilkins, 1989), 1842-1858

13. T.D. Brock and J. Gustafson, "Ferric iron reduction by sulfur- and iron-oxidizing bacteria," Applied and Environmental Microbiology, 32 (1976), 567-571

14. J.T. Pronk, K. Liem, P. Bos and J.G. Kuenen, "Energy transduction by anaerobic ferric iron respiration in Thiobacillus ferrooxidans," Applied and Environmental Microbiology, 57 (1991), 2063-2068

15. A. Das, A.K. Mishra and P. Roy, "Anaerobic growth on elemental sulfur using dissimilar iron reduction by autotrophic Thiobacillus ferrooxidans," FEMS Microbiology Letters, 97 (1992), 167-172

16. J.T. Pronk, J.C. de Bruyn, P. Bos and J.G. Kuenen, "Anaerobic growth of Thiobacillus ferrooxidans," Applied and Environmental Microbiology, 58 (1992), 2227-2230

17. C.M. Corbett and W.J. Ingledew, "Is $Fe^{2+}/Fe^{3+}$ cycling an intermediate in sulphur oxidation by Thiobacillus ferrooxidans?" FEMS Microbiology Letters, 41 (1987), 1-16

18. W.J. Ingledew, "Thiobacillus ferrooxidans: the bioenergetics of an acidophilic chemolithotroph," Biochim. Biophys. Acta, 683 (1982), 89-117.

19. A.B. Roy and P.A. Trudinger, The biochemistry of inorganic compounds of sulphur (Cambridge, University Press, 1970)

20. R. Guay and M. Silver, "Thiobacillus acidophilus sp. nov.; isolation and some physiological characteristics," Canadian Journal of Microbiology, 21 (1975), 281-288

21. P.R. Norris, R.M. Marsh and E.B. Lindstrom, "Growth of mesophilic and thermophilic acidophilic bacteria on sulphur and tetrathionate," Biotechnology and Applied Biochemistry, 8 (1986) 313-329

22. J. Mason, D.P. Kelly and A.P. Wood, "Chemolithotrophic and autotrophic growth of Thermotrix thiopara and some thiobacilli on thiosulphate and polythionates, a reassessment of the growth yields of T. thiopara in chemostat cultures," Journal of General Microbiology, 33 (1987), 1249-1256.

23. A. Kletzin, "Coupled enzymatic production of sulfite, thiosulfate and hydrogen sulfide from sulfur: purification and properties of a sulfur oxygenase reductase from the facultatively anaerobic archaebacterium Desulfurolobus ambivalens," Journal of Bacteriology, 171 (1989), 1638-1643.