Stellingen behorende bij het proefschrift van Jack Pronk:
Physiology of the acidophilic thiobacilli

1. Alexander et al. (1987) stellen ten onrechte dat de vergelijking:
   \[ \text{pH}_e = pK_a + \log[A_e/A_{o_e}(1 + 10^{10^{pH_e-pK_a}})] \]
   de verdeling van zuurrest-anionen over de cytoplasmatische membraan beschrijft.

2. De conclusie van Barros et al. (1984) dat de door hen gebruikte Thiobacillus ferrooxidans in staat is tot mixtrote groei op glucose en tweewaardig ijzer is, gezien de door hen waargenomen groeiregensten, waarschijnlijk onjuist.

3. De karakterisering van Thiobacillus ferrooxidans als obligaat aeroob organism beheeft nader onderzoek.
   Hoofdstuk 8 van dit proefschrift

4. Bij het interpreteren van experimenten betreffende glucosetransport en -metabolisme in Gluconobacter oxydans hadden Pronk et al. de mogelijkheid van gefaciliteerde diffusie van glucose in de discussie moeten betrekken.
   Pronk et al. (1989) Enzyme and Microbial Technology 11: 160-164

5. Van Schie et al. stellen ten onrechte dat de molaire groeiregenstand van acetaatgelimiteerde cultures van Acinetobacter calcoaceticus onafhankelijk is van de pH waarbij het organism gekeukt wordt.
   H.J. Noorman en J.T. Pronk, ongepubliceerde waarnemingen

6. Over het metabolisme van waterstofperoxyde in Acetobacter peroxidans is minder bekend dan de naam van dit organism doet veronderstellen.

7. Recent werk met 16S RNA, geïsoleerd uit natuurlijke populaties, bevestigt de stelling dat het gebruik van een initiële ophopingsstap voor het isoleren van dominante bacteriën uit dergelijke populaties een grote kans tot mislukken in zich draagt.

8. De uitzondering die in de Kernenergiwowet wordt gemaakt voor natuurlijke radio-isotopen kan in isotopenlaboratoria, bijvoorbeeld bij gebruik van niet verrijkt uranylacetaat, aanleiding geven tot verwarring situaties.

9. Recent onderzoek aan Vibrio cholerae laat zien dat de verwensing "krijg de kolere" aan kracht verliest wanneer deze gebezigd wordt tijdens een theekransje.
   Toda et al. (1989) Letters in Applied Microbiology 8: 123-125

10. Gezien de inhoud van dit proefschrift is het wellicht beter te spreken van een "pH3 thesis" dan van een "PhD thesis".
Physiology of the acidophilic thiobacilli
Physiology of the acidophilic thiobacilli

Proefschrift

ter verkrijging van de graad van doctor aan de Technische Universiteit Delft, op gezag van de Rector Magnificus, prof. drs. P.A. Schenck, in het openbaar te verdedigen ten overstaan van een commissie aangewezen door het College van Dekanen op 15 oktober 1991 te 14.00 uur.

door

Jacobus Thomas Pronk
geboren te Broek op Langendijk

doctorandus biologie
Dit proefschrift is goedgekeurd door de promotor
prof. dr. J.G. Kuenen
Eh, Ernie, waarom heb jij die banaan in je oor?
Dat is om de krokodillen weg te jagen, Bert
Maar er zijn hier toch helemaal geen krokodillen, Ernie?
Zie je wel dat het werkt, Bert!

Bert en Ernie uit Sesamstraat

aan mijn ouders
voor Patricia
Voorwoord

Het boekje dat voor U ligt is het resultaat van vier jaar ploeteren. Als alle proefjes echter in één keer gelukt waren, had het misschien ook wel in twee jaar gekund. Deze vaststelling toont al aan dat promoveren een kwestie van vallen en (af en toe) opstaan is. Gelukkig heb ik the growing pains of a PhD student niet alleen hoeven doormaken.

Het niet aflatende enthousiasme van Hans van Dijken is voor mij tijdens m’n promotietijd de grootste wetenschappelijke inspiratiebron geweest. Hans, de manier waarop jij een -op een prettige manier-fanatieke beoefening van de wetenschap kunt combineren met een warme belangstelling voor zaken buiten het werk, heeft mijn diepe bewondering. De door jou georganiseerde trips naar Engeland waren hoogtepunten in de afgelopen vier jaar (en niet aléén in gastronomisch opzicht).

Gijs Kuenen en Piet Bos bedankt ik voor het scheppen van de voorwaarden voor het onderzoek en voor het kritisch bekijken van mijn manuscripten. Gijs, door je dekanaat moest er soms wat geïmproviseerd worden (ik herinner me een “werkbesperking” op een hotelkamer, rond middernacht), maar je interesse was steeds zeer stimulerend. Piet, bedankt voor het telkens weer schrijven van de bonnen en, veel belangrijker, voor je betrokkenheid bij het onderzoek en de patent-verwikkelingen.

Bij technische storingen en ongelukjes deed ik nooit tevergeefs een beroep op instrumentmakerij, glasblazerij, elektronische dienst of (when all else fails) Jos Lispet. Max, Nico, Pascal en Astrid, bedankt voor het verzorgen van schoon glaswerk, chemicaïën en het steriliseren van apparatuur en media.

Dick, Karel, Peter, Prisca, Rogier en Wilmar, bedankt voor de bijdragen die jullie (dag en nacht) als studenten hebben geleverd aan dit boekje en aan de prettige sfeer op de kamer (en) daarbuiten.

Mijn onderzoek richtte zich op bacteriën, maar op het lab was ik gehuisvest temidden van gistfysiologen. Diepgaande meningsverschillen over de voor- en nadelen van beide typen microorganismen waren aan de orde van de dag, maar de sfeer op de kamer bleef prima: gesprekken over het werk werden naadloos afgewisseld met nabeschouwingen van sportevenementen, moppen en limericks van zeer uiteenlopende kwaliteit, cryptogrammen, plannen voor het opruimen van de kamer en vooral veel koffie. Van de kamervanhouders wil ik speciaal Ruud *5.1* Weusthuis bedanken voor al zijn hulp met the lay-out van dit boekje.

Alle vaste en tijdelijke medewerkers van het lab: bedankt voor alle adviezen, hulp en gezelligheid!

Mijn huisgenoten in Leiden slaagden er steeds in mijn gedachten op andere zaken te zetten, wanneer ik me weer eens *’iets’* te veel door de proefjes liet meeslepen (ik zal dit maar niet als excuus aanvoeren voor mijn geringe bijdrage aan het corvee-gebeuren). Berna, Daan, Ellie, Helen, Joost en Maarten: Bedankt!

Dit proefschrift is opgedragen aan mijn ouders. Pap en mam, hardstikke bedankt voor alle steun en interesse, beslissende duwtjes in de rug en, af en toe, de was.

Veel belangrijker dan de proeven die in dit boekje beschreven staan was voor mij de *spin-off* van mijn promotie. Lieve Patricia, woorden schieten tekort. Ik citeer daarom je (één na?) favoriete huisgenoot: miauw!

Jack Pronk
Delft, 8 juli 1991
## Contents

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 1</td>
<td>General Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Chapter 2</td>
<td>Oxidation of reduced inorganic sulphur compounds by acidophilic thiobacilli</td>
<td>11</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>Heterotrophic growth of <em>Thiobacillus acidophilus</em> in batch and chemostat cultures</td>
<td>24</td>
</tr>
<tr>
<td>Chapter 4</td>
<td>Mixotrophic and autotrophic growth of <em>Thiobacillus acidophilus</em> on glucose and thiosulphate</td>
<td>32</td>
</tr>
<tr>
<td>Chapter 5</td>
<td>Energetics of mixotrophic and autotrophic C$_4$-metabolism by <em>Thiobacillus acidophilus</em></td>
<td>41</td>
</tr>
<tr>
<td>Chapter 6</td>
<td>Growth of <em>Thiobacillus ferrooxidans</em> on formic acid</td>
<td>51</td>
</tr>
<tr>
<td>Chapter 7</td>
<td>Growth of <em>Thiobacillus ferrooxidans</em> on formic acid: a patent application</td>
<td>59</td>
</tr>
<tr>
<td>Chapter 8</td>
<td>Energy transduction by anaerobic ferric iron respiration in <em>Thiobacillus ferrooxidans</em></td>
<td>61</td>
</tr>
<tr>
<td>Summary</td>
<td></td>
<td>68</td>
</tr>
<tr>
<td>Samenvatting</td>
<td></td>
<td>70</td>
</tr>
</tbody>
</table>
CHAPTER 1

General Introduction

Life in extreme environments

Microorganisms have evolved to live, thrive and survive under conditions which may be harmful and even life-threatening to higher life forms. Perhaps the most striking example of microbial endurance is provided by certain extremely thermophilic bacteria which have been isolated from volcanic thermal waters. Growth of such organisms at temperatures above 100 °C is now well documented (Stetter et al. 1990).

In addition to microorganisms equipped to withstand the physical stress imposed by very high (or low, [Barros and Morita 1978]) temperatures, hydrostatic pressure (Marquis and Matsumura 1978) or radiation (Nasim and James 1978), others are capable of growth in environments of which the chemical composition is completely different from that of their cytoplasm. In this respect, one should realize that the maintenance of large gradients of solutes across the cytoplasmic membrane is essential for all microorganisms, including those living under conditions which are not generally regarded as 'extreme' (Konings and Michels 1980). Microorganisms have been isolated from environments containing high concentrations of salts (Kushner 1978), organic solvents (Inoue and Horikoshi 1989) and other potentially toxic compounds (Ehrlich 1978). A special group of extremophiles comprises organisms which grow optimally in either very alkaline environments (alkaliphiles) or in acidic environments (acidophiles).

The nomenclature used in the literature to describe microorganisms capable of growth under extreme conditions is complex and often confusing. For example, in a recent review no less than ten group names have been suggested to 'label' organisms according to the temperature spans that permitted growth (Wiegel 1990). The situation is further complicated by the fact that some microorganisms combine various extremophilic characteristics (e.g. 'extremely acidophilic hyperthermophiles'; Stetter et al. 1990). In this thesis, the term 'acidophilic' will be used for organisms which grow optimally below pH 4 and are not capable of growth at neutral pH.

Acidophilic microorganisms have received much attention in microbiological research. This interest is partially fuelled by the economical significance of these organisms for industrial processes such as microbial leaching of metal ores (Norris and Kelly 1988, Brierley 1990) and microbial desulphurization of coal (Bos et al. 1988, Klein et al. 1988). A more fundamental problem which has attracted attention is the question how these organisms perform membrane-associated energy-transducing processes while maintaining a neutral cytoplasm.

Energy transduction and cytoplasmic pH homeostasis in acidophiles

Microorganisms depend upon external energy sources to drive processes in the cell which require a net input of energy. Depending on the type of microorganism, energy may be derived from light (phototrophic growth) or from the conversion of a variety of organic or inorganic substrates (chemotrophic growth).

The energy budget of microbial cells consists of two major 'currencies' (not considering storage materials). First, energy may be stored in the phosphate ester bonds of compounds like adenosine triphosphate, 1,3-bisphosphoglycerate or phosphoenolpyruvate. Secondly, energy may be stored as electrochemical gradients of solutes (in particular protons) across biological membranes (Mitchell 1966). Electrochemical gradients and chemical bond energy can be interconverted via membrane-associated solute translocation systems (Mitchell 1966). Evidently, the bacterial membrane plays a central role in these energy-transducing processes.

It is now generally accepted that several energy-transducing processes, including respiration and phototrophic light absorption (Mitchell 1966), can be coupled to an outward translocation of protons across the cytoplasmic membrane (in eukaryotes, these so-called primary transport mechanisms are located in membranes of specialized organelles: mitochondria and chloroplasts). In some prokaryotes, efflux of metabolic end products across the cytoplasmic membrane can also be coupled to a net translocation of protons (Michels et al. 1979). Since protons are monovalent cations, their translocation leads to the formation of both a chemical potential difference (\( \ln 10 \, \text{RT/F} \, \Delta \text{pH} \)) and an electrical potential difference (\( \Delta \psi \)) across the energy-coupling membrane. Both contribute to the so-called proton motive force according to the equation:

\[
\Delta \rho = \Delta \psi - (\ln 10 \, \text{RT/F}) \, \Delta \text{pH} \, (\text{mV})
\]
(R is the universal gas constant, F is Faraday's constant and T is the temperature in K). In bacteria, the magnitude of the proton motive force is usually between -150 and -220 mV (Kashket 1985).

Specialized membrane-associated proteins couple the influx of protons down the electrochemical gradient to various energy-requiring processes, including transport of various solutes (Konings and Michels 1980), motility (Manson et al. 1977) and the phosphorylation of adenosine diphosphate (Futai and Kanazawa 1983).

It is an interesting question whether growth in acidic environments may interfere with the central role of proton gradients in energy-transducing processes. Indeed, the mechanisms involved in cytoplasmic pH homeostasis in acidophiles have received much attention (for reviews see Ingledew 1982, Cobley and Cox 1983, Krulwich and Guffanti 1983, Booth 1985, Matin 1990). The intracellular pH of a large number of acidophilic bacteria has been measured, using a variety of methods. In all cases investigated, the cytoplasmic pH was between 5 and 7 over a wide range of external pH values (Kashket 1985, Matin 1990). This implies that, at an external pH of 3, the chemical potential difference of protons across the cytoplasmic membrane is between approximately -120 and -240 mV. Also when the external pH is decreased to 2 or lower, the bacteria are capable of maintaining a near-neutral cytoplasmic pH (Ingledew 1982, Cobley and Cox 1983, Michels and Bakker 1985, Matin 1990). In such cases, the pH gradient across the cytoplasmic membrane may exceed 5 pH units, corresponding to a chemical potential difference of -300 mV. In the absence of a Δψ, this would already lead to an extremely high proton motive force. However, in the acidophiles which have been investigated this situation does not occur. On the contrary, the overall magnitude of the proton motive force of energized cells is essentially independent of the external pH (Michels and Bakker 1985, Matin 1990). Since, as mentioned above, the intracellular pH also remains constant, this implies that Δψ must change. Indeed, the magnitude of this component of the proton motive force has been demonstrated to decrease with decreasing external pH. At very low external pH values, Δψ may even change polarity, becoming inside positive (Ingledew 1982, Cobley and Cox 1983, Michels and Bakker 1985, Matin 1990).

An example of the regulation of ΔpH and Δψ in an acidophilic bacterium is given in Fig. 1 (Michels and Bakker 1985). The acidophile Bacillus acidocaldarius maintains a cytoplasmic pH of around 6.8 over a range of extracellular pH values from 2 to 5 (Michels and Bakker 1985). At pH values above 3, Δψ is inside negative, thus adding up to the already highly negative contribution of ΔpH to the proton motive force. At pH values below 3 however, Δψ changes polarity and becomes inside negative. Thus, at these very low external pH values Δψ compensates the high ΔpH and, consequently, the proton motive force does not increase any further. Clearly, growth at low pH values affects the composition, but not the overall magnitude of the proton motive force. Similar observations have been made with other acidophilic bacteria (Ingledew 1982, Matin 1990).

In bacteria which grow at near-neutral pH values, a temporary decrease of the proton motive force, e.g. as a result of starvation, will not lead to excessive acidification of the cytoplasm. In acidophiles however, dissipation of the chemical component of the proton motive force would lead to a dramatic decrease of the cytoplasmic pH and, consequently, to irreversible denaturation of cytoplasmic proteins.

Extensive studies have revealed that acidophilic bacteria are capable of maintaining large pH gradients over the cytoplasmic membrane, even after having been metabolically compromised by starvation or by the addition of respiratory inhibitors or ATPase inhibitors (Matin 1990). Since under these conditions the cytoplasmic pH cannot be poised at near-neutral values by active proton translocation, energy-independent mechanisms are apparently involved in intracellular pH homeostasis. Indeed, in energy-depleted cells of acidophilic bacteria, Δp becomes virtually zero (Matin 1990). The ΔpH that could still be measured under these conditions was completely compensated by an inside positive Δψ (Matin 1990).
Two mechanisms have been proposed to explain these observations (Matin 1990): i) the presence in the cytoplasm of impermeant proton-accepting groups may lead to the generation of an inside positive Donnan potential (Donnan 1924), and ii) influx of protons into the cells may rapidly generate a counterforce in the form of a positive $\Delta \psi$ (proton influx potential) and thus become self-limiting before $\Delta p$ is fully collapsed. These inside positive electrical potentials would prevent further influx of protons by balancing $\Delta p$. Recent work (reviewed by Matin (1990)) suggests that both mechanisms are involved in $\Delta p$ homeostasis in energy-deprived cells.

Effects of organic acids on acidophilic bacteria

It is well-known that small organic acids exert a negative effect on growth and substrate oxidation by acidophilic bacteria (Schmitt and Lundgren 1965, Rao and Berger 1970, Ingledew 1982, Alexander et al. 1987). In addition to this, heterotrophic acidophiles are generally unable to utilize compounds like acetate, pyruvate and glycolate as carbon sources for growth in batch cultures (Darland and Brock 1971, Guay and Silver 1975, Harrison 1981).

At the low pH values at which acidophiles grow, weak organic acids occur almost completely in the non-dissociated (protonated) form. On the contrary, at the near-neutral cytoplasmic pH, these acids occur mainly in the dissociated form. Since the non-dissociated (electronaut) form is much more membrane-permeable than the dissociated (negatively charged) molecule, a net influx of the non-dissociated molecule will occur (Fig. 2). The subsequent dissociation of the translocated acid in the cytoplasm leads to a decrease of $\Delta p$, explaining the toxicity of these compounds (Ingledew 1982, Alexander et al. 1987). The toxicity of organic acids may be further augmented if the anion interferes with cellular metabolism (Booth 1985).

In addition to their toxic effects, organic acids are of interest because of their application for $\Delta p$ measurements (for a review see Kashket 1985). If only the non-dissociated (non-charged) form of the organic acid is membrane-permeable, accumulation of the organic acid can be described by the equation:

$$\frac{A_{in}}{A_{out}} = \frac{10^{pH_{in} - pK_a + 1}}{10^{pH_{out} - pK_a + 1}}$$

where $A_{in}$ and $A_{out}$ are the internal and external concentration of the organic acid (non-dissociated plus dissociated form) and $pK_a$ is the dissociation con-

stant of the acid. Only when $pK_a << pH_{out} - pH_{in}$ and $pH_{out} \geq 2$, this equation can be simplified to $A_{in}/A_{out} = 10^{[a/(a+1)]}$. Therefore, the common idea that organic acids are accumulated n-fold at $\Delta p = n$ is not always correct.

When accumulation of organic acids is used to calculate $\Delta p$, a number of points should be taken into account (Booth 1985, Kashket 1985):

- the concentration of the organic acid should be sufficiently low to avoid dissociation of $\Delta p$ by influx of the non-dissociated acid.
- the observed accumulation ratio should be corrected for (aspecific) binding to cellular components
- the organic acid used for $\Delta p$ measurements may not be transported by specific (active) carrier systems
- the acid should not be metabolized by the cells.

A special situation occurs when a weak organic acid is metabolized after its entry in the cytoplasm. When the rate of influx exceeds the rate of metabolism, dissociation of the acid in the cytoplasm will lead to a net import of protons and therefore to a decrease of $\Delta p$. $\Delta \psi$ will not be affected by influx of the organic acid. When, for example in substrate-limited chemostat cultures, the rate of organic acid metabolism equals the rate of its influx, $\Delta p$ will not be affected. Therefore, diffusion of non-dissociated organic acids

![Diagram](image-url)
Table 1. Characteristics of the acidophilic *Thiobacillus* species (Kelly and Harrison 1989)

<table>
<thead>
<tr>
<th></th>
<th><em>T. ferrooxidans</em></th>
<th><em>T. thiooxidans</em></th>
<th><em>T. albertii</em></th>
<th><em>T. acidophilus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>metabolism</td>
<td>obligately autotrophic</td>
<td>obligately autotrophic</td>
<td>obligately autotrophic</td>
<td>facultatively autotrophic</td>
</tr>
<tr>
<td>pH optimum</td>
<td>ca. 2.5</td>
<td>2.3</td>
<td>3.5-4.0</td>
<td>2.5-3.0</td>
</tr>
<tr>
<td>pH range for growth</td>
<td>1.3-4.5</td>
<td>0.5-5.5</td>
<td>2.0-4.5</td>
<td>1.5-5.5</td>
</tr>
<tr>
<td>substrates for autotrophic growth</td>
<td>inorganic sulphur compounds, ferrous iron, sulphidic minerals</td>
<td>inorganic sulphur compounds</td>
<td>inorganic sulphur compounds</td>
<td>inorganic sulphur compounds</td>
</tr>
<tr>
<td>substrates for heterotrophic growth</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>various simple organic compounds</td>
</tr>
</tbody>
</table>

is not an energy-requiring process when influx and subsequent metabolism are tightly coupled.

The acidophilic thiobacilli

The genus *Thiobacillus* (Beijerinck 1904, Kelly and Harrison 1989) consists of Gram-negative bacteria which share the ability to grow autotrophically and derive energy from the oxidation of reduced inorganic sulphur compounds to sulphate. The genus is extremely heterogeneous, as is evident from G + C contents of total DNA, DNA homologies, optimal growth conditions and other physiological characteristics (Kelly and Harrison 1989).

In the latest edition of *Berger’s Manual for Determinative Bacteriology* four acidophilic *Thiobacillus* species are recognized: *T. ferrooxidans*, *T. thiooxidans*, *T. acidophilus* and *T. albertii* (Kelly and Harrison 1989). Some general characteristics of these organisms are given in Table 1. The acidophilic thiobacilli are all mesophiles, with temperature optima around 30°C. The pH optima for growth are around 2.5 - 3.0, with the exception of *T. albertii*, which has a higher pH optimum of 3.5 - 4.0.

*T. ferrooxidans* was first isolated from acidic mine drainage water (Temple and Colmer 1951) and has since been isolated from a variety of acidic environments. *T. ferrooxidans* is unique among the acidophilic thiobacilli in its ability to oxidize ferrous iron and sulphidic minerals. A further interesting feature of this organism is its ability to oxidize elemental sulphur anaerobically with ferric iron as an electron acceptor (Brock and Gustafson 1976). It is as yet unclear if this process has any physiological significance, e.g. by generating a proton motive force.

Over the years, there have been numerous reports claiming heterotrophic or mixotrophic growth of *T. ferrooxidans* (Shafia and Wilkinson 1969, Shafia et al. 1972, Tabita and Lundgren 1971a,b, Barros et al. 1984). However, these claims have not been substantiated and have probably been caused by contamination of the *T. ferrooxidans* cultures with (facultatively) heterotrophic acidophiles (Kelly and Harrison 1989). Indeed, there are numerous reports of the isolation of such acidophilic heterotrophs from reputedly pure cultures of *T. ferrooxidans* (Zavarzin 1973, Markosyan 1973, Guay and Silver 1975, Arkesteyn and de Bont 1980, Harrison et al. 1980). These heterotrophs survive during prolonged cultivation of *T. ferrooxidans* in inorganic media, probably by scavenging low concentrations of organic compounds excreted by *T. ferrooxidans*. In this respect it has been suggested that the removal of (toxic) organic compounds by the heterotrophs may also be advantageous to *T. ferrooxidans* (Harrison 1984, Wichlacz and Thompson 1988).

A facultative autotroph, *T. acidophilus* was initially isolated as a contaminant of a ferrous iron-grown *T. ferrooxidans* culture (Guay and Silver 1975). It resembles *T. ferrooxidans* in its ability to grow autotrophically on a variety of inorganic sulphur compounds (Kelly and Harrison 1989). However, *T. acidophilus* is unable to oxidize ferrous iron and sulphidic minerals. Another facultatively autotrophic isolate, *T. organoparas*, was originally isolated from acidic mine water (Markosyan 1973). Based on DNA homology and physiological characteristics, *T. organoparas* is now regarded as a strain of *T. acidophilus* (Kelly and Harrison 1989). Judged by the homology of SS RNA sequences, *T. acidophilus* is more related to the obligate acidophilic heterotroph *Acidiphilium cryptum* than to the obligate autotrophic thiobacilli (Lane et al. 1985).

The obligate chemolithoautotroph *T. thiooxidans* can only use inorganic sulphur compounds as electron
donors for growth (Kelly and Harrison 1989). Some strains of this organism were originally designated as *T. concretivorus* (Kelly and Harrison 1989). *T. albertis* (Bryant *et al.* 1983) shows little DNA homology with *T. thiopxidans*, but its physiological characteristics seem to be very similar to those of the latter organism (Table 1, Kelly and Harrison 1989).

The biological reactions involved in the oxidation of inorganic sulphur compounds by the acidophilic thiobacilli are still poorly understood. An overview of the relevant literature is given in Chapter 2 of this thesis (Pronk *et al.* 1990). Apart from being of fundamental interest because of the pathways involved in inorganic sulphur metabolism and the physiological mechanisms of acidophilic growth, the acidophilic thiobacilli have a substantial economic impact, which will be discussed below.

**Economic applications and environmental implications**

The best known and economically most significant application of acidophilic bacteria is the microbial leaching of metal ores. This process accounts for approximately 18% of the production of copper in the USA (Torma 1986). Microbial leaching has also been successfully applied for the recovery of uranium (Torma 1988, Acharya 1990). Application of this technique for the recovery of other metals, including gold, nickel and cadmium is under investigation (Lawrence *et al.* 1986) or has recently been used in industrial scale operations (Torma 1988, Acharya 1990).

The main aim of microbial leaching is the release of metals in solution. Use of this method is usually limited to metal ores that can not be treated economically by conventional physical or chemical techniques. For example, microbial leaching is used on a large scale to treat so-called 'tailings'; low-grade ores which remain after conventional mining (Torma 1988). A large number of reactions may be involved in microbial leaching of metal ores. The multi-disciplinary research field studying the chemistry, biology and technological applications of these reactions has been named biohydrometallurgy. Some of the key reactions involved in microbial leaching are catalysed directly by the microorganisms, others involve chemical reactions of products formed by the bacteria (Fig. 3). Many metals are present in ore bodies as sulphide minerals. Such metal ions can be released by biological oxidation of the sulphur moiety of the mineral (Fig. 3, Torma 1988). Some metals (e.g. gold) may occur as inclusions in pyrite (FeS2). The latter mineral can be oxidized by the bacteria, leading to disclosure of the precious metal, which facilitates further processing and recovery by hydro-metallurgical techniques (Torma 1988). By oxidizing ferrous iron present in the ores (e.g. in the form of pyrite), the microorganisms produce ferric iron. This may subsequently act as an oxidant, causing the solubilization of metal ions (Fig. 3, Torma 1988). The metals can be recovered from solution by a process known as cementation. This encompasses a redox reaction of the dissolved metal ions, for example copper, with a base metal (e.g. scrap iron), leading to its deposition (Lawrence *et al.* 1986). Alternatively, electrowinning and solvent extraction methods may be used (Lawrence *et al.* 1986).

Microbial leaching of metals is mostly carried out under poorly defined conditions. Often, sulphide minerals present in mine waste dumps are leached by spraying with water or slightly acidic solutions, sometimes containing bacteria (dump leaching). In some cases, the geometry of the low-grade ore heaps is designed to optimize parameters like oxygen and heat transfer (heap leaching). Leaching under strictly controlled and optimized conditions (tank leaching) is currently under investigation (Acharya 1990).

As evident from the application of microbial leaching techniques, the oxidation of sulphidic minerals by acidophilic thiobacilli may be economically profitable. However, this process may also have negative environmental consequences. For example, the highly acidic process waters generated during microbial leaching operations, which generally contain high concentrations of toxic metals, have to be treated prior to their release into the environment (Lawrence *et al.* 1986).

Also coal, one of the major fossil fuel sources, is often rich in inorganic sulphur, which occurs mainly in the form of pyrite. Drainage waters of coal mines may become very acidic, leading to substantial environmental problems (Lundgren *et al.* 1972). A related environmental problem is that of the so-called 'acid sulphate soils'. These are soils which, prior to agricultural exploitation, were rich in pyrite. When such soils are drained and brought into culture,

<table>
<thead>
<tr>
<th>Rate Equation</th>
<th>1</th>
<th>CuS + O2 → CuSO4</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>FeS2 + H2O + 7/2 O2 → Fe2(SO4)3 + H2SO4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>UO2 + Fe3(SO4)2 → UO2SO4 + 2FeSO4</td>
<td></td>
</tr>
</tbody>
</table>

III: Chemical oxidation of metal salts by ferric iron formed as a result of microbial action (e.g. from reaction II). In the case of sulphidic minerals, also the sulphur moiety of the mineral may be oxidized by Fe3+ (not shown). A common feature of these reactions is that essentially insoluble metal salts are converted to products which are soluble in acidic solutions.
microbial and chemical oxidation of pyrite lead to extreme acidification and formation of acid hydroxy ferric sulphate (jarosite) which cements clay particles to form impermeable clumps. Both processes lead to loss of crop productivity (Alexander 1977).

The high sulphur content of some coals is a major problem associated with their use as a fuel. During combustion, sulphur dioxide is produced. This compound is one of the major causative agents of the current 'acid rain' problem. Methods are currently being developed to remove pyritic sulphur from coal by microbial oxidation (Bos and Kuenen 1990). In particular, the microbial process may be a serious candidate for coal desulphurization when coal-water mixtures are used (Armson 1986, Bos and Kuenen 1990). Grinding of coal increases the area of the coal susceptible to microbial action. T. ferrooxidans is generally considered to be one of the major catalysts of pyrite oxidation in metal ores and sulphur-containing ores. Indeed, the organism can be readily isolated from acid mine drainage waters and from sulphidic ores and coals which have been exposed to moisture. Furthermore, pure cultures of the organism are effective in oxidizing pyrite. However, it has been demonstrated that during experiments with non-sterilized coal samples, T. ferrooxidans did not become a dominant organism, even when the samples were inoculated with T. ferrooxidans (Muyzer et al. 1987).

Control experiments with sterilized coal samples showed that T. ferrooxidans could grow well on the pyrite present in this coal. These observations suggest that bacteria other than T. ferrooxidans may be the prime bacteria involved in pyrite oxidation. In this respect, it is interesting to note that pyrite oxidation can also be catalysed by defined mixed populations of acidophilic sulphur-oxidizing thiobacilli (T. thiooxidans, T. acidophilus) and the ferrous iron-oxidizing acidophile Leptospirillum ferrooxidans (Norris and Kelly 1978).

Since the oxidation of pyrite is an exergonic process, oxidation of high concentrations of pyrite in large-scale operations, where loss of process heat is minimal, may cause the temperature to rise to values above 50 °C. Therefore, in addition to the acidophilic thiobacilli, thermophilic pyrite-oxidizing microorganisms are also candidates for a microbial coal desulphurization process. Also the microbial removal of organic sulphur compounds from coal is currently under investigation. However, these studies generally focus on neutrophilic, heterotrophic bacteria (Bos and Kuenen 1990).

The formation of sulphuric acid and ferric iron by the acidophilic thiobacilli has often been implicated in corrosion phenomena (Ford and Mitchell 1990). Typical in this context is the name Thiobacillus concretivorus ('concrete-eating thiobacillus'), which was originally given to a strain of T. thiooxidans (Parker 1945).

Studies of the physiology of the acidophilic thiobacilli are required for an increased understanding of their role in natural environments and in industrial leaching operations. Such fundamental knowledge may be applicable both for the optimization of industrial processes and for control of the adverse effects of the biological activities of these bacteria.

Chemostat cultures as a tool for studies of the acidophilic thiobacilli

Over the past decades, chemostat cultivation has become an indispensable technique for studies into the physiology of microorganisms. The growth rate of microorganisms in chemostat cultures can be controlled by manipulating the rate of addition of a growth-limiting substrate. Since other growth conditions can also be strictly defined, the effect of individual growth parameters can be accurately determined.

The principles and general advantages of chemostat cultivation have been discussed in many papers (for an overview see Pirt 1975). Therefore, no general discussion of this technique will be given here. Some specific advantages of chemostat cultures for the cultivation of the acidophilic thiobacilli are summarized below.

Some of the inorganic sulphur compounds utilized by the acidophilic thiobacilli are not stable under acidic conditions. For example, in aqueous solutions, thiosulphate readily decomposes at low pH (Johnston and McAmish 1973). The rate of such chemical decomposition reactions strongly depends on the concentration of the inorganic sulphur compounds. In substrate-limited chemostat cultures, the very low residual substrate concentrations enable the bacteria to compete successfully with the chemical reactions. Problems with instability do not occur in the reservoir media, since these can be kept at neutral pH. The pH of the cultures is automatically kept at a low value by the biological production of sulphuric acid from the reduced sulphur compounds. In fact, this production of acid is often so vigorous that the cultures have to be titrated with alkali to maintain the culture pH at 3.

Apart from the instability of sulphur compounds at low pH, also the toxicity of some of these compounds may be a problem. For example, growth inhibition by tetraionate has been reported for the acidophiles T. ferrooxidans and T. acidophilus (Eccleston and Kelly 1978, Mason and Kelly 1988). The use of substrate-limited chemostat cultures prevents accumulation of toxic growth substrates or intermediates in the culture medium and thereby permits growth.
Under substrate-limited growth conditions, sequential utilization of substrates (diauxic growth) can be prevented. Chemostat cultures are therefore ideally suited to study the simultaneous utilization of substrates by microorganisms. Such studies may provide insight into the energetics of microbial growth. Furthermore, growth on carefully defined mixtures of substrates may be used to optimize the production of enzymes (e.g. for biochemical studies), by increasing both the amount of biomass produced and its enzyme content.

Simultaneous utilization of ferrous iron and reduced inorganic sulphur compounds by substrate-limited chemostat cultures of *T. ferrooxidans* has been studied by Hazeu et al. (1987). This work has led to the conclusion that the bioenergetic value of reduct equivalents derived from sulphur oxidation is higher than that of ferrous iron reduct equivalents (see also Chapter 2).

Facultatively autotrophic bacteria grown in chemostats can simultaneously utilize organic and inorganic substrates. In many cases, the growth yields of such mixotrophic cultures are higher than the sum of the growth yields in autotrophic and heterotrophic cultures. This can be explained from the fact that the bacteria use the inorganic substrate to energize the assimilation of the organic substrate. Only under conditions where the assimilation of the organic substrate reaches its theoretical maximum, the inorganic substrate is used to energize the energetically less favourable process of CO$_2$ assimilation (Gommers et al. 1988, see also Chapters 4 and 5). Simultaneous utilization of organic and inorganic substrates has been studied with the facultatively autotrophic thiobacilli *T. versutus* and *T. novellus* (Gottschal and Kuenen 1980, Beudeker et al. 1982, Leefeldt and Matin 1980, Perez and Matin 1980). However, growth of the acidophilic facultative autotroph *T. acidophilus* on mixed substrates in chemostat cultures has not been studied in detail (Mason and Kelly 1988).

If, in growing cells of acidophilic bacteria, an increase of the energy demand occurs in order to maintain a neutral cytoplasm, this will most probably result in an increased maintenance energy requirement for growth. As discussed above, experimental work by various authors with resting cell suspensions of acidophilic bacteria (reviewed by Matin 1990) suggests that maintenance of ΔpH in acidophiles does not increase the energy demand on the cells. Determination of the maintenance energy requirement of acidophilic bacteria grown at different pH values in chemostat cultures may give an indication whether this conclusion also holds for growing cells.

**Scope and context of this thesis**

Within the framework of the Environmental Biotechnology group of the Universities of Delft and Leiden, a multidisciplinary research project focuses on the industrial application of acidophilic bacteria for metal leaching, coal desulphurization and the treatment of sulphur-containing waste streams. In this context, the Department of Microbiology and Enzymology of the Delft University of Technology is involved in fundamental research into the physiology of the acidophilic thiothrix. Research in this field is focused at two topics: the oxidation of inorganic sulphur compounds by these bacteria and, more in general, the physiology of growth in acidic environments. The current knowledge on sulphur compound oxidation by the acidophilic thiobacilli and the relevance of this process for the industrial applications of these bacteria is discussed in Chapter 2.

Until recently, the obligately chemolithoautotrophic acidophile *Thiobacillus ferrooxidans* has been used as a model organism for these studies. Although studies on intact cells of *T. ferrooxidans* have increased our understanding of inorganic sulphur compound metabolism, cell-free experimental systems (e.g. purified enzymes) are essential to identify intermediates and to elucidate pathways involved in sulphur metabolism. A major drawback of the use of *T. ferrooxidans* for such studies is that biomass yields of this organism are very low due to its autotrophic lifestyle.

The present study was initiated to investigate possibilities of circumventing the problems associated with the low biomass yields of *T. ferrooxidans*. One approach to this problem is the use of a facultatively autotrophic model organism. *Thiobacillus acidophilus* seems a good candidate, since this organism can oxidize a wide variety of inorganic sulphur compounds and shares its natural habitat with *T. ferrooxidans* (in fact, the organism was first isolated as a contaminant from a *T. ferrooxidans* culture). Alternatively, research can be directed at developing methods for growing *T. ferrooxidans* at high densities. In this thesis, both approaches have been followed.

Apart from a characterization of *T. acidophilus* and *T. ferrooxidans* in view of their potential use as model organism for laboratory studies, the fundamental physiological studies described in this thesis are aimed at understanding the role of these organisms in their natural environment and in industrial metal-leaching and coal desulphurization operations.

**Outline of this study**

Chapter 2 of this thesis gives an overview of the literature on the oxidation of sulphur compounds by
acidophilic thiobacilli. Since this literature survey suggested at least some 'unity in biochemistry' among the acidophilic thiobacilli, it was decided to investigate the physiology of *Thiobacillus acidophilus* in more detail.

In Chapter 3, heterotrophic growth of *T. acidophilus* is studied. Apart from a characterization of the organism in view of its potential use as a model organism, this study was directed at studying the energetics of growth in acidic environments in order to obtain more information on the possible role of (facultative) heterotrophs in acidophilic environments.

Mixotrophic and autotrophic utilization of inorganic sulphur compounds by *T. acidophilus* is studied in Chapter 4. This chapter mainly deals with the energetics of mixotrophic growth and the regulation of 'autotrophic potential' (inorganic carbon fixation and inorganic sulphur oxidation).

Experimental work described in Chapter 3 indicated that *T. acidophilus* can utilize potentially toxic organic acids as carbon and energy sources for growth in substrate-limited chemostat cultures. In Chapter 5, utilization of formate by *T. acidophilus* in mixotrophic and autotrophic formate-limited chemostat cultures is studied.

Since high cell densities could be realised in autotrophic, formate-limited chemostat cultures of *T. acidophilus*, it was investigated whether this compound could also be used by other acidophiles. A screening of a variety of acidophiles and a detailed study of the autotrophic growth of *T. ferrooxidans* on formate are described in Chapter 6. The results presented in this chapter have led to a patent application. A brief summary of the claims contained in this application is given in Chapter 7.

The use of ferric iron as an electron acceptor for the oxidation of elemental sulphur and formate by *T. ferrooxidans* is studied in Chapter 8. The role of ferric iron respiration in energy metabolism is investigated and the possible environmental significance of this process is discussed.

**Literature**


Introduction


Norris, P.R., and Kelly, D.P. (1978) Dissolution of pyrite (FeS_2) by pure and mixed cultures of some acidophilic bacteria. FEMS Microbiol. Lett. 4: 143-146


CHAPTER 2

Oxidation of reduced inorganic sulphur compounds by acidophilic thiobacilli

Introduction

Acidophilic sulphur-oxidizing bacteria were first isolated from acidic mine effluents (Colmer et al. 1950), where they are the causative agents of the environmental problem acid mine drainage. Furthermore, acidophilic thiobacilli are at least partially responsible for the development of acid sulphate soils (Arkesteyn 1980). Over the past decades, there has been a growing interest in the application of this type of organisms in the biological leaching of metal ores (Norris and Kelly 1988) and the biological desulphurization of coal (Bos et al. 1988, Klein et al. 1988).

The key reaction in the processes mentioned above is the biological oxidation of pyrite (FeS₂) to ferric sulphate and sulphuric acid. *Thiobacillus ferrooxidans* is widely used as a model organism to study the biological oxidation of pyrite. *T. ferrooxidans* is an obligately chemolithoautotrophic, aerobic, Gram-negative bacterium. Energy sources for autotrophic growth include ferrous iron and a number of reduced inorganic sulphur compounds (Kelly and Harrison 1989). Most studies into the physiology and bio-energetics of *T. ferrooxidans* have focused on the oxidation of ferrous iron (for a review, see Ingledew 1982).

During the complete oxidation of pyrite, only one electron is derived from the ferrous iron part of the mineral, whereas fourteen electrons are derived from the sulphur moiety (Fig. 1). Apart from this quantitative difference between the reduction equivalents derived from ferrous iron and sulphur, there is also a qualitative difference. The growth yield of *T. ferrooxidans* (expressed as the amount of biomass produced per mole of electrons) is much higher during growth on reduced sulphur compounds than during growth on ferrous iron (Table 1). Also the growth yield per mole of electrons on pyrite is significantly higher than the molar growth yield on ferrous iron (Hazeu et al. 1987). This observation suggests that the sulphur part of the mineral is oxidized biologically to a significant extent, rather than exclusively via an indirect non-biological oxidation with ferric iron.

In view of the apparent importance of the reduction equivalents derived from the sulphur moiety of pyrite, work in our group is focused on the physiology and enzymology of the oxidation of reduced inorganic sulphur compounds by *T. ferrooxidans*.

Due to its autotrophic lifestyle, the biomass yields of *T. ferrooxidans* in batch and chemostat cultures are very low. This is a major disadvantage during biochemical studies, where large amounts of biomass are frequently required (e.g. for enzyme purification procedures).

To overcome the practical problems associated with the use of *T. ferrooxidans* as a model organism, we recently decided to introduce *Thiobacillus acidophilus* as a second model organism. This organism was initially isolated as a contaminant of a ferrous iron-grown culture of *T. ferrooxidans* (Guay and Silver 1975). Like *T. ferrooxidans*, *T. acidophilus* is an acidophilic bacterium with a pH optimum of approximately 3. Substrates for autotrophic growth

![Figure 1. Oxidation of pyrite. Note that for every 15 electrons available from the oxidation of pyrite, only one electron is derived from the ferrous iron part of the mineral.](image)

<table>
<thead>
<tr>
<th>Growth limitation</th>
<th>Mode of cultivation</th>
<th>Growth yield (g dry weight/mole electrons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrous Iron</td>
<td>Chemostat (D=0.034 h⁻¹)</td>
<td>0.23</td>
</tr>
<tr>
<td>Tetraionate</td>
<td>Chemostat (D=0.030 h⁻¹)</td>
<td>0.92</td>
</tr>
<tr>
<td>Pyrite</td>
<td>Batch</td>
<td>0.35-0.5</td>
</tr>
</tbody>
</table>
include a number of reduced sulphur compounds, but not ferrous iron (Kelly and Harrison 1989, Guay and Silver 1975). In contrast to *T. ferrooxidans*, the organism is also capable of heterotrophic growth on a number of simple organic compounds (Guay and Silver 1975). It has recently been demonstrated that growth of *T. acidophilus* on mixtures of glucose and reduced sulphur compounds leads to an increase both of the biomass yields and the specific oxidation rates with a number of reduced sulphur compounds (Mason and Kelly 1988, Pronk et al. 1990). These properties make *T. acidophilus* an attractive model organism to study the oxidation of reduced sulphur compounds in acidic environments.

From work on sulphur oxidation by various neutrophilic and acidophilic thiobacilli, it becomes increasingly clear that there is no uniformity in the pathways employed by different *Thiobacillus* species and that, in fact, vast differences may be found in the pathways involved (Kelly 1985, Kelly 1989). This explains why many previous attempts to formulate a unifying route for the observed biological reactions have failed.

The acidophilic thiobacilli can thrive in environments with pH values as low as pH 1.5. From bioenergetic considerations, it can be expected that at least some of the reactions involved in the oxidation of reduced sulphur compounds will take place extracelluoplastically (Hooper and DiSpirito 1985), implying that the enzymes involved should be able to function at low pH values. Studies of sulphur compound oxidation by these bacteria would not only be useful from an academic and applied point of view, but also may yield useful information on the mechanisms underlying biological activity in this type of ‘extreme environment’.

Aim of the present paper is to provide an overview of the current knowledge on the oxidation of a number of reduced inorganic sulphur compounds by three acidophilic thiobacilli: *T. ferrooxidans*, *T. thiooxidans* and *T. acidophilus*. The sulphur compounds that will be discussed are sulphide, elemental sulphur, thiosulphate, tetrathionate and sulphite. Apart from a review of the relevant literature, some as yet unpublished results from work in our laboratory are discussed.

**Oxidation of sulphide**

Sulphide is the most reduced species of the inorganic sulphur compounds. At concentrations in the millimolar range it reacts spontaneously and at significant rates with oxygen, in particular at pH values above 6 (Chen and Morris 1972). The spontaneous oxidation of sulphide may produce a number of products, including thiosulphate, sulphite and elemental sulphur (Chen and Morris 1972, Zehnder and Zinder 1980). Particularly at low sulphide concentrations and in acidic environments, the biological oxidation of sulphide can compete effectively with the chemical process. The reactivity of sulphide is a major problem in physiological studies into the mechanism of sulphide oxidation. Perhaps for this reason, publications on sulphide oxidation by acidophilic thiobacilli are few and far between.

In contrast to the growth of acidophilic thiobacilli on insoluble sulphides such as ferrous sulphide and pyritic minerals, growth on free sulphides has received little or no attention (Kelly and Harrison 1989), although it is known that *T. ferrooxidans*, *T. thiooxidans* and *T. acidophilus* readily oxidize sulphide (Hazeu et al. 1986, London and Rittenberg 1964, Meulenberg; unpublished). This is probably due to the experimental problems related to the high rate of spontaneous oxidation of sulphide in combination with its low solubility at low pH values. In our laboratory we have recently focused on the oxidation of sulphide by *T. ferrooxidans* and *T. acidophilus*. Both organisms exhibit a high affinity towards sulphide. The *Km* for sulphide of both organisms is approximately 5 μM (Hazeu et al., 1986; Meulenberg, unpublished). Intact cells exhibit sulphide-oxidizing activity over a broad range of pH values (pH 1-6), with an optimum at pH 2-4 (Hazeu et al. 1986; Meulenberg, unpublished).

Oxidation of sulphide by cell-free extracts was first mentioned by London and Rittenberg (1964). Cell-free extracts of *T. concreitivorus* (now *T. thiooxidans*) were reported to catalyse the complete oxidation of sulphide to sulphate with thiosulphate, trithionate and tetrathionate as intermediates. However, these observations should be interpreted cautiously, since high sulphide concentrations were used at near-neutral pH values. Furthermore, sample preparation involved heat-deproteinization.

A high affinity sulphide-oxidizing system (*Km* = 2 μM) associated with the cell envelope of *T. thiooxidans* was studied by Moriarty and Nicholas (1969, 1970a,b). Cell-free extracts catalysed the oxidation of sulphide with oxygen as an electron acceptor. Sulphide oxidation by cell-free extracts proceeded at high rates and could be coupled to the reduction of respiratory chain components and the phosphorylation of ADP. The initial step in sulphide oxidation was the formation of a conjugated sulphur compound, which was bound to a membrane fraction. Since this initial step led to the consumption of approximately one mole of oxygen for each two moles of sulphide oxidized, it was concluded that the initial product probably consisted of either elemental sulphur or long polysulphide chains. Copper-chelating compounds inhibited the initial phase of sulphide
oxidation, suggesting a role of copper in the sulphide-oxidizing enzyme system. The sulphide-oxidizing system was active between pH 5 and pH 9. However, since oxygen was used as an electron acceptor in these studies, these properties can not be attributed exclusively to a sulphide oxidoreductase enzyme, but may also reflect properties of the respiratory chain which couples the oxidation of sulphide to the reduction of molecular oxygen.

Also in *T. ferrooxidans* and *T. acidophilus* the oxidation of sulphide proceeds via some form of conjugated sulphur (Hazeu *et al.* 1988). Formation of intermediary sulphur by cell suspensions can be monitored by following the optical density in the UV region (Figure 2). Accumulation of significant amounts of intermediary sulphur also leads to an increase in the turbidity of cell suspensions. After staining with silver nitrate, formation of intermediary sulphur can be visualized by electron microscopy (Hazeu *et al.* 1988, Gromova *et al.* 1983). Close examination of thin sections of silver-stained *T. ferrooxidans* cells revealed that intermediary sulphur was mainly localized between the outer membrane and the cytoplasmic membrane. This observation suggests that in *T. ferrooxidans* the formation of intermediary sulphur from sulphide may be a periplasmic process.

Fresh cell suspensions of *T. ferrooxidans* oxidize sulphide completely to sulphate, with a transient accumulation of intermediary sulphur. Upon the addition of the inhibitors N-ethylmaleimide or CCCP, sulphide is incompletely oxidized with the uptake of approximately 1 mole of oxygen for each two moles of sulphide. A similar oxidation pattern is observed after freezing and thawing of cell suspensions (Hazeu *et al.* 1988). These observations are compatible with the formation of intermediary sulphur consisting of elemental sulphur (mainly S₈) and higher polythionates, as found during the oxidation of tetrathionate by *T. ferrooxidans* (Steudel *et al.* 1987). Although the possibility that during sulphide oxidation polysulphides rather than polythionates are formed can not be excluded, since in both cases the bulk of the compound should be in the state of elemental sulphur, this seems unlikely in view of the instability of polysulphides at low pH.

Also in *T. acidophilus* the oxidation of intermediary sulphur can be inhibited by the addition of NEM (Meulenbreg, unpublished). Interestingly, the ability of *T. acidophilus* to oxidize sulphide to sulphate is also dependent on its growth history. Upon the addition of sulphide, cells from mixotrophic chemostat cultures rapidly produce sulphate with only a minor transient accumulation of intermediary sulphur which is subsequently oxidized to sulphate. In contrast, cells from heterotrophic cultures rapidly oxidize sulphide to intermediary sulphur, but the further oxidation to sulphate proceeds only at very low rates (Meulenbreg, unpublished).

**Oxidation of elemental sulphur**

Elemental sulphur occurs mainly in the form of S₈ rings and, in contrast to the inorganic sulphur anions, its water solubility (approximately 5 μg/l) is very low (Steudel *et al.* 1987, Roy and Trudinger 1970). In view of the low water solubility and hydrophobicity of elemental sulphur it seems likely that surface effects play a role in its biological oxidation. The presence of surface-active agents ("wetting agents") has been demonstrated in culture supernatants of *T. thiooxidans* and *T. ferrooxidans* grown on elemental sulphur (Cook 1964, Agate *et al.* 1969). Furthermore, growth of *T. thiooxidans* and *T. acidophilus* on elemental sulphur can be stimulated by the addition of surface-active agents to the growth media (Cook 1964, Kingma and Silver 1979).

Although the accessibility of sulphur particles may be enhanced by the addition of surface-active agents or by increasing the area available for microbial action, the S₈ molecule as such is rather inert. Therefore, biological oxidation of elemental sulphur is likely to include an initial activation step, in which the S₈ structure is made susceptible to
Figure 3. pH optima of elemental sulphur (●) and sulphite (○) oxidation by *T. ferrooxidans* (A) and *T. acidophilus* (B). *T. acidophilus* was pregrown in a mixotrophic chemostat culture (5 mM glucose, 20 mM thiosulphate, D = 0.05 h⁻¹, pH = 3.0, T = 30 °C). *T. ferrooxidans* was pregrown in a thiosulphate-limited chemostat culture (D = 0.02 h⁻¹; Hazeu *et al.* 1986).

Further microbial oxidation. In a recent publication, Bacon and Ingledeiw (1989) have demonstrated that *T. ferrooxidans* releases small amounts of hydrogen sulphide during aerobic and anaerobic incubation with elemental sulphur, suggesting that the activation step may involve an initial reductive step.

Oxidation of elemental sulphur has been studied extensively in *T. thiooxidans*. Sulphur oxidation by intact cells occurs over a broad pH range, with an optimum at around pH 4 (Kodama and Mori 1968a). Oxidation of elemental sulphur is inhibited by freezing and thawing of cells, by inhibitors of respiration like cyanide, carbon monoxide and azide and by a number of metal-chelators, including diethylthiocarbamate. Organic acids were found to inhibit sulphur oxidation when added at pH values below their pKₐ (Iwatsuka and Mori 1960).

Intact cells of *T. ferrooxidans* and *T. acidophilus* oxidize elemental sulphur over a broad range of pH values (Figure 3). In *T. ferrooxidans*, the rate of elemental sulphur oxidation is stimulated by the presence of sulphate ions. This sulphate effect is very specific. The only anion which can replace sulphate is selenate (Hazeu *et al.* 1988). The oxidation of elemental sulphur by *T. ferrooxidans* cells can be inhibited by the addition of -SH reagents like N-ethylmaleimide, by freezing and thawing of cells and by the addition of uncouplers (Arkesteyn 1980, Hazeu *et al.* 1988). Published schemes for the oxidation of reduced sulphur compounds proposed in the literature involve sulphite as an essential intermediate. However, formation of sulphite from elemental sulphur by intact cells of acidophilic thiobacilli has not been reported in the literature. When *T. ferrooxidans* cells are incubated with elemental sulphur at pH values above 5.0, significant amounts of sulphite are formed (Figure 4), demonstrating that indeed sulphite can be an intermediate of elemental sulphur oxidation. Oxidation of elemental sulphur at lower pH values does not result in the accumulation of sulphite. This observation can be explained by the low pH optimum for sulphite oxidation by *T. ferrooxidans* (Figure 3).

Low rates of thiosulphate formation from elemental sulphur have been observed with cell-free extracts of *T. thiooxidans* prepared under a nitrogen atmosphere (Suzuki 1965b). The enzyme activity, which required reduced glutathione (GSH) for activity, was partially purified and exhibited optimum activity at pH 7.5. Catalase stimulated the reaction after prolonged incubation times, whereas KCN was inhibitory (Suzuki 1965a). The initial product of sulphur oxidation by cell-free extracts of *T. thiooxidans* was identified as sulphite (Suzuki and Silver 1966). The formation of thiosulphate can be explained from a chemical reaction of sulphite with elemental sulphur. Based on experiments with ¹⁸O, Suzuki (1965b) concluded that an oxygenase was involved in the formation of thiosulphate. However, ¹⁸O incorporation in thiosulphate did not occur with all preparations tested and the overall incorporation of ¹⁸O was very low in all experiments. From a bioenergetic point of view, direct oxygenation of sulphur...
with molecular oxygen seems wasteful, since such a process does not involve transfer of electrons via an electron transport chain.

Using a crude cell-free extract of *T. thiooxidans*, containing a particulate fraction, Kodama and Mori (1968b) observed GSH-independent oxidation of sulphur. Anaerobic conditions during cell disruption were necessary to obtain active cell-free extracts. The active components could be separated in an oxygen-sensitive, particulate fraction and a soluble fraction, both of which were needed for catalytic activity. The soluble fraction contained both high molecular weight components and a low molecular weight component. The latter could be replaced by either NAD or NADP (Kodama 1969). The high molecular weight soluble fraction contained two essential proteins, a 120,000 Da non-heme iron protein and a 23,000 Da non-heme iron flavoprotein (Takakuwa 1975).

*T. thiooxidans* cell-free sulphur-oxidizing systems have been reported to be sensitive to metal-chelating compounds, CO and thiol-binding agents (Kodama and Mori 1968b, Takakuwa 1975, Adair 1966).

Oxidation of elemental sulphur by a cell-free preparation of *T. ferroxidans* was first described by Sliver and Lundgren (1968). Like the *T. thiooxidans* cell-free system described by Suzuki (1965a), this system required GSH for activity and oxidized elemental sulphur to sulphite. The partially purified enzyme had a pH-optimum of 7.8 and contained non-heme iron and acid-labile sulphide.

With intact cells of both *T. ferroxidans* and *T. thiooxidans*, ferric ions can act as electron acceptors for the oxidation of elemental sulphur (Brock and Gustafson 1976, Kino and Usami 1982). Based on these observations, Sugio et al. (1985) proposed a mechanism for elemental sulphur oxidation by *T. ferroxidans*. In this Fe$^{3+}$/Fe$^{2+}$ cycling model, Fe$^{2+}$ is the initial electron acceptor for the oxidation of elemental sulphur. According to the model, ferrous iron formed during sulphur oxidation is then oxidized by the ferric iron-oxidizing enzyme system present in *T. ferroxidans*. An enzyme catalyzing the oxidation of elemental sulphur to sulphite with ferric iron as an electron acceptor was detected in cell-free extracts and purified to homogeneity (Sugio et al. 1987). Since osmotic shock treatment resulted in the release of enzyme activity from the cells, a periplasmic localization was proposed, although the purified enzyme preparation exhibited a pH-optimum of 6.5. Enzyme activity was strictly dependent on the addition of GSH. The purified enzyme consisted of two 23,000 Da subunits (Sugio et al. 1987).

The physiological significance of this sulphur-ferric iron oxidoreductase has been questioned. In *T. ferroxidans*, the oxidation of sulphur, but not the oxidation of ferrous iron is inhibited by HOQNO (2-n-heptyl-4-hydroxyquinoline N-oxide). This suggests involvement of the bc1 complex in electron transfer from elemental sulphur to oxygen. This respiratory chain component is apparently not involved in electron transfer from ferrous iron to oxygen which, as pointed out by Corbett and Ingledew (1987), is in contradiction with the Fe$^{3+}$/Fe$^{2+}$ cycling model. Secondly, these authors demonstrated that the transfer of electrons from elemental sulphur to ferric iron involves participation of cytoplasmic components, which is not in agreement with the presence of a periplasmic elemental sulphur-ferric iron oxidoreductase as proposed by Sugio et al. (1987). Also the observation that the rates of elemental sulphur-dependent oxygen uptake by *T. ferroxidans* are independent of the presence of Fe$^{3+}$-ions seems difficult to reconcile with the Fe$^{3+}$/Fe$^{2+}$ cycling model (Corbett and Ingledew 1987). When *T. ferroxidans* is grown in tetraionate-limited chemostat cultures, the ability to oxidize ferrous iron is completely repressed. However, cells grown under these conditions retain the ability to oxidize elemental sulphur (Hazeu et al. 1986). Also this observation is in contradiction with an Fe$^{3+}$/Fe$^{2+}$ cycling mechanism. As mentioned above, growth yields (expressed as g biomass per mole of electrons) of *T. ferroxidans* grown on reduced sulphur compounds are higher than the molar growth yields on ferrous iron (Hazeu et al. 1987). If oxidation of (intermediary) sulphur would be coupled to the electron transport chain via the reduction of ferric iron, lower growth yields on reduced sulphur compounds would be expected.

Recently, Sugio and coworkers have reported that sulphide, rather than elemental sulphur is the actual substrate for the ferric iron-reducing enzyme (Sugio et al. 1989). In this recent model, elemental sulphur is first reduced to sulphide with GSH. Subsequently, sulphide is oxidized directly to sulphite with ferric iron as an electron acceptor. If long-chain intermediary sulphur compounds are true intermediates during the oxidation of sulphide (Hazeu et al. 1988), it seems difficult to envisage sulphide as an intermediate during the oxidation of elemental sulphur.

**Oxidation of thiosulphate**

Thiosulphate is relatively unstable in acidic environments. At pH values below pH 4, decomposition to sulphur and sulphite may occur (Johnston and McAmish 1973). For this reason, batch cultures are ill-suited to study growth of acidophilic thiobacilli on thiosulphate. The rate of chemical decomposition strongly depends on the thiosulphate concentration. In thiosulphate-limited chemostat cultures, the rate of chemical decomposition is negligible in comparison
Figure 5. Formation of tetrathionate (○) during the oxidation of thiosulphate (●) by a *T. ferrooxidans* cell suspension. *T. ferrooxidans* was pregrown in a thiosulphate-limited chemostat culture (D = 0.02 h⁻¹; Hazeu *et al.* 1986).

with the biological oxidation rates, due to the low residual substrate concentrations.

Growth on thiosulphate as a sole source of energy has been demonstrated with *T. ferrooxidans*, *T. thiooxidans* and *T. acidophilus* (Kelly and Harrison 1989). Different groups have shown that in these three organisms, the initial step in thiosulphate metabolism is its oxidation to tetrathionate (Sinha and Walden 1966, Okuzumi and Kita 1965, Pronk *et al.* 1990, respectively). When low concentrations (< 1 mmol L⁻¹) of thiosulphate are added to cell suspensions of *T. ferrooxidans*, transient accumulation of tetrathionate occurs (Figure 5). Near-quantitative conversion of thiosulphate to tetrathionate can be observed during the first phase of thiosulphate oxidation. This strongly suggests that the oxidation of thiosulphate via tetrathionate is the major, if not the only route of thiosulphate metabolism in *T. ferrooxidans*.

Oxidation of thiosulphate with oxygen as an electron acceptor has been demonstrated in crude cell-free extracts of *T. thiooxidans* (Okuzumi and Kita 1965). Tetrathionate was formed as an intermediate. Thiosulphate oxidation by these extracts exhibited a low pH optimum.

A thiosulphate-oxidizing enzyme system was purified from cell-free extracts of *T. ferrooxidans* by Silver and Lundgren (1968). The enzyme catalysed the oxidation of thiosulphate to tetrathionate with ferricyanide as an artificial electron acceptor. Enzyme activity was not measured at pH values below 4.5 because of the instability of thiosulphate at lower pH values. However, enzyme activities were very low at near-neutral pH values, suggesting that the initial step in thiosulphate oxidation by *T. ferrooxidans* occurs extracellularly. The *Kₐ* of the purified enzyme preparation, measured at pH 4.5, was 0.9 mM. Kinetic analysis of thiosulphate oxidation by intact cells of *T. ferrooxidans* suggests that the affinity for thiosulphate may be higher at low pH values (Hazeu *et al.* 1986). The purified enzyme preparation described by Silver and Lundgren (1968) showed three bands after acrylamide gel electrophoresis. Further research is needed to assess whether thiosulphate-acceptor oxidoreductase from *T. ferrooxidans* is a multi-subunit enzyme. Although similar enzymes in other thiobacilli have been demonstrated to use cytochrome c as an electron acceptor, this has not yet been demonstrated for the *T. ferrooxidans* enzyme. Also with *T. acidophilus* cell-free extracts, oxidation of thiosulphate with ferricyanide as an artificial electron acceptor has been demonstrated (Guay and Silver 1975).

Cell-free extracts of *T. ferrooxidans* also contain rhodanese (thiosulphate-cyanide sulphur transferase, EC 2.8.1.1). *T. ferrooxidans* rhodanese has been purified (Silver and Lundgren 1968). Rhodanese activity is not restricted to sulphur-oxidizing bacteria. The enzyme has also been demonstrated in mammalian tissues and in some heterotrophic bacteria (Cerletti 1986). A significant role of rhodanese in thiosulphate oxidation by *T. ferrooxidans* seems unlikely in view of the near-quantitative formation of tetrathionate from thiosulphate observed with cell suspensions (Figure 5).

**Oxidation of tetrathionate**

Tetrathionate formed as an intermediate during the oxidation of thiosulphate is readily oxidized by *T. ferrooxidans*, *T. thiooxidans* and *T. acidophilus*. These three organisms are also capable of autotrophic growth with tetrathionate as a sole energy source (Kelly and Harrison 1989).

In contrast to thiosulphate, tetrathionate is relatively stable in acidic environments. However, in the presence of other reduced sulphur compounds, formation of pentathionate may occur (Sinha and Walden 1966).

Cell suspensions of *T. ferrooxidans* and *T. acidophilus* exhibit very high affinities towards tetrathionate, with affinity constants of 4 µM and 0.6 µM, respectively (Hazeu *et al.* 1986, Meulenberg unpublished).

When low concentrations of tetrathionate are added to cell suspensions of *T. ferrooxidans*, a
transient increase of the turbidity can be observed (Hazeu et al. 1988). Analysis of acetone extracts of cell suspensions revealed that the increase in turbidity is due to the accumulation of some form of intermediary sulphur. The formation of long-chain sulphur compounds from tetrathionate could be monitored as an increase of the optical density in the UV region. Electron microscopic analysis revealed that intermediary sulphur globules were deposited between the inner and outer membrane of *T. ferrooxidans* cells during the oxidation of tetrathionate.

A detailed chemical analysis of the intermediary sulphur formed from tetrathionate by *T. ferrooxidans* was performed by Steudel and coworkers in collaboration with our laboratory (Steudel et al. 1987). The intermediary sulphur was found to consist of long-chain (up to $S_9$) polythionates and some elemental sulphur, the latter mainly in the form of $S_8$. The combination of these compounds might produce the form of hydrophilic sulphur shown in Figure 6.

A set of reactions have been proposed to explain the formation of polythionates and elemental sulphur from tetrathionate (Figure 7; Steudel et al. 1987). Key intermediates in this pathway of intermediary sulphur formation are the sulphane-mono sulphonic acids. These are highly reactive compounds, which may undergo spontaneous elongation reactions to eventually form elemental sulphur and sulphite. The formation of polythionates can be explained from an oxidative condensation of two sulphane-mono sulphonylic acids. Formation of polythionates from sulphane-mono sulphonic acids is also possible under anaerobic conditions. In this case hydrogen sulphide is formed (Figure 7; Steudel et al. 1987).

This proposed scheme for the formation of elemental sulphur and polythionates from tetrathionate has a number of attractive properties:

- The scheme explains the formation and the observed composition of intermediary sulphur during tetrathionate oxidation by *T. ferrooxidans*. The scheme is in agreement with the observation that formation of intermediary sulphur from tetrathionate may also occur under anaerobic conditions (Hazeu, unpublished).
- Formation of trithionate as an intermediate during tetrathionate oxidation has been reported for *T. ferrooxidans* (Sinha and Walden 1966) and *T. thiooxidans* (Okuzumi 1965, Okuzumi 1966a). Trithionate can be formed chemically by a reaction of tetrathionate with sulphite (Trudinger 1964), which is formed during the chain elongation reactions. Alternatively, trithionate might be formed by (biological) oxidation of $S_3$-sulphane-monosulphonlic acid.
- The reactions leading to the formation of polythionates and elemental sulphur from $S_3$-sulphane-monosulphonlic acid are reversible. Therefore, although this scheme explains the transient accumulation of intermediary sulphur after the pulse-wise addition of tetrathionate to *T. ferrooxidans*, it does not imply that long-chain polythionates or elemental sulphur are obligatory.
intermediates during tetrathionate oxidation. If trithionate can be formed by the biological oxidation of $S_2$-sulphane-monosulphonic acid, a cyclic pathway similar to that proposed by Sinha and Walden (1966) can be envisaged (Figure 8). Hydrolysis of trithionate, yielding thiosulphate, has been reported for *T. thiooxidans* (Okuzumi 1966b). As discussed earlier, the biological oxidation of thiosulphate to tetrathionate is well established in the acidophilic thiobacilli. The formation of intermediary sulphur from sulphane-monosulphonic acids according to the scheme proposed by Steudel et al. (1987) is reversible. Therefore, these reactions could catalyse the entry of sulphur atoms from elemental sulphur into the cyclic pathway described above. However, such a pathway of elemental sulphur oxidation would require a net input of three sulphite molecules for each $S_2$ molecule entering the cycle. As yet, there is no experimental evidence to support such a mechanism of sulphur oxidation.

The key enzyme in the scheme suggested by Steudel et al. (1987) catalyses the hydrolysis of tetrathionate to yield $S_2$-sulphane-monosulphonic acid (Figure 7). We recently observed that cell-free extracts of *T. ferrooxidans* catalyse the formation of long-chain sulphur compounds from tetrathionate (Hazeu, unpublished). The enzyme activity catalysing this reaction did not require oxygen and was strictly dependent on the presence of sulphate anions (Figure 9). The only anion which could replace sulphate was selenate. We propose the name tetrathionate hydrolase for this enzyme activity. The product of the reaction, sulphate, has not been measured quantitatively due to the high background sulphate concentrations. The enzyme is active at low, but not at near-neutral pH values (Hazeu, unpublished). This is in agreement with the observation that intermediary sulphur formation occurs between the inner and outer membranes of *T. ferrooxidans* cells (Hazeu et al. 1988).

**Oxidation of sulphite**

Sulphite solutions are readily auto-oxidized in air, a process catalyzed by a number of metal ions. The catalytic effect of metal ions can be prevented by the addition of metal-chelating compounds (Roy and Trudinger 1970).

Sulphite is an essential intermediate in proposed pathways for the oxidation of reduced sulphur compounds by thiobacilli (Kelly 1985, Kelly 1989). However, autotrophic growth of the acidophilic thiobacilli on sulphite has not been demonstrated (Kelly and Harrison 1989). At pH 3, cell suspensions of *T. ferrooxidans* exhibit significant rates of sulphite oxidation. The $K_s$ for sulphite of thiosulphate-grown *T. ferrooxidans* cells is approximately 50 μM (Hazeu et al. 1986). Oxidation of sulphite by this organism can be coupled to the phosphorylation of ADP (Hazeu, unpublished). These observations suggest that sulphite-limited growth of *T. ferrooxidans* is possible.
when practical problems with sulphite auto-oxidation can be overcome.

Intact cells of \( T. \text{thiooxidans} \) (Kodama and Mori 1968a) and \( T. \text{acidophilus} \) (Figure 4) exhibit only very low rates of sulphite oxidation at pH 3. Only when the pH of cell suspensions is increased to approximately pH 6, significant rates of sulphite oxidation can be observed. Sulphite oxidation at these non-physiological pH values may be an (aspecific) periplasmic reaction. Alternatively, oxidation of exogenous sulphite in these organisms may be catalysed by a cytoplasmic enzyme. In the latter case, the increase in activity at elevated pH values might be explained from an increased accessibility of the enzyme for sulphite.

At the enzyme level, two mechanisms have been proposed for the biological oxidation of sulphite. First, sulphite may be oxidized directly to sulphate by a cytochrome \( c \)-linked oxidoreductase. Alternatively, sulphite may undergo an oxidative condensation with adenosine 5'-monophosphate (AMP) to form adenosine 5'-phosphosulphate (APS). The liberation of sulphate from APS is coupled to the formation of ADP. AMP can subsequently be regenerated by the action of adenylate kinase. This substrate-level phosphorylation mechanism yields half a molecule of ATP for each molecule of sulphite oxidized. Of course, it must be assumed that the electrons from the AMP-dependent pathway are also fed into the electron transport chain and thus may contribute to the formation of a proton-motive force.

Literature data on the enzymes involved in sulphite oxidation by the acidophilic thiobacilli are sparse and sometimes conflicting. The presence of an APS reductase system in \( T. \text{thiooxidans} \) has been reported by Peck (1961). However, AMP-independent oxidation of sulphite by cell-free membrane preparations of this organism has also been reported (Adair 1966, Kodama and Mori 1968a, Moriarty and Nicholas 1970b). In the latter papers, sulphite-dependent oxygen uptake was measured at near-neutral pH values.

An AMP-independent, sulphite-oxidizing enzyme has been partially purified from cell-free extracts of \( T. \text{ferrooxidans} \) (Vestal and Lundgren 1971). Oxidation of sulphite by this enzyme could be coupled to reduction of ferricyanide or horse heart cytochrome \( c \). The \( K_m \) of the enzyme for sulphite was 0.58 mM. The affinity constant of intact \( T. \text{ferrooxidans} \) cells for sulphite is about ten-fold lower (Hazeu et al. 1986). The enzyme was active over a broad range of pH values (pH 5.4 - pH 9.0). No data are available on the activity of this sulphite oxidoreductase at low pH values.

**Figure 10.** Scheme for the oxidation of reduced sulphur compounds by the acidophilic thiobacilli. The boxed set of compounds and reactions represents a pool of intermediary sulphur compounds (sulphane-monosulphonic acids, polythionates, elemental sulphur and possibly polysulphides). The nature of the intermediary sulphur compounds formed from sulphide and elemental sulphur are unknown. Also the mechanism of sulphite formation from elemental sulphur remains to be elucidated. \( \delta \) sulphur may combine with polythionates to form hydrophobic sulphur complexes as shown in Figure 6.

**Conclusions**

Literature data suggest that there is little unity in the catabolic reactions employed by different sulphur-oxidizing bacteria, even within the genus \( T. \text{thiobacillus} \) (Kelly 1985, Kelly 1989). Based on the data discussed above, it is not possible to construct a definite scheme for the oxidation of reduced inorganic sulphur compounds by the acidophilic thiobacilli. In Figure 10, an attempt has been made to summarize our view on the current knowledge in this field. This model has been constructed from one hand intact cell studies and on the other hand work with cell-free extracts and purified enzymes. In the following, we will discuss these two aspects separately.

**Intact cells**

Experiments with intact cells of the acidophilic thiobacilli demonstrate that tetra-thionate is the first intermediate during the oxidation of thiosulphate.

There is now ample evidence demonstrating that some form of long-chain intermediary sulphur can be formed during the oxidation of sulphide and tetra-thionate. In both cases, the intermediary sulphur is hydrophilic in nature (Hazeu et al. 1988). Intermediary sulphur formed from tetra-thionate by \( T. \text{ferrooxidans} \) consists mainly of long-chain polythionates and elemental sulphur (Steudel et al. 1987). The chemical composition of the intermediary sulphur formed from sulphide has not been investigated in detail. It seems likely that, after an initial activation step (Bacon and Ingledew 1989), elemental sulphur also enters an intermediary sulphur 'pool'. Further research is needed to study the
composition of intermediary sulphur compounds formed during the oxidation of elemental sulphur and sulphone. Detailed analysis of the chemical structure of the intermediary sulphur may also provide more insight in the reactions involved in the oxidation of sulphone and elemental sulphur.

Chemical analysis of hydrophilic sulphur produced by other microorganisms is needed to see whether its composition is similar to the intermediary sulphur produced by *T. ferrooxidans*. It has been suggested that the amphipatic character of the long-chain polythionates would, in addition to the structures shown in Figure 6, also allow the formation of micelles or vesicle-like structures, which would be filled with water (Steudel 1989). Such structures, or an analogous type of vesicles, might explain the low buoyant density of sulphur globules formed by *Chromatium* species (Guerro et al. 1984).

An attractive model to explain accumulation of intermediary sulphur compounds during tetrathionate oxidation has been proposed by Steudel et al. (1987). In this model, sulphane-monomosulfonic acids are key intermediates. Although formation of long-chain intermediary sulphur compounds can be explained by this model, it is not clear whether or not these are obligate intermediates. Alternatively, accumulation long-chain sulphur compounds may reflect a bottleneck, resulting in overflow, in the sulphur-metabolizing pathways. If long-chain sulphur compounds are not obligate intermediates, accumulation of a reactive intermediate (for example $S_2$-sulphane-monomosulfonic acid in the model of Steudel et al. (1987) might set off a series of chemical reactions leading to chain elongation and, eventually, the formation of elemental sulphur. At sub-optimal substrate concentrations, oxidation of substrates might then proceed via a cyclic pathway as shown in Figure 8. Interestingly, this hypothetical cyclic pathway does not necessarily involve sulphite as an intermediate. It should be stressed that direct enzymatic evidence for the involvement of a cyclic pathway in sulphur compound oxidation by the acidophilic thiobacilli is at present lacking.

Oxidation of elemental sulphur via the cyclic pathway mentioned above would require a net input of sulphite. In contrast to this, production of sulphite can be observed during the oxidation of elemental sulphur by *T. ferrooxidans* cells at elevated pH values (Figure 4). Although this observation suggests that sulphite is an intermediate during the oxidation of elemental sulphur, the molecular mechanism of sulphite formation remains unclear.

With respect to the oxidation of sulphone, there seems to be a significant difference between on one hand *T. ferrooxidans* and on the other hand *T. acidophilus* and *T. thiooxidans*. In the latter two species, added sulphone is only oxidized at significant rates at elevated, non-physiological pH values. This implies that sulphone generated extra-cytoplasmically can not be oxidized by growing cells of these bacteria. Since *T. acidophilus* does not accumulate sulphone during thiosulphate-limited growth (Fronk, unpublished), sulphone is either formed intracellularly or not involved as an intermediate in thiosulphate oxidation by this organism.

**Cell-free systems**

Although oxidation of all reduced sulphur compounds discussed here has been demonstrated in cell-free systems, the data obtained are often incomplete and deserve further attention. Experimental work with cell-free systems is complicated by the reactivity of many substrates and intermediates and by the lack of suitable (acid-resistant) artificial electron acceptors.

Sulphone oxidation has been demonstrated with cell-free membrane preparations of *T. thiooxidans* (Moriarty and Nicholas 1969, 1970a,b). However, the enzyme involved has not been purified, nor has its cellular localization been studied.

Considerable effort has been put into the isolation and characterization of cell-free systems capable of oxidizing elemental sulphur. For various reasons discussed above, the oxygenase reaction reported by Suzuki (1965b) and the Fe$^{3+}$/Fe$^{2+}$ cycling mechanism proposed by Sugio et al. (1985, 1987, 1989) are unlikely pathways to account for the bulk of elemental sulphur oxidation in any of the acidophilic thiobacilli. Further research is needed to assess the physiological significance of the enzyme activities described by these authors. A detailed study of elemental sulphur oxidation was performed with cell-free preparations of *T. thiooxidans* (Kodama and Mori 1968b, Kodama 1969, Takakuwa 1975). The results demonstrate that oxidation of elemental sulphur by *T. thiooxidans* requires various enzyme activities, both soluble and particulate. The exact catalytic function of the proteins involved and their cellular localization are still unknown.

In *T. ferrooxidans* and *T. acidophilus* cell-free extracts, the oxidation of thiosulphate to tetrathionate can be coupled to the reduction of ferricyanide (Silver and Lundgren 1968, Guay and Silver 1975). The enzyme activity responsible for this reaction has been purified to a significant extent from *T. ferrooxidans* (Silver and Lundgren 1968). Although the available data suggest a periplasmic localization and coupling to the electron transport chain at the level of cytochrome c, conclusive evidence is still lacking.

Formation of long-chain sulphur compounds from tetrathionate has been demonstrated with cell-free extracts of *T. ferrooxidans* (Hazeu, unpublished).
A tetrionate hydrolase, producing S₄-sulphane-
monosulphonic acid, has been implicated as the
enzyme activity responsible for this reaction. Further
characterization of this enzyme activity is currently
in progress in our laboratory.

Data obtained with intact cells suggest that the
mechanism of sulphite oxidation may differ
significantly among the acidophilic thiobacilli. A
detailed study of the enzyme activities involved in
sulphite oxidation by the different species may
provide more insight in a possible role of sulphite as
an intermediate during the oxidation of the more
reduced inorganic sulphur compounds. Sulphite is a
very reactive compound. When studying the enzymo-
logy of sulphite oxidation, it is important to rule out
aspecific reactions of sulphite (e.g. with electron
transport chain components). To assess the
physiological significance of sulphite oxidation in cell-
free systems, the catalytic behaviour of such systems
should always be compared to that of intact cells.

As discussed above, a cyclic pathway as shown in
Figure 8 might be involved in the oxidation of reduced
sulphur compounds by the acidophilic
thiobacilli. One of the possible key reactions in this
process is the oxidative conversion of tetrathonate into
triothionate. This conversion may involve S₄-
sulphane-monosulphonic acid as an intermediate.
Additionally, a number of other reactive intermediates
may be involved. Detailed studies of sulphur
compound oxidation by purified, cell-free systems may
either prove or disclaim the involvement of the set of
reactions shown in Figure 8.

The characterization of enzymes involved in
inorganic sulphur metabolism by the acidophilic
thiobacilli is an intriguing, but complicated field of
research. An understanding of the reactions involved
in the biological oxidation of simple inorganic sulphur
species may eventually yield useful information for the
application of these organisms for the leaching of
metal ores and the desulphurization of coal.

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CHAPTER 3

Heterotrophic growth of *Thiobacillus acidophilus* in batch and chemostat cultures

Abstract

Heterotrophic growth of the facultatively chemolithoautotrophic acidophile *Thiobacillus acidophilus* was studied in batch cultures and in carbon-limited chemostat cultures. The spectrum of carbon sources supporting heterotrophic growth in batch cultures was limited to a number of sugars and some other simple organic compounds. In addition to ammonium salts and urea, a number of amino acids could be used as nitrogen sources. Pyruvate served as a sole source of carbon and energy in chemostat cultures, but not in batch cultures. Apparently the low residual concentrations in the steady-state chemostat cultures prevented substrate inhibition, which was already observed at 150 \( \mu \text{M} \) pyruvate. Molar growth yields of *T. acidophilus* in heterotrophic chemostat cultures were low. The \( Y_{\text{gen}} \) and maintenance coefficient of *T. acidophilus* grown under glucose limitation were 69 g biomass.mol\(^{-1}\) and 0.10 mmol.g\(^{-1}.\text{h}^{-1}\), respectively. Neither the \( Y_{\text{gen}} \) nor the maintenance coefficient of glucose-limited chemostat cultures changed when the culture pH was increased from 3.0 to 4.3. This indicates that in *T. acidophilus* the maintenance of a large pH gradient is not a major energy-regquiring process. Significant activities of ribulose-1,5-bisphosphate carboxylase were retained during heterotrophic growth on a variety of carbon sources, even under conditions of substrate excess. Also thiosulphate- and tetrathionate-oxidizing activities were expressed under heterotrophic growth conditions.

Introduction

Acidophilic, sulphur-oxidizing bacteria play an important role in the biological leaching of metal ores (Norris and Kelly 1988). Furthermore, these organisms may be applicable for the biological desulphurization of coal (Bos et al. 1988; Klein et al. 1988).

The obligate chemolithoautotroph *Thiobacillus ferrooxidans* has been used frequently as a model organism to study the physiology and bioenergetics of growth in acidic environments (for a review see Ingledew 1982). Due to its autotrophic lifestyle, biomass yields of *T. ferrooxidans* grown on ferrous iron or reduced sulphur compounds are low. Furthermore, the nature of the substrates supporting autotrophic growth prevents the use of high substrate concentrations for obtaining high biomass concentrations. During biochemical studies large amounts of biomass are frequently required. In these cases, facultatively autotrophic acidophiles may provide an attractive alternative model system.

The facultative autotroph *Thiobacillus acidophilus* was first isolated as a contaminant of a ferrous-iron grown *T. ferrooxidans* culture (Guay and Silver 1975). Growth substrates for its autotrophic growth include elemental sulphur (Guay and Silver 1975), tetrathionate (Norris et al. 1986), thiosulphate and trithionate (Mason et al. 1987). Recently, mixotrophic growth of *T. acidophilus* on glucose and tetrathionate has been reported (Mason and Kelly 1988). The organism can not grow autotrophically on ferrous iron but is capable of maintaining itself in ferrous iron-grown cultures of *T. ferrooxidans* (Guay and Silver 1975, Arkesteijn and de Bont 1980). Apparently *T. acidophilus* can grow on an hitherto unidentified excretion product of *T. ferrooxidans*.

*T. acidophilus* is an attractive model organism to study the bioenergetics of acidophilic growth (Matin et al. 1982, Matin and Matin 1982, Zychlinsky and Matin 1983a,b), but little attention has so far been paid to its heterotrophic potential.

Carbon sources supporting heterotrophic growth of *T. acidophilus* include a number of monosaccharides, TCA-cycle intermediates and amino acids (Guay and Silver 1975). With two exceptions (Arkesteijn and de Bont 1980, Mason and Kelly 1988) studies on the heterotrophic growth of *T. acidophilus* have been performed with batch cultures.

*T. acidophilus* and similar acidophilic heterotrophs have been isolated from bioclogging populations. The presence of heterotrophs has been demonstrated to stimulate growth and leaching of
metal ores by autotrophic cultures (Tsuchya et al. 1974, Nerkar et al. 1977). It has been suggested that in this respect the scavenging of toxic organic compounds is of key importance.

In the present study, the heterotrophic growth of *T. acidophilus* was investigated in carbon-limited chemostat cultures. The aim of this study was twofold. First, to further characterize *T. acidophilus* in view of its potential usefulness as a model acidophile. Secondly, to obtain more insight in the ecological role of *T. acidophilus* and related organisms in mixed bioleaching populations.

**Material and Methods**

*Organism and Growth Conditions*

*Thiobacillus acidophilus* DSM 700 was obtained from the Deutsche Sammlung von Mikroorganismen as a liquid culture on glucose. A sample was plated on mineral medium supplemented with glucose and solidified with 0.8 % agarose. A single colony was inoculated in 200 ml mineral medium plus glucose (20 mM). The resulting culture was made 10 % (v/v) with dimethylsulphoxide and stored at -70 °C in 1 ml aliquots. These frozen samples were used as inocula for continuous culture studies.

*Thiobacillus acidophilus* ATCC 27807 was obtained from the American Type Culture Collection as a freeze-dried culture and used directly as an inoculum for a glucose-limited chemostat culture in a control experiment (see Discussion).

*Mineral medium*

*T. acidophilus* was grown in a mineral medium containing per litre of demineralized water: (NH₄)₂SO₄, 3.0 g; KH₂PO₄, 0.3 g; MgSO₄·7H₂O, 0.5 g; Na₂SO₄, 1.4 g; CaCl₂·2H₂O, 0.26 g; FeSO₄·7H₂O, 11 mg; ZnSO₄·7H₂O, 0.9 mg; MnCl₂·2H₂O, 2.0 mg; CoCl₂·6H₂O, 0.6 mg; CuSO₄·5H₂O, 0.6 mg; NaMoO₄·8H₂O, 0.8 mg; H₃BO₃, 2.0 mg; KI, 0.2 mg; EDTA, 30 mg; nitrilotriacetic acid, 5 mg and silicon antifoaming agent (BDH Chemicals, Poole, Dorset, UK), 25 μl. The medium was adjusted to pH 3.0 with 1 M H₂SO₄ and autoclaved at 120 °C. Carbon sources were either autoclaved at 110 °C or filter-sterilized before addition to the autoclaved mineral medium.

*Growth conditions*

Batch cultures for the screening of carbon sources were grown in 300 ml Erlenmeyer flasks containing 100 ml of mineral medium, adjusted to pH 3.5. Organic compounds were added to a final concentration of 10 mM. The cultures were inoculated with 1 ml samples from glucose-limited chemostat cultures and incubated on a rotary shaker for 10 days at 30 °C.

Batch cultures for screening of nitrogen sources were performed in 300 ml Erlenmeyer flasks containing 100 ml ammonium-free mineral medium adjusted to pH 3.5 and supplemented with 20 mM glucose. Nitrogen-containing compounds were added to a final concentration of 10 mM. The cultures were inoculated with 1 ml of a cell suspension obtained from a nitrogen-limited chemostat culture of *T. acidophilus* and incubated on a rotary shaker for ten days at 30 °C.

Continuous cultivation was performed in Applikon laboratory fermenters with a working volume of 1 l. The pH was automatically titrated with either 1 M KOH or 0.5 M H₂SO₄. The cultures were continuously gassed with water-saturated air (1 l min⁻¹) and stirred at 600 rpm. The dissolved oxygen concentration in the cultures was monitored with a steam-sterilizable Clark-type electrode. Unless otherwise indicated, chemostat cultures were grown at a dissolved oxygen concentration of above 75 % of air saturation, at 30 °C, at pH 3.0 and at a dilution rate of 0.35 h⁻¹.

*Control of culture purity*

The purity of the chemostat cultures was routinely checked by phase-contrast microscopy and by plating on mineral medium plus glucose, solidified with 0.8 % (w/v) agarose. Additionally, immuno-fluorescence assays were performed. Antisera against *T. acidophilus* were obtained as described previously (Muyzer et al. 1987).

*Dry weight*

The dry weight of cell suspensions was determined by filtering culture samples over nitrocellulose filters (pore diameter 0.45 μm, Schleicher and Schüll, Dassel, FRG). The cells were washed three times with demineralized water and dried to constant weight at 70 °C.

*Protein determination*

The protein content of whole cells was assayed with a modified biuret method: cells were harvested from continuous cultures, washed with demineralized water and resuspended to a concentration of approximately 2.5 mg dry weight ml⁻¹. The concentrate was boiled in 1 M KOH for 10 min and subsequently cooled on ice. CuSO₄·5H₂O was then added to a final concentration of 25 mM. After 5 min, the mixture was centrifuged in an Eppendorf bench-top centrifuge (13,000 g) for 2 min. The absorbance of the supernatant was measured at 550 nm. The protein content of cell-free extracts was determined by the method of Bradford (1976). In both assays,
bovine serum albumin (fatty acid free, Sigma, St. Louis, USA) was used as a standard.

**Organic carbon content**

A Beckman model 915B Tocamaster total organic carbon analyser was used to determine the carbon content of whole cultures and culture supernatants, the carbon content of the bacteria being obtained from the difference. Cell suspensions were acidified with H₂PO₄ prior to analysis in order to expel carbon dioxide accumulated inside the cells.

**Substrate determinations**

Glucose concentrations in media and culture supernatants were measured with the GOD-PAP method (Boehringer, Mannheim, FRG). Glycerol and fructose were assayed with Boehringer test-kits 148270 and 139106, respectively. Pyruvate and malate were determined by HPLC on a HPX-87H column (300 x 7.8 mm, Bio-Rad, USA) at room temperature. Samples were eluted with 0.01 N H₂SO₄ at a flow rate of 0.6 ml.min⁻¹. Detection was by means of a Waters 441 UV-meter at 210 nm, coupled to a Waters 741 data module. Peak areas were proportional to concentrations.

**Measurement of substrate-dependent oxygen consumption**

Respiration rates of cells were assayed polarographically with a Clark-type oxygen electrode (Yellow Springs Instruments Inc., Yellow Springs, Ohio, USA) at 30 °C. Cells from chemostat culture were assayed directly, without dilution, in the culture fluid. The determinations were carried out at the temperature at which the cells had been grown. Calculations were made on the basis of an oxygen concentration of 236 μM in air-saturated water at 30 °C. The values presented here have been corrected for the (low) endogenous respiration rates.

**Ribulose-1,5-bisphosphate carboxylase (RuBPCase)**

Cells were harvested from chemostat cultures by centrifugation (10,000 x g, 10 min), washed with a buffer containing 100 mM Tris-HCl, 20 mM MgSO₄, 2.0 mM NaHCO₃ and 5 mM dithiothreitol (pH 8.2). Cells were resuspended in the same buffer to a concentration of approximately 10 mg dry weight.ml⁻¹ and were disrupted by sonication at 4 °C in an MSE 150 W sonifier (6 bursts of 30 seconds with intermittent cooling). Intact cells and debris were removed by centrifugation (40,000 x g, 20 min). RuBPCase was assayed as described by Beudeker et al. (1980). The rate of ¹⁴C incorporation was proportional to the amount of cell-free extract added. Incorporation of ¹⁴C was dependent on addition of ribulose-1,5-bisphosphate.

**Chemicals**

Ribulose-1,5-bisphosphate was obtained from Sigma Chemical Co. (St. Louis, MO, USA). [¹⁴C]-NaHCO₃ (2.11 TBq.mol⁻¹) was obtained from Amersham International PLC. D-xylulose was prepared as described by Pronk et al. (1988). All other chemicals were reagent grade and obtained from commercial sources.

**Results**

**Carbon and energy sources for heterotrophic growth in batch cultures**

In the original description of the organism, a small number of organic compounds were reported to support heterotrophic growth of *Thiobacillus acidophilus* (Guay and Silver 1975). In order to obtain more insight into the metabolic versatility of this facultative chemolithoautotroph, a wide variety of organic compounds were screened as possible carbon sources for heterotrophic growth in batch cultures.

The organism was able to grow on: L-arabinose, L-aspartate, citrate, D-fructose, D-galactose, D-glucose, L-glutamate, L-malate, mannitol, D-ribose and D-xylulose, in accordance with the results of Guay and Silver (1975). Growth also occurred in batch cultures containing L-fucose, D-glucuronate, glycerol or D-xylulose as sole carbon sources.

*T. acidophilus* failed to grow in mineral medium supplemented with acetate, ascorbate, benzoate, D-cellobiose, glycollate, DL-lactate, D-lactose, D-malone, DL-mandelate, D-mannose, D-melibiose, phenol, phenylalanine, L-proline, pyruvate, D-raffinose, ramnose, sorbose, succinate, trehalose, tryptophan or L-tyrosine, in accordance with Guay and Silver (1975). Other substrates, which did not support growth of *T. acidophilus* in batch cultures were acetone, acetoain, adipate, adonitol, L-alanine, p-aminobenzoate, L-arginine, L-asparagine, butanol, butyrate, L-citulline, creatine, cyclohexane carboxylate, cyclohexanol, L-cysteine, 2-deoxy-D-glucose, dulcitol, ethanol, ethyldacetate, ethylyamine, formaldehyde, fumarate, gentiose, glucuronate, L-glutamine, glutarate, glycine, glycollate, n-hexadecane, L-histidine, o-hydroxybenzoate, p-hydroxybenzoate, L-isoleucine, 2-ketogluconate, 5-ketogluconate, α-oxoglutarate, L-leucine, L-lysine, D-melezitose, methanol, methylacetate, methylyamine, L-methionine, α-methylglucoside, L-ornithine, oxalate, palmitinose, phenylethylamine, pimelate, propionate, propylene glycol, L-serine, sucrose, L-threonine and xylitol.
Table 1. Growth of *Thiobacillus acidophilus* in chemostat cultures (D = 0.05 h⁻¹, pH = 3.0, T = 30 °C) under various growth limitations. RuBPCase activities and oxidation rates represent the means of two independent assays.

<table>
<thead>
<tr>
<th>Growth-limiting substrate</th>
<th>Molar growth yield g dry weight mol⁻¹</th>
<th>RuBPCase nmol [min. mg protein]⁻¹</th>
<th>Oxidation rate nmol O₂ [min. mg dry weight]⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Thiosulphate Tetrathionate</td>
</tr>
<tr>
<td>Glucose</td>
<td>60</td>
<td>5.1</td>
<td>48 39</td>
</tr>
<tr>
<td>Fructose</td>
<td>60</td>
<td>4.7</td>
<td>46 31</td>
</tr>
<tr>
<td>L-Malate</td>
<td>25</td>
<td>4.6</td>
<td>80 44</td>
</tr>
<tr>
<td>Glycerol</td>
<td>41</td>
<td>5.0</td>
<td>48 40</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>24</td>
<td>n.d.ᵃ</td>
<td>45 30</td>
</tr>
<tr>
<td>Nitrogenᵇ</td>
<td>n.d.ᵃ</td>
<td>2.2</td>
<td>57 9</td>
</tr>
<tr>
<td>Thiosulphateᵇ</td>
<td>5.5</td>
<td>40</td>
<td>350 130</td>
</tr>
</tbody>
</table>

ᵃ n.d. = not determined
ᵇ Ammonium-limited chemostat cultures were grown with glucose as a carbon source. The residual glucose concentration in the culture was 3.8 mM. Enzyme activities were measured at least 10 volume exchanges after a shift to ammonium limitation.
ᶜ Thiosulphate-limited cultures were grown at a dilution rate of 0.03 h⁻¹

According to Guay and Silver (1975), *T. acidophilus* can use D-sucrose as a carbon source for heterotrophic growth. In our hands, growth on D-sucrose was dependent on non-biological acid hydrolysis of this disaccharide.

**Nitrogen sources for heterotrophic growth**

*T. acidophilus* can use ammonium salts and urea, but not nitrate as a sole source of cell nitrogen (Guay and Silver 1975). We screened a number of nitrogen-containing compounds as possible nitrogen sources for glucose-grown *T. acidophilus*. The following compounds could be used as nitrogen sources: L-alanine, L-asparagine, L-aspartate, L-citruilne, creatine, L-cysteine, L-cystine, L-glutamate, L-glutamine, glycine, L-lysine, L-methionine, L-ornithine, L-serine and L-tyrosine. Growth did not occur when nitrogen-free mineral medium was supplemented with glucose and either one of the following compounds: L-arginine, glucosamine, L-histidine, L-isoleucine, L-leucine, methylyamine, L-phenylalanine, L-threonine or L-valine. Weak growth was observed with L-proline and L-tryptophan.

**Growth yields and cellular composition in carbon-limited chemostat cultures**

When grown on glucose in batch cultures at pH 3.0 and at 30 °C, *T. acidophilus* exhibits a specific growth rate of 0.084 h⁻¹ (Guay and Silver 1975; Pronk, unpublished). Growth in glucose-limited chemostat cultures was initially studied at a dilution rate of 0.05 h⁻¹. The mineral medium used in this study is a modification of the medium described by Bounds and Colmer (1972). Apart from the addition of sodium sulphate and trace elements, the medium differs from the Bounds and Colmer medium by a ten-fold lower potassium phosphate concentration. With the original phosphate concentration, *T. acidophilus* accumulated significant amounts of polyphosphate as judged by electron microscopy (data not shown). Polyphosphate accumulation did not occur with the modified medium. A similar observation has been made by Gommers and Kuenen (1988) with *Thiobacillus* strain Q.

To check whether the mineral medium and growth conditions used throughout this study could support carbon-limited growth, several parameters
were studied in chemostat cultures grown on mineral medium supplemented with increasing concentrations of glucose. The carbon and protein contents of the biomass were (48 ± 1) % and (67 ± 2) %, respectively. Electron microscopy of thin sections did not reveal any significant accumulation of storage polymers. The dry weight of the cultures increased linearly with the glucose concentration in the influent medium and residual glucose could not be detected in the growth medium with the analysis methods used. These results demonstrate that the cultures were indeed glucose-limited.

At a dilution rate of 0.05 h⁻¹, the molar growth yield of *T. acidophilus* was 60 ± 1 g biomass (mol glucose)⁻¹. This growth yield is low as compared to the growth yields observed with many other heterotrophic microorganisms on this substrate (Linton and Stephenson 1978). Low growth yields were also observed with carbon-limited chemostat cultures grown on fructose, glycerol and malate (Table 1).

**Maintenance energy requirement**

To check whether the low growth yields observed in carbon-limited chemostat cultures were influenced by maintenance requirements, molar growth yields of glucose-limited chemostat cultures (pH 3, 30 °C) were determined at various dilution rates. The specific glucose consumption rate plotted against the dilution rate yielded a straight line (Fig. 1). From this plot, a theoretical maximum growth yield of 69 g dry weight (mol glucose)⁻¹ was calculated. The maintenance coefficient derived from this plot was 0.10 mmol glucose (g dry weight h)⁻¹. This value is lower than the maintenance coefficients observed for various neutrophilic bacteria grown on glucose (Table 2), suggesting that growth of *T. acidophilus* at low pH values does not require excessive proton-pumping activity.

In acidophilic microorganisms, the pH of the cytoplasm is poised at near-neutral values (Matin et al. 1982). Therefore, growth at low pH values requires the maintenance of a large pH gradient over the cytoplasmic membrane. To investigate whether the maintenance of this gradient imposes a major energy requirement on growing cells, glucose-limited cultures were grown at pH 4.3. This increase of the culture pH affected neither the maximum growth yield nor the maintenance coefficient (Fig. 1). This also indicates that the maintenance of a large pH gradient does not impose a major energy requirement on growing *T. acidophilus* cells.

**Growth of *T. acidophilus* on pyruvate**

When studying the heterotrophic growth of *T. acidophilus*, we were particularly interested in growth of the organism on pyruvate. Pyruvate can be excreted in significant amounts by *Thiobacillus ferrooxidans*. Concentrations of up to 100 μM have been reported in iron-grown batch cultures of a
strain of this organism (Schnaitman and Lundgren 1965). In our hands, pyruvate concentrations of approximately 10 μM could be reproducibly detected in thiosulphate-limited chemostat cultures of *T. ferrooxidans* ATCC 19859 (J.T. Prnk and W. Hazen, unpublished results). Growth of *T. acidophilus* on pyruvate might offer an explanation for the presence of this organism in ferrous iron-grown *T. ferrooxidans* cultures (Guay and Silver 1975, Arkesteyn and de Bont 1980).

As reported above, *T. acidophilus* did not grow in batch cultures supplemented with 10 mM pyruvate. However, when added at low concentrations pyruvate was readily oxidized by cells from glucose-limited chemostat cultures. Pyruvate oxidation was strongly inhibited at concentrations above 200 μM (Fig. 2). To investigate whether the organism was capable of growth on pyruvate under substrate-limited growth conditions, the influent medium of a glucose-limited chemostat culture (D = 0.05 h⁻¹, pH = 3.0) was exchanged for mineral medium supplemented with pyruvate as a sole carbon source. Indeed, under such conditions *T. acidophilus* exhibited pyruvate-limited growth, with a molar growth yield of 24 g biomass.(mol pyruvate)¹ (Table 1). The residual substrate concentration in pyruvate-limited chemostat cultures was below the detection level, 5 μM. The substrate inhibition kinetics of cells grown in pyruvate-limited chemostat cultures were similar to those of glucose-grown cells (data not shown).

As mentioned above, α-ketoglutarate and succinate did not support heterotrophic growth in batch cultures. However, when added at low concentrations, these substrates could be used for respiration of *T. acidophilus* cells pregrown on glucose. It remains to be investigated whether the organism can grow on these and other organic acids in carbon-limited chemostat cultures.

**Table 2. Maintenance coefficient of *Thiobacillus acidophilus* and of a number of neutrophilic bacteria. All data were obtained from glucose-limited chemostat cultures with ammonium as a nitrogen source.**

<table>
<thead>
<tr>
<th>Organism</th>
<th>mₚ</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Beneckea natrigenes</em></td>
<td>0.35</td>
<td>Linton <em>et al.</em> 1977</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>0.31</td>
<td>Schulze and Lipe 1964</td>
</tr>
<tr>
<td><em>Rhodobacter sphaeroides</em></td>
<td>0.19</td>
<td>Nishizawa <em>et al.</em> 1974</td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em></td>
<td>0.24</td>
<td>Frankena <em>et al.</em> 1985</td>
</tr>
<tr>
<td><em>T. acidophilus</em></td>
<td>0.10</td>
<td>this study</td>
</tr>
</tbody>
</table>

**Regulation of thiosulphate- and tetrahionate-oxidizing capacity**

Cells from carbon-limited chemostat cultures were capable of oxidizing reduced sulphur compounds, including thiosulphate and tetradionate. The oxidation rates observed with these inorganic electron donors did not depend upon the carbon source used (Table 1). Thiosulphate and tetradionate oxidation rates were three- to eight-fold lower than the activities measured with cells grown in autotrophic, thiosulphate-limited chemostat cultures. In all carbon-limited chemostat cultures, the residual substrate concentrations were below the detection limits of the analysis methods used. Therefore, these results do not exclude the possibility that thiosulphate- and tetradionate-oxidizing activities might be repressed in the presence of excess organic carbon in the growth medium. To examine this possibility, oxidation of thiosulphate and tetradionate was studied in cells grown on glucose in nitrogen-limited chemostat cultures. Oxygen consumption rates with thiosulphate were not reduced under nitrogen-limited growth conditions. However, the ability to oxidize tetradionate was significantly lower than in glucose-limited chemostat cultures (Table 1).

**Regulation of Ribulose-1,5-bisphosphate carboxylase**

Cell-free extracts from cells grown in carbon-limited chemostat cultures exhibited significant activities of ribulose-1,5-bisphosphate carboxylase (RuBPCase; EC 4.1.1.39), the key enzyme of the Calvin cycle for carbon dioxide fixation. The RuBPCase activities found in carbon-limited heterotrophic cultures were approximately eight-fold lower than the activities found in extracts prepared from cells grown autotrophically under thiosulphate limitation (Table 1). The RuBPCase activities observed in cell-free extracts from autotrophic cul-
tures grown at a dilution rate of 0.03 h\(^{-1}\) were sufficient to account for the biomass production observed in these cultures.

RuBPCase activities in cell-free extracts prepared from cells grown in nitrogen-limited chemostat cultures were significantly lower than those found in carbon-limited cultures (Table 1). However, even after prolonged cultivation under nitrogen-limited growth conditions (over 15 volume exchanges in the chemostat), significant RuBPCase activities were detectable in cell-free extracts. Apparently, the regulation of inorganic carbon fixation and inorganic sulphur oxidation in *T. acidophilus* is less strict than in the neutrophilic facultative chemolithoautotroph *T. versutus* (Gottschal *et al.* 1981).

**Discussion**

**Ecology of Thiobacillus acidophilus**

Although *T. acidophilus* is capable of heterotrophic growth, its metabolic versatility is considerably less developed than that of the neutrophilic chemolithoautotrophs *T. novellus* and *T. versutus* (Taylor and Hoare 1969). The spectrum of carbon sources supporting heterotrophic growth is restricted to a small number of sugars and some simple intermediates of central metabolic pathways. Our results confirm and extend the original observations of Guay and Silver (1975) and may be useful for the identification of new acidophilic isolates.

The results obtained with pyruvate as a carbon source indicate that growth data from batch cultures should be interpreted cautiously. Toxicity of organic acids like pyruvate to acidophilic bacteria is a well-known phenomenon. The effects of these compounds have been explained from their accumulation in the cytoplasm, leading to a decrease of the internal pH (Ingledew 1982, Alexander *et al.* 1987).

Kingma and Silver (1981) reported that pyruvate, when added at low concentrations, could be cometabolized by glucose- and elemental sulphur-growing *T. acidophilus*. However, in their batch experiments no growth was observed on pyruvate as the sole carbon source. Growth of *T. acidophilus* in pyruvate-limited chemostat cultures (Table 1) sheds new light on the possible role of this organism in natural bioleaching populations and on its occurrence in ferrous iron-grown *T. ferrooxidans* cultures (Guay and Silver 1975, Arkesteyn and de Bont 1980). Pyruvate inhibits growth and respiration of the obligate autotrophs *T. ferrooxidans* and *T. thiooxidans* (Schnaitman and Lundgren 1965, Alexander *et al.* 1987, Rao and Berger 1970). By scavenging pyruvate that is excreted by *T. ferrooxidans*, *T. acidophilus* may avoid inhibition of the autotrophic, 'catalytic' population and thereby increase the stability and performance of bioleaching populations.

**Energetics of growth**

The growth yields of *T. acidophilus* on glucose reported here (Table 1, Fig. 1) are much lower than the yield figure reported by Mason and Kelly (1988). We have checked both the growth medium and the *T. acidophilus* strain used in our studies. However, neither the use of the mineral medium of Mason and Kelly (1988) nor the use of *T. acidophilus* ATCC 27807 resulted in the high growth yield (112.5 g dry weight, mol glucose\(^{-1}\) at a dilution rate of 0.03 h\(^{-1}\) reported in the latter paper. Our yield data are in good agreement with those observed by Arkesteyn and de Bont (1980). The reason for the discrepancy between our yield data and those of Mason and Kelly (1988) remains unclear.

Neither the maximum growth yield nor the maintenance coefficient of *T. acidophilus* changed significantly when the culture pH was increased from pH 3.0 to pH 4.3, a step corresponding with a twenty-fold decrease of the external free proton concentration (Figure 1). This observation indicates that the maintenance of a large \(\Delta pH\) by this acidophilic bacterium is not a major energy-requiring process. From experiments done with non-growing cells and spheroplasts of *T. acidophilus*, Matin and coworkers concluded that growth at low external pH values influenced the magnitude of the electrical component of the proton motive force (\(\Delta \Phi\)), but not the magnitude of the total proton motive force (Matin *et al.* 1982, Matin and Matin 1982, Zychlinsky and Matin 1983a,b). These authors therefore concluded that the maintenance of a near-neutral intracellular pH is not an energy-requiring process. Our results obtained with growing cultures of *T. acidophilus* are in full agreement with this conclusion.

**Acknowledgements**

We are grateful to Wilma Batenburg-van der Vegte and Anke de Bruyn for performing the electron microscopy and the immunofluorescence experiments, respectively. We thank Robert Rozenhout and Rogier Meulenberg for critical reading of the manuscript. Peter de Bruijn skilfully performed some of the experimental work.

**Literature**


CHAPTER 4

Mixotrophic and autotrophic growth of Thiobacillus acidophilus on glucose and thiosulphate

Abstract

Mixotrophic growth of the facultatively autotrophic acidophile *Thiobacillus acidophilus* on mixtures of glucose and thiosulphate or tetrathionate was studied in substrate-limited chemostat cultures. Growth yields in mixotrophic cultures were higher than the sum of the heterotrophic and autotrophic growth yields. Pulse experiments with thiosulphate indicated that tetrathionate is an intermediate during thiosulphate oxidation by cell suspensions of *T. acidophilus*. From mixotrophic growth studies, the energetic value of thiosulphate and tetrathionate redox equivalents was estimated to be 50% of that of redox equivalents derived from glucose oxidation. RuBPCase activities in cell-free extracts and rates of sulphur compound oxidation by cell suspensions increased with increasing thiosulphate to glucose ratios in the influent medium of the mixotrophic cultures. Significant RuBPCase and sulphur compound-oxidizing activities were detected in heterotrophically grown *T. acidophilus*. Polyhedral inclusion bodies (carboxysomes) could be observed at low frequencies in thin sections of cells grown in heterotrophic, glucose-limited chemostat cultures. Highest RuBPCase activities and carboxysome abundance were observed in cells from autotrophic, CO₂-limited chemostat cultures. The maximum growth rate at which thiosulphate was still completely oxidized was increased when glucose was utilized simultaneously. This, together with the fact that even during heterotrophic growth, the organism exhibited significant activities of enzymes involved in autotrophic metabolism, indicates that *T. acidophilus* is well adapted to a mixotrophic lifestyle. In this respect, *T. acidophilus* may have a competitive advantage over autotrophic acidophiles with respect to sulphur compound oxidation in environments where organic compounds are present.

Introduction

The acidophilic thiobacilli are capable of autotrophic growth in extremely acidic environments. Their ability to oxidize various inorganic sulphur compounds is of fundamental interest because of the extreme acid-tolerance of some of the periplasmic enzymes involved in these reactions. The biochemical activities of these bacteria are also of considerable economical importance. For example, oxidation of metal sulphides by *Thiobacillus ferrooxidans* is applied on a large scale for the biological leaching of metal ores (Norris and Kelly 1988). However, the pathways involved in the oxidation of sulphur compounds by the acidophilic thiobacilli are still poorly understood (for a review see Pronk et al. 1990a).

Studies into the physiological mechanisms involved in growth and substrate oxidation by the obligately autotrophic *Thiobacillus* species are hampered by the low growth yields of these organisms. Faculative autotrophs provide an attractive alternative model system for physiological studies. The facultative autotroph *T. acidophilus* is capable of heterotrophic growth on glucose and various other simple organic compounds (Guay and Silver 1975, Pronk et al. 1990b). Autotrophic growth can be supported by a variety of inorganic sulphur compounds (Guay and Silver 1975, Norris et al. 1986, Mason et al. 1987) and formate (Pronk et al. 1990c). The organism has been used to study mechanisms involved in ΔpH maintenance under acidic growth conditions (Matin and Matin 1982, Matin et al. 1982, Zychlinski and Matin 1983a,b). Its physiological characteristics also make *T. acidophilus* an attractive model organism to study the enzymology of acidophilic sulphur compound oxidation (Pronk et al. 1990a).

Facultatively autotrophic acidophiles like *T. acidophilus* are not only interesting because of their suitability as model organisms. The presence of acidophilic heterotrophs can increase the performance of metal-leaching operations (Wichlacz and Thompson 1988), probably by preventing the accumulation of toxic organic compounds (Harrison 1984, Wichlacz and Thompson 1988, Pronk et al. 1990b).

We have recently studied the mixotrophic growth of *T. acidophilus* on glucose and the C₃ compounds formate and formaldehyde (Pronk et al. 1990c). As observed with other facultative
autotrophs, mixotrophic growth yields were higher than the sum of the heterotrophic and autotrophic growth yields. Mason and Kelly (1988) reported that mixotrophic growth of *T. acidophilus* on glucose and tetrahionate also led to an increase of the growth efficiency. However, their mixotrophic chemostat studies were limited to one glucose to tetrahionate ratio. Therefore, no quantitative comparison could be made of the energetics of the mixotrophic utilization of inorganic sulphur compounds and C$_1$-compounds.

Aim of the present study was to investigate the energetics of mixotrophic growth of *T. acidophilus* on mixtures of glucose and thiosulphate or tetrahionate. Furthermore, attention was paid to the regulation of sulphur compound oxidation and inorganic carbon metabolism during mixotrophic and autotrophic growth.

**Materials and Methods**

*Organism and maintenance*

*Thiobacillus acidophilus* DSM 700 was obtained from the Deutsche Sammlung von Mikroorganismen as a liquid culture on glucose. A sample was plated on mineral medium (pH 3.5) supplemented with 0.8 % agarose. A single colony was inoculated in 200 ml mineral medium plus glucose (20 mM). The resulting culture was made 10 % (v/v) with dimethylsulphoxide and stored at -70 °C in 1 ml aliquots. These frozen samples were used as inocula for continuous culture studies.

*Mineral medium*

Mixotrophic chemostat cultures of *T. acidophilus* were fed with a mineral medium containing per litre of demineralized water: (NH$_4$)$_2$SO$_4$, 3.0 g; KH$_2$PO$_4$, 0.15 g; K$_2$HPO$_4$, 0.19 g; MgSO$_4$·7H$_2$O, 0.5 g; Na$_2$SO$_4$, 1.4 g; CaCl$_2$·2H$_2$O, 0.26 g; FeSO$_4$·7H$_2$O, 11 mg; ZnSO$_4$·7H$_2$O, 0.9 mg; MnCl$_2$·2H$_2$O, 2.0 mg; CoCl$_2$·6H$_2$O, 0.6 mg; CuSO$_4$·5H$_2$O, 0.6 mg; NaMoO$_4$, 0.8 mg; H$_3$BO$_3$, 2.0 mg; KI, 0.2 mg; EDTA, 30 mg; nitrilo-triacetic acid, 5 mg and silicon antifoaming agent (BDH Chemicals, Poole, Dorset, UK), 25 μl, and variable amounts of sodium thiosulphate or potassium tetrahionate. Prior to the addition of MgSO$_4$ and CaCl$_2$, the mineral medium was adjusted to pH 7.5 with 5 M KOH and autoclaved at 120 °C. MgSO$_4$, CaCl$_2$, and glucose were sterilized separately at 110 °C. Heterotrophic cultures were grown in the same mineral medium (without thiosulphate), adjusted to pH 3.0 with H$_2$SO$_4$.

**Growth conditions**

Continuous cultivation was performed in Applikon laboratory fermenters with a working volume of 1 l. The pH was automatically titrated with 2 M KOH. The cultures were continuously gassed with water-saturated air (1 l.min$^{-1}$) and stirred at 800 rpm. The dissolved oxygen concentration in the cultures was monitored with a steam-sterilizable Clark-type electrode. Chemostat cultures were grown at a minimum dissolved oxygen concentration of 75 % air saturation, at 30 °C, and at a dilution rate of 0.05 h$^{-1}$. Biomass concentrations in the chemostat cultures were linearly proportional to the concentrations of the growth-limiting substrates in the reservoir media.

**Control of culture purity**

The purity of chemostat cultures was routinely checked by phase-contrast microscopy and by plating on mineral medium plus glucose, solidified with 0.8 % (w/v) agarose. Additionally, immuno-fluorescence microscopy with specific antisera against *T. acidophilus* was performed as described by Muys et al. (1987).

**Dry weight determination**

The dry weight of cell suspensions was determined by filtering aliquots over nitrocellulose filters (pore diameter 0.45 μM, Schleicher and Schüll, Dassel, FRO). The cells were washed three times with demineralized water and dried to constant weight at 70 °C.

**Protein determination**

The protein content of whole cells was assayed with a modified biuret method: cells were harvested from continuous cultures, washed with demineralized water and resuspended to a concentration of approximately 2.5 mg dry weight.ml$^{-1}$. The concentrate was boiled in 1 M KOH for 10 min and subsequently cooled on ice. CuSO$_4$·5H$_2$O was then added to a final concentration of 25 mM. After 5 min, the mixture was centrifuged in an Eppendorf bench-top centrifuge (13,000 x g) for 2 min. The absorbance of the supernatant was measured at 550 nm. The protein concentration in cell-free extracts was determined by the method of Bradford (1976). In both assays, bovine serum albumin (fatty acid free, Sigma, St. Louis, USA) was used as a standard.

**Organic carbon determination**

A Beckman model 915B Tocamaster total organic carbon analyser was used to determine the
carbon content of whole cultures and culture supernatants. The carbon content of the bacteria was obtained from the difference. Cell suspensions were acidified with H₃PO₄ prior to analysis in order to expel carbon dioxide accumulated inside the cells.

**Substrate determinations**

Since thiosulphate interfered with the GOD-PAP method (Boehringer, Mannheim, FRG), glucose concentrations in media and culture supernatants were measured with a commercial hexokinase/glucose 6-phosphate dehydrogenase kit (Boehringer Mannheim, test combination no. 676543). Thiosulphate and tetrathionate were determined according to Sorbø (1957). The analyses were carried out at room temperature. Separate calibration curves were made for thiosulphate and tetrathionate.

**Measurement of substrate-dependent oxygen consumption**

Respiration rates of cells were assayed polarographically with a Clark-type oxygen electrode (Yellow Springs Instruments Inc., Yellow Springs, Ohio, USA). Cells from carbon-limited chemostat cultures were assayed directly in the culture fluid or after appropriate dilution in mineral medium without a carbon source (pH 3.0). When cell suspensions were diluted with mineral medium or culture supernatant, the observed oxygen uptake rates were linearly proportional to the biomass concentration (data not shown). Calculations were made on the basis of an oxygen concentration of 236 µM in air-saturated water at 30 °C. The values presented here have been corrected for the (low) endogenous respiration rates.

**Ribulose 1,5-bisphosphate carboxylase (RuBPCase)**

Cell-free extracts for RuBPCase assays were prepared as described previously (Prönk et al. 1990b). RuBPCase was assayed according to Beudeker et al. (1980).

**Electron microscopy**

Preparation of culture samples for electron microscopy was done according to Handley et al. (1988). Ultra-thin sections were studied in a Philips EM 201.

**Chemicals**

Ribulose 1,5-bisphosphate was obtained from Sigma Chemical Co. (St. Louis, Missouri, USA), [¹⁴C]-NaHCO₃ (2.11 TBq.mol⁻¹) from Amersham International P.L.C. Sodium thiosulphate pentahydrate was obtained from J.T. Baker Chemicals, Deventer, the Netherlands. Anhydrous potassium tetrathionate was obtained from Fluka AG, Buchs, Switzerland. All other chemicals were reagent grade and obtained from commercial sources.

**Results**

**Autotrophic and mixotrophic growth on thiosulphate**

Mixotrophic growth of *T. acidophilus* was studied in substrate-limited chemostat cultures grown at a dilution rate of 0.05 h⁻¹ and at pH 3.0. At this pH, thiosulphate is unstable at millimolar concentrations (Johnston and McAmish 1973). The residual thiosulphate and tetrathionate concentrations in the chemostat cultures were lower than the detection limit of the cyanolysis assay (approximately 10 µM). At these low concentrations, chemical decomposition of thiosulphate was negligible (data not shown). The thiosulphate concentration in the reservoir medium, which was adjusted to pH 7.5, did not change during the experiments (data not shown).

Attempts to grow *T. acidophilus* autotrophically on thiosulphate at D = 0.05 h⁻¹ were unsuccessful, in accordance with the observation of Mason et al. (1987) that the maximum growth rate of *T. acidophilus* on thiosulphate was below 0.05 h⁻¹. However, steady state cultures could be obtained at D = 0.03 h⁻¹. The observed biomass yield at this dilution rate was 6.0 g.(mol thiosulphate)⁻¹. This yield figure is in good agreement with a yield of 5.5 g.mol⁻¹ found at D = 0.025 h⁻¹ by Mason et al. (1987). No effect on the biomass yields was observed when the cultures were sparged with air containing 5 % (v/v) carbon dioxide, indicating that the cultures were not carbon-limited.

In contrast to autotrophic cultures, which washed out at dilution rates above 0.03 h⁻¹, mixotrophic utilization of thiosulphate was observed at a dilution rate of 0.05 h⁻¹. Thiosulphate was completely oxidized to sulphate at this dilution rate up to a molar ratio of thiosulphate to glucose of 14.

Addition of thiosulphate to the reservoir medium of glucose-limited chemostat cultures resulted in an increase of the biomass concentration in the cultures. At thiosulphate to glucose ratios below 5, the biomass density in the cultures increased linearly with the influent thiosulphate concentration (Fig. 1). At thiosulphate to glucose ratios above 5, the increase of the biomass concentration corresponded with the autotrophic growth yield on thiosulphate (Fig. 1). The influent glucose and thiosulfate concentrations of the chemostat cultures did not significantly influence the carbon and protein content of the biomass, which remained at 49 ± 1 % and 69 ± 2 %,
Figure 1. Effect of increasing concentrations of thiosulphate in the reservoir medium on biomass concentrations in mixotrophic, substrate-limited chemostat cultures of *T. acidophilus* (2.5 mM glucose; D = 0.05 h⁻¹; pH 3.0; T = 30 °C). The dotted line indicates the theoretical upper limit of carbon conversion during heterotrophic growth on glucose (Gommers et al. 1988). The slope of the dashed line indicates the growth yield in autotrophic, thiosulphate-limited chemostat cultures (D = 0.03 h⁻¹).

**Oxidation of thiosulphate**

Cells of *T. acidophilus* pregrown in heterotrophic, glucose-limited chemostat cultures exhibited significant rates of thiosulphate-dependent oxygen uptake (Pronk et al. 1990b; Fig. 2). Thiosulphate-dependent oxygen uptake rates increased during mixotrophic growth on glucose and thiosulphate (Fig. 2). Thiosulphate oxidation by cell suspensions from mixotrophic chemostat cultures exhibited a typical biphasic pattern. After an initial rapid oxygen uptake, during which approximately 0.25 mol oxygen was consumed per mol thiosulphate, oxygen uptake continued at a lower rate. The oxygen uptake rates in the second phase corresponded to the rates of tetrathionate oxidation by the cell suspensions. The explanation of this phenomenon becomes evident from Fig. 3. During the first phase of thiosulphate oxidation, a near-quantitative conversion to tetrathionate occurred, in accordance with the observed biphasic oxygen uptake patterns.

Mixotrophic growth of *T. acidophilus* on glucose and thiosulphate led to an increase of the tetrathionate-dependent oxygen uptake rates of cell suspensions (Fig. 2). The latter oxygen uptake rates were almost identical to the rates required for complete oxidation of thiosulphate via tetrathionate in the chemostat cultures. In contrast, the maximum rates of thiosulphate-dependent oxygen uptake by cell suspensions were much higher than the actual thiosulphate oxidation rates observed in the chemostat cultures.

Also the rates of substrate-dependent oxygen uptake with the inorganic sulphur compounds trithionate, sulphide and elemental sulphur increased during mixotrophic growth on glucose and thiosulphate (data not shown).

**Mixotrophic utilization of tetrathionate**

As discussed above, experiments with cell suspensions suggested that tetrathionate is an obligatory intermediate during the oxidation of thiosulphate by *T. acidophilus*. Only one of the eight electrons available from the complete oxidation of thiosulphate to sulphate is derived from the initial formation of tetrathionate. If tetrathionate is indeed an obligatory intermediate during thiosulphate oxidation by *T. acidophilus*, it can be expected that the energetic value of the redox equivalents derived from thiosulphate and tetrathionate oxidation will be similar.

In a recent study, Mason et al. (1987) reported that the molar growth yield of *T. acidophilus* in tetrathionate-limited, autotrophic chemostat cultures was 2.3-fold higher than the molar growth yield in thiosulphate-limited chemostat cultures. This would imply that the energetic value of
tetrathionate redox equivalents is 1.3 fold higher than those derived from thiosulphate oxidation. This conclusion is in apparent contradiction with our conclusions regarding the role of tetrathionate as an intermediate during thiosulphate oxidation. To investigate the energetic value of tetrathionate redox equivalents, mixotrophic utilization of glucose and tetrathionate was studied.

The addition of tetrathionate to the reservoir medium of glucose-limited chemostat cultures led to an increase of the biomass yields. The increase of the biomass yield per mole of redox equivalents was identical to that observed with thiosulphate as an energy source (Fig. 4).

Rubisco activities and polyhedral bodies

Many (facultatively) autotrophic bacteria that employ the Calvin cycle for CO₂ fixation contain typical polyhedral inclusion bodies. Since in all species studied, these organelles have been demonstrated to contain active ribulose 1,5-bisphosphate carboxylase (Rubisco, EC 4.1.1.39), they are commonly referred to as carboxysomes (Codd 1988). It has recently been reported that also elemental sulphur-grown T. acidophilus cells contain polyhedral inclusion bodies (Katayama-Fujimura et al. 1984). However, there are no literature data on the regulation of carboxysome synthesis in T. acidophilus.

The Rubisco activity of 43 nmol.min⁻¹.(mg protein)⁻¹ in cell-free extracts of thiosulphate-limited, autotrophic chemostat cultures was sufficient to account for the observed rate of inorganic carbon fixation in the cultures (31 nmol.min⁻¹.(mg protein)⁻¹). Polyhedral inclusion bodies were abundant in cells from thiosulphate-limited chemostat cultures (data not shown). However, the organelles were not observed in all sections. This can be explained from the fact that they were typically located in the centre of the cells (Fig. 5). The mean diameter of the organelles, measured in various thin sections of fixed cells, was 100 ± 10 nm. Both Rubisco activities in cell-free extracts and the abundance of polyhedral bodies increased when autotrophic cultures were grown under CO₂ limitation (Table 1, Fig. 5).

As reported previously (Prönk et al. 1990b), T. acidophilus retained significant activities of Rubisco during heterotrophic growth in glucose-limited chemostat cultures (Table 1). In addition to this, polyhedral inclusion bodies similar to those in
Table 1. Activities of RuBPCase in cell-free extracts of *T. acidophilus* grown in chemostat cultures (pH 3.0, 30 °C) under various growth conditions.

<table>
<thead>
<tr>
<th>Growth-limiting substrate</th>
<th>Growth rate h⁻¹</th>
<th>RuBPCase nmol (mg protein.min)⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (5 mM)</td>
<td>0.05</td>
<td>5</td>
</tr>
<tr>
<td>Glucose (5 mM)</td>
<td>0.05</td>
<td>10</td>
</tr>
<tr>
<td>S₂O₃⁻ (20 mM)</td>
<td>0.05</td>
<td>29</td>
</tr>
<tr>
<td>Glucose (2.5 mM)</td>
<td>0.05</td>
<td>29</td>
</tr>
<tr>
<td>S₂O₃⁻ (34 mM)</td>
<td>0.03</td>
<td>43</td>
</tr>
<tr>
<td>S₂O₃⁻ (20 mM)</td>
<td>0.03</td>
<td>78</td>
</tr>
</tbody>
</table>

* Culture sparged with air (1 L.min⁻¹)

* Thiosulphate-grown chemostat culture (S₂O₃⁻ = 20 mM), sparged with air containing 0.0125 % CO₂. Although CO₂ was limiting, all thiosulphate was oxidized to sulphate. The molar growth yield on thiosulphate was 70 % of that of a thiosulphate-limited autotrophic culture.

autotrophic cultures could be observed at a low frequency in thin sections (Fig. 5). Mixotrophic growth on glucose and thiosulphate led to an increase of the RuBPCase activities in cell-free extracts (Table 1). Furthermore, polyhedral inclusion bodies were more abundant than in cells from heterotrophic chemostat cultures (data not shown).

**Discussion**

**Energetics of mixotrophic growth**

During mixotrophic growth of facultatively autotrophic bacteria, CO₂ assimilation is in most cases strictly regulated (Dijkhuizen and Harder 1979, Gottschal and Kuenen 1980). Under heterotrophic growth conditions, or at low ratios of inorganic and organic substrates, CO₂-assimilating activity is not expressed. In such cultures, the inorganic substrate is used to enhance heterotrophic carbon assimilation. As a result, the cell yields on the organic substrate increase up to the theoretical limit of organic carbon assimilation (88 % conversion of glucose carbon into biomass [Gommers et al. 1988]). A further increase of the inorganic substrate concentration does not lead to a situation of energy excess, since under such conditions autotrophic CO₂-assimilating capacity is induced. As demonstrated by Gommers et al. (1988), this situation is encountered with *Thiobacillus versutus* (growth on acetate and thiosulphate, [Gottschal and Kuenen 1980]) and *Pseudomonas oxalatus* (growth on acetate and formate, [Dijkhuizen and Harder 1979]).

At first sight, the same situation seems to apply for *Thiobacillus acidophilus* grown mixotrophically on glucose and thiosulphate. Also in this case, the relation between the ratio of thiosulphate to glucose and the biomass yields was biphasic (Fig. 1). At thiosulphate to glucose ratios below 5, autotrophic CO₂ fixation did not seem to occur, since the growth yields were higher than the sum of the heterotrophic and autotrophic growth yields (Fig. 1). Autotrophic CO₂ fixation set in before the theoretical limit of glucose assimilation was reached, as judged from the fact that the further increase of the biomass yields paralleled the autotrophic growth yield on thiosulphate (Fig. 1).

When *T. acidophilus* is grown mixotrophically on formate and glucose, autotrophic growth sets in when glucose assimilation reaches its theoretical maximum (Prönk et al. 1990c). The difference between mixotrophic growth on glucose and formate and the present study may be found in the...
fact that redox equivalents from formate can enter the electron transport chain at the level of NADH (Pronk et al. 1990c). Redox equivalents from thiosulphate probably enter the electron transport chain at the level of cytochrome c. With the latter substrate, reversed electron transport is therefore required for NAD or NADP reduction. The fact that, as judged by the growth pattern shown in Fig. 1, maximum assimilation of glucose is not reached with thiosulphate as an additional energy source, may therefore reflect a shortage of low potential redox equivalents for biosynthesis.

Cell-free extracts from heterotrophic cultures and from mixotrophic cultures grown at low ratios of thiosulphate and glucose contained significant RubPCase activities (Table 1). Theoretically, this enzyme could be involved in actual in vivo CO₂ assimilation. However, if these RubPCase activities in cell-free extracts were indicative of in vivo CO₂ assimilation, a strictly biphasic curve as shown in Fig. 1 would not be expected. The apparent contradiction between growth yields and RubPCase activities in cell-free extracts suggests that RubPCase synthesis is not the only site of regulation of autotrophic CO₂ assimilation. For example, in vivo RubPCase activity might be regulated by intracellular metabolite concentrations. Alternatively, another step in inorganic carbon metabolism (CO₂ uptake, phosphoribulokinase) may be the site of control of CO₂ fixation in vivo.

When it is postulated that indeed, at low ratios of thiosulphate to glucose, thiosulphate was used exclusively to increase the efficiency of glucose assimilation, a quantitative comparison can be made of the energetic value of the redox equivalents derived from the oxidation of glucose and thiosulphate. At thiosulphate to glucose ratios below 5, the increase of the biomass concentration as a result of thiosulphate addition was 9.79 g (mol thiosulphate)⁻¹ or 1.22 g (mol redox equivalents)⁻¹ (Fig. 1). The molar growth yield of T. acidophilus in glucose-limited chemostat cultures grown at D = 0.05 h⁻¹ and pH 3.0 was 59 g (mol glucose)⁻¹ or 2.46 g (mol redox equivalents)⁻¹. Thus, the energetic value of thiosulphate redox equivalents was only (1.22 : 2.46) x 100 = 50 % of that of the redox equivalents from glucose oxidation.

The energetic value of tetrahionate redox equivalents did not differ significantly from those derived from thiosulphate (Fig. 4). We have recently also studied the mixotrophic growth of T. acidophilus on glucose and formate (Pronk et al. 1990c). The energetic value of formate redox equivalents was 75 % of that of glucose redox equivalents, or 50 % higher than those from thiosulphate and tetrahionate (Fig. 4). The low growth yields of T. acidophilus in heterotrophic cultures (Pronk et al. 1990b) may be caused by a low efficiency of the proton-translocating respiratory chain. In T. acidophilus, formate redox equivalents may enter the respiratory chain at the level of NAD (Pronk et al. 1990c). If it is assumed that active uptake of formate is coupled to the inward translocation of one proton, the relative efficiency of formate and thiosulphate redox equivalents is compatible with H⁺/O ratios of 4 and 2 for NADH and thiosulphate, respectively.

**Thiosulphate metabolism**

The transient accumulation of tetrahionate during thiosulphate oxidation (Fig. 3) indicated that tetrahionate can be an intermediate of thiosulphate oxidation by T. acidophilus. The near-quantitative conversion of thiosulphate to tetrahionate observed in these experiments suggests that this pathway plays a major role in thiosulphate oxidation. In this respect, thiosulphate metabolism in T. acidophilus is similar to that of the acidophiles T. ferrooxidans (Sinha and Walden 1966) and T. thiooxidans (Okuzumi and Kita 1965). Also the identical energetic values of thiosulphate and tetrahionate calculated from growth yields of mixotrophic chemostat cultures are consistent with oxidation of thiosulphate via tetrahionate.

Tetrahionate is much more stable in acidic environments than thiosulphate. The very high maximum rates of the conversion of thiosulphate to tetrahionate may allow the organism to compete successfully with the chemical decomposition of thiosulphate. Further metabolism of tetrahionate is probably initiated by a hydrolytic cleavage, yielding sulphate and S₇-sulphane monosulphonic acid (Hazeu et al. 1988, Pronk et al. 1990a).

In addition to an increase in the growth efficiency (Fig. 1), simultaneous utilization of glucose and thiosulphate led to an increase of the maximum growth rate at which thiosulphate could be utilized as an energy source, compared to the maximum growth rate of autotrophic, thiosulphate-limited cultures. These factors may be advantageous in the competition of T. acidophilus with obligate autotrophs in environments where both inorganic sulphur compounds and organic substrates are available.

The high specific activities and the possibility to grow dense mixotrophic cultures make T. acidophilus an attractive model organism to study the enzymology of sulphur compound metabolism in acidic environments.

**Carboxysomes and RubPCase activities**

Polyhedral inclusion bodies are widespread
among autotrophic prokaryotes. In all species containing the organelles which have been examined, the inclusion bodies have been shown to contain active RuBPCase (Codd 1988). Although we have not demonstrated the presence of RuBPCase in the organelles, the morphological similarities with carboxysomes from other thiobacilli (Codd 1988, Holthuijzen et al. 1986) and the apparent coordinate regulation of RuBPCase activities and inclusion body abundance suggest that the T. acidophilus inclusion bodies are carboxysomes. Attempts to isolate carboxysomes from T. acidophilus by a procedure described for T. neapolitanus (Holthuijzen et al. 1986) were unsuccessful.

With the exception of T. denitrificans, carboxysomes have been detected in all obligately autotrophic Thiobacillus species studied (Codd 1988). In the facultatively autotrophic thiobacilli, carboxysomes are less ubiquitous. The organelles have been detected in the facultative autotrophs T. intermedius and T. acidophilus, but not in T. versutus and T. novellus (Katayama-Fujimura et al. 1984). Regulation of carboxysome synthesis has been studied in batch cultures of T. intermedius. Both RuBPCase activity and carboxysomes were observed after autotrophic growth on thiosulphate, but not in cultures which had been grown heterotrophically on yeast extract (Purohit et al. 1976). In contrast to these observations, carboxysomes could be observed at low frequencies in heterotrophically grown cells of T. acidophilus (Fig. 5).

The regulation of RuBPCase activity in T. acidophilus is less strict than in other facultatively autotrophic thiobacilli. The retention of significant RuBPCase levels during heterotrophic growth (Pronek et al. 1990b, Table 1) may reduce the time required for adaptation to autotrophic growth. Also the retention of sulphur compound-oxidizing capacity under heterotrophic growth conditions suggests that T. acidophilus is well adapted to growth in rapidly changing environments.

As observed in other thiobacilli (Beudeker et al. 1980, Codd 1988), RuBPCase activities and carboxysome abundances in cell sections appeared to be correlated. This observation suggests a physiological role of carboxysomes in CO₂ assimilation by T. acidophilus. Several physiological functions of carboxysomes have been proposed in the literature, including protection of RuBPCase from oxygen and a function as RuBPCase storage bodies (Codd 1988). Its metabolic versatility (Guay and Silver 1975, Pronk et al. 1990b) makes T. acidophilus well suited for further studies into the regulation and function of carboxysome synthesis.

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Literature


Chapter 4


CHAPTER 5

Energetics of mixotrophic and autotrophic C₄-metabolism by Thiobacillus acidophilus

Abstract

Although the facultatively autotrophic acidophile *Thiobacillus acidophilus* is unable to grow on formate and formaldehyde in batch cultures, cells from glucose-limited chemostat cultures exhibited substrate-dependent oxygen uptake with these C₄-compounds. Oxidation of formate and formaldehyde was unconnector-sensitive, suggesting that active transport was involved in the metabolism of these compounds. Formate- and formaldehyde-dependent oxygen uptake was strongly inhibited at substrate concentrations above 150 and 400 μM, respectively. However, autotrophic formate-limited chemostat cultures were obtained by carefully increasing the formate to glucose ratio in the reservoir medium of mixotrophic chemostat cultures. The molar growth yield on formate (Y = 2.5 g mol⁻¹ at a dilution rate of 0.05 h⁻¹) and RubBPCase activities in cell-free extracts suggested that *T. acidophilus* employs the Calvin cycle for carbon assimilation during growth on formate. *T. acidophilus* was unable to utilize the C₄-compounds methanol and methylamine. Formate-dependent oxygen uptake was expressed constitutively under a variety of growth conditions. Cell-free extracts contained both dye-linked and NAD-dependent formate dehydrogenase activities. NAD-dependent oxidation of formaldehyde required reduced glutathione. In addition, cell-free extracts contained a dye-linked formaldehyde dehydrogenase activity. Mixotrophic growth yields were higher than the sum of the heterotrophic and autotrophic yields. A quantitative analysis of the mixotrophic growth studies revealed that formaldehyde was a more effective energy source than formate.

Introduction

*Thiobacillus acidophilus* is a facultatively autotrophic, acidophilic bacterium. The organism was first isolated as a contaminant of a ferrous iron-grown culture of the obligate autotroph *T. ferrooxidans* (Guay and Silver 1975). Substrates for autotrophic growth of *T. acidophilus* include elemental sulphur, thiosulphate and tetrathionate (Guay and Silver 1975; Norris et al. 1986), but not ferrous iron.

*T. acidophilus* has been used as a model organism to study the energetics of growth in acidic environments (Matin et al. 1982; Matin and Matin 1982; Zychlinski and Matin 1983a,b). It has been suggested that heterotrophic acidophiles like *T. acidophilus* play an important role in natural bioleaching populations by scavenging toxic organic compounds (Tsuchiyama et al. 1974; Nerkar et al. 1977; Pront et al. 1990a).

A number of simple organic compounds can be used as carbon sources for heterotrophic growth of *T. acidophilus*. However, most small organic acids cannot be used as carbon sources for growth in batch cultures (Guay and Silver 1975; Pront et al. 1990a). Toxicity of weak acids in acidic environments has been observed with other acidophilic microorganisms and can be explained by their accumulation in the cytoplasm, leading to a dissipation of the transmembrane pH gradient (Ingledew 1982, Alexander et al. 1987). For example, pyruvate can not be used as a carbon source for heterotrophic growth of *T. acidophilus* in batch cultures due to substrate inhibition. However, this substrate can support growth in carbon-limited chemostat cultures (Pront et al. 1990a).

Although autotrophic growth of neutrophilic thiobacilli on formate has been reported, the acidophilic *Thiobacillus* species are generally considered to be unable to use this compound (Kelly and Harrison 1989). However, we have found that cell suspensions of *T. acidophilus* pregrown on glucose readily oxidize formate. Therefore, the ability of *T. acidophilus* to use formate and other C₄-compounds as energy sources for mixotrophic and autotrophic growth was re-investigated.

Material and Methods

Organism and maintenance

*Thiobacillus acidophilus* DSM 700 was obtained from the Deutsche Sammlung von Mikroorganismen
as a liquid culture on glucose. A sample was plated on mineral medium (pH 3.5) supplemented with glucose and solidified with 0.8% agarose. Agarose was sterilized separately to prevent acid hydrolysis. A single colony was inoculated in 200 ml mineral medium plus glucose (20 mM). The resulting culture was made 10% (v/v) with dimethyl-sulphoxide and stored at -70 °C in 1 ml aliquots. These frozen samples were used as inocula for continuous culture studies.

**Mineral medium**

*T. acidophilus* was grown in a synthetic medium containing per litre of de-mineralized water: (NH₄)₂SO₄ 3.0 g; KH₂PO₄ 0.3 g; MgSO₄·7H₂O 0.5 g; Na₂SO₄ 1.4 g; CaCl₂·2H₂O 0.26 g; FeSO₄·7H₂O 11 mg; ZnSO₄·7H₂O 0.9 mg; MnCl₂·2H₂O 2.0 mg; CoCl₂·6H₂O 0.6 mg; CuSO₄·5H₂O 0.6 mg; NaMoO₄ 0.8 mg; H₂BO₃ 2.0 mg; KI 0.2 mg; EDTA 30 mg; nitro–triacetic acid, 5 mg and silicon antifoaming agent (BDH Chemicals, Poole, Dorset, UK), 25 μl. The medium was adjusted to pH 3.0 with 1 M H₂SO₄ and autoclaved at 120 °C. Solutions of formic acid and glucose were sterilized separately at 110 °C and added to the autoclaved mineral medium. Formaldehyde solutions were prepared by hydrolysis of paraformaldehyde in 10 mM KOH (10 min at 100 °C) and filter-sterilized before addition to the autoclaved mineral medium.

**Growth conditions**

Continuous cultivation was performed in Applikon laboratory fermenters with a working volume of 1 l. The pH was automatically titrated with 1 M KOH. The cultures were continuously gassed with water-saturated air (1 l min⁻¹) and stirred at 800 rpm. The dissolved oxygen concentration in the cultures was monitored with a steam-sterilizable Clark-type electrode. Chemostat cultures were grown at a dissolved oxygen concentration of above 75% of air saturation, at 30 °C, at pH 3.0 and at a dilution rate of 0.05 h⁻¹. Since biomass concentrations in the chemostat cultures were linearly proportional to the substrate concentrations in the reservoir media, it was concluded that the cultures were carbon- and energy-limited.

**Control of culture purity**

The purity of chemostat cultures was routinely checked by phase-contrast microscopy and by plating on mineral medium plus glucose, solidified with 0.8% (w/v) agarose. Additionally, immuno-fluorescence microscopy with specific antisera against *T. acidophilus* was performed as described by Muyzer et al. (1987).

**Dry weight determination**

The dry weight of cell suspensions was determined by filtering aliquots over nitrocellulose filters (pore diameter 0.45 μm, Schleicher and Schüll, Dassel, FRG). The cells were washed three times with demineralized water and dried to constant weight at 70 °C.

**Protein determination**

The protein content of whole cells was assayed with a modified biuret method: cells were harvested from continuous cultures, washed with demineralized water and resuspended to a concentration of approximately 2.5 mg dry weight ml⁻¹. The concentrate was boiled in 1 M KOH for 10 min and subsequently cooled on ice. CuSO₄·5H₂O was then added to a final concentration of 25 mM. After 5 min, the mixture was centrifuged in an Eppendorf bench-top centrifuge (13,000 rpm) for 2 min. The absorbance of the supernatant was measured at 550 nm. The protein content of cell-free extracts was determined by the method of Bradford (1976). In both assays, bovine serum albumin (fatty acid free, Sigma, St. Louis, USA) was used as a standard.

**Determination of organic carbon content**

A Beckman model 915B Tocamaster total organic carbon analyser was used to determine the carbon content of whole cultures and culture supernatants. The carbon content of the bacteria was obtained from the difference. Cell suspensions were acidified with H₂PO₄ prior to analysis in order to expel carbon dioxide accumulated inside the cells.

**Substrate determinations.**

Glucose concentrations in media and culture supernatants were measured with the GOD-PAP method (Boehringer, Mannheim, FRG). The formate concentrations in the reservoir media were determined according to Lang and Lang (1972), formaldehyde was assayed according to Nash (1953).

**Measurement of substrate-dependent oxygen consumption**

Respiration rates of cells were assayed polarographically with a Clark-type oxygen electrode (Yellow Springs Instruments Inc., Yellow Springs, Ohio, USA). Cells from carbon-limited chemostat cultures were assayed directly in the culture fluid or after dilution with mineral medium without carbon source (pH 3.0). Calculations were made on the basis of an oxygen concentration of 0.24 mM in air-saturated mineral medium at 30 °C. The values presented here have been corrected for the (low) endogenous respiration rates.
Preparation of cell-free extracts

Cells were harvested from chemostat cultures by centrifugation (10,000 x g, 10 min) and washed with a buffer containing 100 mM sodium acetate and 5 mM magnesium nitrate (pH 5.0). This buffer proved to be optimal for the extraction of formate dehydrogenase activity. For convenience, the same buffer was used for formaldehyde dehydrogenase assays. In this case, no difference was observed with activities of extracts prepared in Tris-HCl buffer (0.1 M, pH 8.0). Cells were resuspended in the same buffer to a concentration of approximately 10 mg dry weight ml⁻¹ and disrupted by sonication in an MSE 150 W sonifier (12 bursts of 30 s with intermittent cooling). Intact cells and debris were removed by centrifugation (40,000 x g, 15 min). The clear supernatants were used as cell-free extracts.

Enzyme assays

The reduction of NAD and DCPIP were monitored spectrophotometrically at 340 and 600 nm, respectively. Enzyme activities were calculated using extinction coefficients of 6.3 mM⁻¹cm⁻¹ for NADH and 20.6 mM⁻¹cm⁻¹ for DCPIP (Armstrong 1964) and expressed as U (mg protein)⁻¹ (1 U = 1 μmol electron acceptor reduced.min⁻¹). In all enzyme assays, the enzyme activity was linearly proportional to the amount of cell-free extract added. Enzyme assays were performed at 30 °C.

Formate dehydrogenase, NAD-dependent

The reaction mixture (1 ml) contained: potassium phosphate buffer (pH 7.0), 100 μmol; MgSO₄, 10 μmol; Triton X-100, 2 mg NAD, 2 μmol; and cell-free extract. The reaction was started by the addition of 10 μmol potassium formate.

Formate dehydrogenase, dye-linked

The reaction mixture (1 ml) contained: potassium phosphate buffer (pH 7.0), 100 μmol; MgSO₄, 10 μmol; Triton X-100, 2 mg phenazine methosulphate (PMS), 0.3 μmol; dichlorophenol-indophenol (DCPIP), 0.2 μmol; and cell-free extract. The reaction was started by the addition of 10 μmol potassium formate.

Formaldehyde dehydrogenase, NAD-dependent

The reaction mixture (1 ml) contained: potassium phosphate buffer (pH 7.0), 100 μmol; MgSO₄, 10 μmol; Triton X-100, 2 mg; reduced glutathione (GSH), 2 μmol; NAD, 2 μmol; and cell-free extract. The reaction was started by the addition of 5 μmol formaldehyde.

Formaldehyde dehydrogenase, dye-linked

The reaction mixture was identical to that employed for assaying dye-linked formate dehydrogenase. The reaction was started by the addition of 5 μmol formaldehyde.

Ribulose 1,5-bisphosphate carboxylase

Cell-free extracts for RuBPCase assays were prepared as described by Pronk et al. (1990a). RuBPCase was assayed according to Beudeker et al. (1980).

Chemicals

Ribulose 1,5-bisphosphate was obtained from Sigma Chemical Co. (Boston, Missouri, USA). [¹⁴C]-NaHCO₃ (2.11 TBq.mol⁻¹), from Amersham International PLC. agarose (medium electrophorosmosis) and formic acid (p.a. quality) were from Merck, Darmstadt, FRG. All other chemicals were reagent grade and obtained from commercial sources.

Results

Oxidation of C₄-compounds by Thiobacillus acidophilus

In batch cultures, T. acidophilus can grow on formate as a sole source of energy (Pronk et al. 1990a). However, cells from glucose-limited chemostat cultures exhibited high rates of formate-dependent oxygen uptake. At a formate concentration of 100 μM, the pH optimum for formate oxidation by intact cells was approximately 3.0. This pH optimum is comparable with the pH optima for the oxidation of various reduced sulphur compounds by T. acidophilus (results not shown). In all oxygen uptake studies, the stoichiometry of formate and oxygen consumption was as expected (2:1), indicating that the observed oxygen uptake rates were not caused by stimulation of endogenous respiration.

At substrate concentrations below 150 μM, formate oxidation by cell suspensions obeyed Monod kinetics (Fig. 1). The apparent Kₐ and Vₘₐₓ, for formate were 57 μM and 0.37 μmol O₂.min⁻¹.(mg dry weight)⁻¹, respectively. At formate concentrations higher than 150 μM, substrate inhibition occurred (Fig. 1). The oxidation rates observed with formate as a substrate were significantly higher than with the inorganic sulphur compounds thiosulphate (Vₘₐₓ = 70 nmol O₂.min⁻¹.mg⁻¹) and tetrathionate (Vₘₐₓ = 50 nmol O₂.min⁻¹.mg⁻¹).

In addition to formate, T. acidophilus could also respire formaldehyde. Cells from glucose-limited chemostat cultures exhibited formaldehyde-dependent respiration rates of 35-40 nmol O₂.min⁻¹.(mg dry weight)⁻¹ at a formaldehyde concentration of 200 μM. Substrate inhibition was observed at formaldehyde concentrations above 400 μM. The pH optimum for formaldehyde oxidation was approximately pH 3. The kinetics of formaldehyde oxidation were not studied in detail.
Oxidation of formate and formaldehyde by cell suspensions was inhibited by the protonophores 2,4-dinitrophenol (Fig. 2) and carbonyl cyanide m-chlorophenylhydrazone (data not shown). In contrast, tetraionate-dependent oxygen uptake was not inhibited by uncouplers (Fig. 2).

Heterotrophically grown cells of *T. acidophilus* did not exhibit significant oxygen uptake rates with methylamine and methanol.

**Autotrophic growth on C₁-compounds**

In a facultative autotroph like *T. acidophilus*, it may be expected that the oxidation of formate and formaldehyde can provide the metabolic energy for mixotrophic and autotrophic growth. For mixotrophic growth of *T. acidophilus* on glucose and formate, the formate concentration in the reservoir medium was chosen in such a way that the rate of formate addition to the culture did not exceed its formate-oxidizing capacity. This was routinely calculated from formate-dependent oxygen consumption rates of culture samples.

When mixotrophic chemostat cultures of *T. acidophilus* grown on glucose and formate were switched to a medium containing formic acid as a sole energy source, stable autotrophic formate-limited cultures were obtained. The use of formic acid in the reservoir media does not lead to accumulation of mineral salts in the cultures, since all substrate is converted into carbon dioxide and water. Formate-limited chemostat cultures were grown with influent formic acid concentrations of 75 - 250 mM, resulting in biomass concentrations of up to 0.6 g l⁻¹. The molar growth yield of *T. acidophilus* in formate-limited chemostat cultures was 2.5 g (mol formate)⁻¹. This growth yield is similar to the growth yields of other bacteria that employ the Calvin cycle for carbon assimilation during growth on formate (Dijkhuizen et al. 1977a, van Verseveld and Stouthamer 1978, Kelly et al. 1979). Molar growth yields of 7-8 g (mol formate)⁻¹ have been reported for bacteria that employ the serine pathway of formate assimilation (Rokem et al. 1978). The growth yields observed in the present study therefore strongly suggest that *T. acidophilus* employs the Calvin cycle for carbon assimilation during formate-limited growth.

Analogous to the growth experiments with formate, it was possible to grow substrate-limited chemostat cultures on glucose and formaldehyde. We have not investigated growth of *T. acidophilus* on formaldehyde as a sole source of energy.

The carbon and energy sources used in mixotrophic and autotrophic chemostat cultures did not significantly influence the carbon and protein content of the biomass, which remained at 48 ± 1
Figure 3. Effect of increasing concentrations of formic acid in the reservoir medium on the biomass concentrations in glucose-limited chemostat cultures of T. acidophilus (5 mM glucose, D = 0.05 h⁻¹, pH 3.0, T = 30 °C). The dashed line indicates the growth yield in autotrophic, formate-limited chemostat cultures, the dotted line indicates the theoretical maximum growth yield on glucose as a carbon source (130 g.mol⁻¹; Gommers et al. 1988)

% and 67 ± 2 %, respectively. The residual concentrations of glucose, formaldehyde and formate in the chemostat cultures were below the detection limits of the analytical procedures used.

Attempts to induce methanol-oxidizing activity by growth on mixtures of glucose and methanol were unsuccessful. This is in accordance with previous reports that T. acidophilus is unable to utilize methanol as a sole energy source in batch cultures (Pronk et al. 1990a).

Mixotrophic growth on C₁-compounds

Addition of formic acid to the reservoir medium of glucose-limited chemostat cultures resulted in an increase of the biomass density of the cultures. At formate to glucose ratios up to 19, the biomass density increased linearly with the formate concentration in the reservoir medium. At these formate to glucose ratios, the cell yields of the cultures were higher than the sum of the autotrophic and heterotrophic growth yields (Fig. 3). At formate to glucose ratios above 19, the increase of the biomass density corresponded with the autotrophic growth yield of T. acidophilus on formate (Fig. 3).

T. acidophilus exhibits a low growth yield during growth in glucose-limited chemostat cultures (Y_max = 69 g.mol⁻¹; Pronk et al. 1990a). The actual growth yield at a dilution rate of 0.05 h⁻¹ (59 g.mol⁻¹) was lower due to maintenance effects (Pronk et al. 1990a). In bacteria, the theoretical upper limit of carbon conversion during growth on glucose is 88 % (Gommers et al. 1988), equivalent to a molar growth yield of 130 g.(mol glucose)⁻¹. The biomass density observed at a formate to glucose ratio of 19 corresponded almost exactly with this theoretical value (Fig. 3).

Also the addition of formaldehyde to the reservoir media of glucose-limited chemostat cultures led to an increase of the biomass yields. On a molar basis, this yield increase was more than twice that observed with formate as the auxiliary energy source (Fig. 4).

Enzymology of C₁-metabolism

T. acidophilus grown in glucose-limited chemostat cultures exhibited high rates of formate-dependent oxygen uptake (Table 1). Neither the V_max nor the K_s values calculated from oxygen uptake experiments changed significantly when the cells were grown mixotrophically on glucose and formate or in autotrophic, formate-limited chemostat cultures (Table 1). Formate-oxidizing activity was not repressed in heterotrophic, nitrogen-limited
Table 1. Kinetic parameters of formate-dependent oxygen uptake by cell suspensions of *Thiobacillus acidophilus* from substrate-limited chemostat cultures. Growth conditions: $D = 0.05$ h$^{-1}$, pH 3.0, $T = 30$ °C.

<table>
<thead>
<tr>
<th>Carbon and energy source</th>
<th>Growth limitation</th>
<th>$K_s$ μM</th>
<th>$V_{max}$ mmol O$_2$ [min mg]$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose 10 mM</td>
<td>glucose</td>
<td>57</td>
<td>366</td>
</tr>
<tr>
<td>Glucose 20 mM</td>
<td>nitrogen</td>
<td>54</td>
<td>334</td>
</tr>
<tr>
<td>Glucose 5 mM; formate 100 mM</td>
<td>glucose/formate</td>
<td>58</td>
<td>291</td>
</tr>
<tr>
<td>Formate 250 mM</td>
<td>formate</td>
<td>51</td>
<td>305</td>
</tr>
</tbody>
</table>

The residual glucose concentration in the culture was 3.8 mM.

chemostat cultures (Table 1). Apparently the formate-oxidizing activity was not influenced by the presence of organic substrates in the culture supernatant. Also thiosulphate-limited, autotrophic cultures of *T. acidophilus* exhibited high formate-oxidizing activities. These data suggest that formate oxidation by *T. acidophilus* is constitutive and not subject to regulation.

Cell-free extracts prepared from chemostat cultures of *T. acidophilus* contained both NAD-dependent and dye-linked formate dehydrogenase activities (Table 2). No activity was observed with NADP as an electron acceptor. The activity with the artificial electron-accepting couple PMS-DCPIP was approximately six-fold higher than with DCPIP alone. In all extracts tested, the activities of formate dehydrogenase assayed with PMS-DCPIP were comparable with the NAD-dependent activities. Both enzyme activities exhibited maximum activity at pH 7.

In contrast to the formate-dependent oxygen uptake rates observed with intact cells (Table 1), NAD- and dye-linked formate dehydrogenase activities in cell-free extracts were dependent on the culture conditions (Table 2). NAD- and dye-linked formate dehydrogenase activities in cell-free extracts prepared from heterotrophic chemostat cultures were roughly one order of magnitude too low to account for the rates of formate oxidation observed with cell suspensions (Tables 1 and 2). Attempts to increase the formate-oxidizing activities in extracts from heterotrophic cultures by changing the assay conditions or the procedures for cell-free extract preparation were unsuccessful. Activities of mixed cell-free extracts (combined extracts from heterotrophic and autotrophic chemostat cultures or extracts prepared from mixed cell suspensions) were additive, indicating that the different formate dehydrogenase activities were not due to the presence of soluble stimulatory or inhibitory compounds (data not shown). The experimental data suggest that the stability of formate dehydrogenase during cell-free extract preparation is positively influenced by growth conditions that require formate dehydrogenase activity. We have no satisfactory explanation for this phenomenon.

Formaldehyde-dependent oxygen uptake rates by cell suspensions increased only slightly to approximately 55 mmol O$_2$min$^{-1}$mg$^{-1}$ during growth of *T. acidophilus* on mixtures of glucose and formaldehyde. NAD-dependent oxidation of formaldehyde by cell-free extracts required the presence of reduced glutathione. The rates of formaldehyde-dependent NAD reduction in the absence of added GSH were approximately 30-fold lower than in its presence. GSH could not be replaced by β-mercaptoethanol or dithiothreitol. No activity was observed with NADP as an electron acceptor. Cell-free extracts also contained a dye-linked formaldehyde dehydrogenase activity (Table 2).

Table 2. Enzymes of C$_3$-metabolism in cell-free extracts of *T. acidophilus* pregrown in chemostat cultures (D = 0.05 h$^{-1}$, pH 3.0, T = 30 °C). Enzyme activities are expressed as U (mg protein)$^{-1}$.

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>Growth limitation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heterotrophic 10 mM glucose</td>
</tr>
<tr>
<td>Formate dehydrogenase NAD-dependent</td>
<td>0.03</td>
</tr>
<tr>
<td>Formate dehydrogenase dye-linked</td>
<td>0.03</td>
</tr>
<tr>
<td>Formaldehyde dehydrogenase NAD/GSH-dependent</td>
<td>0.20</td>
</tr>
<tr>
<td>Formaldehyde dehydrogenase dye-linked</td>
<td>0.11</td>
</tr>
<tr>
<td>Ribulose-1,5-bisphosphate carboxylase</td>
<td>0.005</td>
</tr>
</tbody>
</table>
2). Formaldehyde-dependent reduction of DCPIP could not be detected in the absence of PMS.

In contrast to the formaldehyde-dependent oxygen uptake rates observed with intact cells, NAD-dependent formaldehyde dehydrogenase activities in cell-free extracts increased substantially when cells were grown on mixtures of glucose and formaldehyde (Table 2). The dye-linked formaldehyde dehydrogenase activity did not vary significantly with the culture conditions (Table 2).

Cell-free extracts prepared from carbon-limited, heterotrophic chemostat cultures of *T. acidophilus* contained significant activities of RuBPCase (Ribulose-1,5-bisphosphate carboxylase, EC 4.1.1.39), the key enzyme of the Calvin cycle for CO₂ fixation (Table 2, Pronk et al. 1990a). The activity of RuBPCase in cell-free extracts of cells grown in mixotrophic cultures increased with the formate to glucose ratio (Table 2). Cell-free extracts prepared from cells grown autotrophically on formate contained RuBPCase activities of 53 nmol min⁻¹ (mg protein)⁻¹. This corresponds well with the activity of approximately 50 nmol min⁻¹ (mg protein)⁻¹ that is required to realize the carbon fixation rates observed in the autotrophic chemostat cultures.

**Discussion**

Ecophysiological significance of C₁-compound utilization

Autotrophic or mixotrophic utilization of formate by acidophilic bacteria has not been reported previously. Due to the toxicity of formate and formaldehyde, substrate-limited growth conditions were required for utilization of these compounds by *Thiobacillus acidophilus*. It is well-known that formaldehyde is toxic at millimolar concentrations (Attwood and Quayle 1984). Formate is even more toxic to *T. acidophilus*, as demonstrated by the substrate inhibition of formate-dependent oxygen uptake (Fig. 1). A further demonstration of the toxicity of formate was encountered during mixotrophic growth experiments. When the rate of formate addition to a chemostat culture only slightly exceeded its formate-oxidizing capacity, this resulted in cell lysis and washout (data not shown). Autotrophic cultures on formate could only be obtained by careful manipulation of the influent formate concentration.

The ability to oxidize formate is expressed constitutively in *T. acidophilus* (Tables 1 and 2). Furthermore, the dilution rate of 0.05 h⁻¹ used throughout the present study is higher than the maximum growth rate of *T. acidophilus* on the reduced sulphur compounds thiosulphate and tetrathionate. This suggests that the ability to oxidize these C₁-compounds is of physiological significance for the organism.

Little is known about the fluxes of organic carbon in the natural environment of the acidophilic thiobacilli. If formate and formaldehyde are generated either biologically or non-biologically, the ability of *T. acidophilus* to metabolize these toxic compounds is a prerequisite for survival. This could also explain the constitutive nature of formate oxidation.

Enzymology of formate and formaldehyde metabolism

The uncoupler sensitivity of formate and formaldehyde-dependent oxygen uptake by *T. acidophilus* (Fig. 2) suggests that the uptake of these compounds is energy-dependent. Energy-dependent formaldehyde uptake has recently also been reported in the RuMP-type methylotroph T15 (Bussineau and Papoutsakis 1988) and *Paracoccus denitrificans* (Köstler and Kleiner 1989). Since protonophore uncouplers do not abolish ΔpH in *T. acidophilus* (Matin et al. 1982), the uncoupler sensitivity also implicates involvement of the electrical component of the proton motive force in both uptake processes.

It has often been assumed in the literature that passive diffusion of non-dissociated formic acid across the cytoplasmic membrane is by definition an energy-requiring process, equivalent to the inward translocation of one proton (Dijkhuizen et al. 1977b; van Verseveld and Stouthamer 1978; Papoutsakis et al 1981). However, this assumption neglects the fact that the proton associated with the carboxyl moiety is consumed again during formate oxidation. Passive diffusion of formate can only exert a negative influence on the membrane potential when the rate of formate influx exceeds the rate of formate oxidation. The latter situation does not occur in substrate-limited chemostat cultures. Thus, in case of energy-dependent formate transport, the formate anion has to be symported with more than one proton. This is likely to be the case in *T. acidophilus*.

Both NAD-dependent and dye-linked formate dehydrogenase activities could be detected in cell-free extracts of *T. acidophilus* (Table 2). Some purified NAD-dependent bacterial formate dehydrogenases can also use dyes as artificial electron acceptors (Müller et al. 1978). Although the formate dehydrogenase activities in different cell-free extracts varied substantially, the ratio of the NAD-dependent and dye-linked activities was always close to unity (Table 2). Moreover, the two formate dehydrogenase activities in cell-free extracts of *T. acidophilus* had identical pH optima. We have no
evidence to suggest that different enzymes were responsible for the NAD-dependent and dye-linked activities.

Cell-free extracts of *T. acidophilus* grown on mixtures of glucose and formate and formaldehyde contained high activities of an NAD-dependent, GSH-requiring formaldehyde dehydrogenase (Table 2). This type of formaldehyde dehydrogenase is widespread among bacteria and yeasts (Attwood and Quayle 1984). The increase of the formaldehyde dehydrogenase activity as a result of mixotrophic growth on glucose and formaldehyde (Table 2) was not paralleled by the formaldehyde-dependent oxygen uptake rates of cell suspensions. Apparently, the amount of formaldehyde dehydrogenase was not limiting the rate of formaldehyde oxidation by cell suspensions. Instead, the uncoupler sensitivity of the latter process suggests that formaldehyde transport may be the rate-limiting step. The dye-linked formaldehyde dehydrogenase activity did not increase during mixotrophic growth on glucose and formaldehyde and is therefore probably of minor importance for in vivo formaldehyde oxidation.

**Energetics of mixotrophic growth on C₅-compounds**

In most facultatively autotrophic microorganisms, inorganic carbon fixation is strictly regulated during mixotrophic growth. When the concentration of the inorganic substrate is low relative to the concentration of the organic substrate, RuBPCase is not synthesized and the inorganic substrate is used exclusively for heterotrophic carbon assimilation. As a result, a larger fraction of the organic substrate can be converted into biomass. With increasing concentrations of the inorganic substrate, the cell yields on the organic substrate increase until the theoretical limit of carbon conversion is reached (Gomers et al. 1988). A further increase of the inorganic substrate concentration would lead to a situation of energy excess. However, this does not occur because in such situations the capacity to assimilate CO₂ is induced. As demonstrated by Gomers et al. (1988), this situation is encountered with *Thiobacillus versutus* (growth on glucose and thiosulfate; Gottschal and Kuken 1980) and *Pseudomonas oxalaticus* (growth on acetate and formate; Dijkhuizen and Harder 1979).

The same pattern seems to apply for mixotrophic utilization of formate by *T. acidophilus*. Also in this case the relation between the ratio of formate to glucose and the biomass concentration was biphasic (Fig. 3). In the first phase, autotrophic CO₂ fixation seemed not to occur, since the growth yields were higher than the sum of the heterotrophic and autotrophic growth yields. Only when maximum assimilation of glucose was reached (Gomers et al. 1988), CO₂ fixation set in, as judged from the fact that the further increase of the cell yields paralleled the autotrophic growth yield on formate (Fig. 3). However, the RuBPCase activities in cell-free extracts were in apparent contradiction with this conclusion. Since the enzyme was present in cells grown heterotrophically and mixotrophically at low formate to glucose ratios, it could be argued that autotrophic CO₂ assimilation occurred under these growth conditions (Table 2). However, if the RuBPCase activities in cell-free extracts would reflect in vivo Calvin cycle activity, a strictly biphasic curve (Fig. 3) would not be expected. One of the possible explanations is that other key enzymes of autotrophic CO₂ assimilation are more strictly regulated than RuBPCase. Alternatively, in vivo RuBPCase activity may be regulated at other levels than enzyme synthesis.

When indeed, at low formate to glucose ratios, formate was used exclusively to increase the efficiency of glucose assimilation, a quantitative comparison can be made of the energetic value of the redox equivalents derived from the oxidation of glucose and formate. At formate to glucose ratios below 19, the increase in biomass concentration as a result of formate addition was 3.67 g.mol⁻¹ or 1.84 g.(mol redox equivalents)⁻¹ (Fig. 3). The molar growth yield of *T. acidophilus* in glucose-limited chemostat cultures grown at the same dilution rate was 59 g.mol⁻¹ or 2.46 g.(mol redox equivalents)⁻¹. Thus, the energetic value of the formate redox equivalents is only (1.84 : 2.46) x 100 % = 75 % of that of the redox equivalents from glucose oxidation. This difference is too large to be explained satisfactorily by substrate level phosphorylation occurring during glucose catabolism (data not shown). However, this calculation does not take into account the energy requirement of formate transport. For example, with an assumed the H⁺/O ratio of 4 for glucose and formate (this value is consistent with the low growth yields of *T. acidophilus*), the apparent H⁺/O ratio for formate would only be 3 in the case of an HCOOH/H⁺ symport mechanism. This could explain the lower energetic value of formate redox equivalents.

The increase of the biomass concentration as a result of formaldehyde addition to glucose-limited chemostat cultures was 8.9 g.(mol formaldehyde)⁻¹ (Fig. 4). Analogous to the calculations mentioned above, the energetic value of formaldehyde redox equivalents was estimated to be approximately 90 % of that of glucose redox equivalents. The energetic value of formaldehyde redox equivalents was higher than the value of formate redox equivalents (Fig. 4).
Also this observation can be explained in terms of energy requirement for transport of these C₄-compounds. For example, if both formate and formaldehyde transport occur via symport mechanisms with one proton, an H⁺/O ratio of 4 for both substrates would result in apparent H⁺/O ratios for formate and formaldehyde oxidation of 3 and 3.5, respectively (net translocation of 3 mol H⁺/[mol formate]¹ and 7 mol H⁺/[mol formaldehyde]¹).

The low growth yields of *T. acidophilus* in glucose-limited chemostat cultures can not be explained from energy-dependent maintenance of the intracellular pH (Pronk et al. 1990a). The present study suggests that the low growth yields of the organism are due to low stoichiometries of respiration-linked proton translocation. Unfortunately, the acidophilic nature of *T. acidophilus* prevented the use of direct proton translocation assays (Mitchell and Moyle 1967) to confirm or renounce this conclusion.

**Acknowledgements**

We thank Prof. Dr. W.N. Konings for stimulating discussions. We are grateful to Anke de Bruijn for performing immunofluorescence assays and to Rogier Meulenbergh for critical reading of the manuscript.

**Literature**


CHAPTER 6

Growth of *Thiobacillus ferrooxidans* on formic acid

Abstract

A variety of acidophilic microorganisms were shown to be capable of oxidizing formate. This included *Thiobacillus ferrooxidans* ATCC 21834 which, however, could not grow on formate in normal batch cultures. However, the organism could be grown on formate when the substrate supply was growth-limiting, e.g. in formate-limited chemostat cultures. The cell densities achieved by the use of the latter cultivation method were higher than cell densities reported for growth of *T. ferrooxidans* on ferrous iron or reduced sulfur compounds. Inhibition of formate-dependent oxygen consumption by cell suspensions, but not cell-free extracts, of formate-grown *T. ferrooxidans* occurred at formate concentrations above 100 $\mu$M. This observation explains the inability of the organism to grow on formate in batch cultures. Cells grown in formate-limited chemostat cultures retained the ability to oxidize ferrous iron at high rates. RuBPCase activities in cell-free extracts indicated that *T. ferrooxidans* employs the Calvin cycle for carbon assimilation during growth on formate. Oxidation of formate by cell-free extracts was NAD(P)-independent.

Introduction

The toxicity of small organic acids for acidophilic microorganisms is a well-known phenomenon. The toxicity of organic acids is due to the fact that at low pH values of the growth medium, most of the organic acid is present in the non-dissociated form, which can diffuse easily over the cytoplasmic membrane. As the internal pH of the acidophiles is near neutral (Cobley and Cox 1983), the acid molecules will dissociate upon entering the cytoplasm. In this way, influx of the non-dissociated acid down its concentration gradient will drive the transport of protons into the cell, leading to dissipation of the transmembrane pH gradient essential for growth in acidic environments (Ingledeuw 1982, Alexander et al. 1987).

Batch cultures are ill-suited for growth studies on the metabolism of organic acids by acidophilic bacteria. For example, the acidophile *Thiobacillus acidophilus* is unable to grow on pyruvate in batch cultures. However, growth on this substrate was possible under substrate-limited growth conditions in chemostat cultures (Prönk et al. 1990a). Apparently, the toxic effects of this organic acid can be prevented by keeping the residual substrate concentration low, for example by carbon-limited growth. This result prompted us to study the metabolism of organic acids by acidophilic microorganisms in more detail.

Formate can be used by a wide range of neutrophilic microorganisms as an energy source for both heterotrophic and autotrophic growth (Quayle 1972). In autotrophic organisms, carbon dioxide generated during the oxidation of formate can subsequently be used as a carbon source (Quayle and Kecceh 1959). According to the literature, the acidophilic thiobacilli are unable to grow on formate (Kelly and Harrison 1989). In taxonomic studies however, growth on formate is usually tested in batch cultures. At low pH values, formate (pK$_a$ = 3.8) will occur mainly in the non-dissociated form. Therefore, the high substrate concentrations used in such experiments are likely to inhibit growth due to substrate toxicity. Earlier studies in our laboratory revealed that the facultatively autotrophic acidophile *Thiobacillus acidophilus* could utilize formate as an energy source when this substrate was provided at low concentrations (Prönk et al. 1990b). It therefore seemed of interest to reexamine the ability of acidophilic bacteria to utilize formate.

The obligate chemolithoautotrophi *T. ferrooxidans* is of great economical importance because of its role in the microbial leaching of metal ores (Norris and Kelly 1988). Laboratory cultures of *T. ferrooxidans* are routinely grown on ferrous iron or reduced sulfur compounds (Ingledew 1982). The low growth yields on these substrates require the use of high substrate concentrations for the production of biomass. The accumulation of mineral salts in the growth medium limits the attainable biomass densities. In addition to this, growth on ferrous iron leads to the accumulation of ferric ions in the growth medium. The latter easily form precipitates, in particular at pH values above 2. Since oxidation of formic acid leads to the stoichiometric formation of water and carbon dioxide, autotrophic growth of microorganisms on this substrate does not cause accumulation of mineral salts in the growth
medium.

The aim of the present study was to investigate the ability of various acidophiles to utilize formate and in particular the possibility of formate-limited growth of *T. ferrooxidans*. Attention was focused on *T. ferrooxidans* since growth of dense cultures of this organism may be relevant both for its industrial applications and for its use as a model acidophile in fundamental studies.

**Materials and methods**

*Organisms and culture conditions*

Pure cultures of the micro-organisms used in this study were obtained from the culture collection of the Department of Microbiology and Enzymology, Delft. Batch cultivation methods and growth media were as described by Kuenen and Tuovinen (1981).

For growth of *T. ferrooxidans* on evaporated formate in 'fed batch' cultures, modified 500 ml erlenmeyer shake flasks were used. A glass tube (5 cm, 1 cm diameter) was fitted on the bottom of the flasks. The flasks were filled with 100 ml mineral medium (see below) without carbon source and inoculated with *T. ferrooxidans* cell suspension (either cells harvested from ferrous iron-grown batch cultures or cell suspension from formate-limited chemostat culture) to a density of approximately 15 mg dry weight ml⁻¹. Subsequently, 2 ml of a concentrated formic acid solution (5 - 50 % v/v) was added to the central reservoir tube, from which it could only enter the medium via evaporation. The cultures were incubated at 30 °C on a rotatory shaker at 200 rpm.

Chemostat cultures were started by batch cultivation at pH 1.6 in mineral medium (Mackintosh 1978), supplemented with 180 mM FeSO₄·7H₂O. The mineral medium used for continuous cultivation of *T. ferrooxidans* on formic acid consisted of the following components (per litre of demineralized water): (NH₄)₂SO₄, 3.0 g; KH₂PO₄, 1.0 g; MgSO₄·7H₂O, 0.5 g; CaCl₂·2H₂O, 0.05 g; Na₂SO₄, 1.0 g; FeSO₄·7H₂O, 5 mg; ZnSO₄·7H₂O, 1 mg; CuSO₄·5H₂O, 2 mg; MnSO₄·H₂O, 1 mg; NaMoO₄·2H₂O, 0.5 mg; CoCl₂·6H₂O, 0.5 mg; Na₂SeO₄·10H₂O, 1 mg; NiCl₂·6H₂O, 1 mg. The medium was adjusted to pH 1.8 with H₂SO₄ and autoclaved at 120 °C. Formic acid solutions were sterilized separately by autoclaving at 110 °C.

Chemostat experiments were performed at 30 °C in a 20 l laboratory fermenter with a working volume of 18 l. Cultures were aerated (2.5 l min⁻¹) and stirred at 500 rpm. During batch growth on ferrous iron, the pH of the culture was maintained at 1.6 by automatic titration of 1 M H₂SO₄. Continuous cultivation on formic acid did not require titration of the cultures, which remained at pH 1.8.

*Gas analysis*

Measurements of CO₂ production by the cultures and calculation of specific CO₂ production rates were performed as described previously (van Urk et al. 1988).

*Control of culture purity*

The purity of formate-grown chemostat cultures of *T. ferrooxidans* was regularly examined by immunofluorescence microscopy, using specific antisera raised against pure cultures of this organism (Muyzer et al. 1987).

*Measurement of substrate-dependent oxygen uptake*

Cells from batch cultures were harvested either by centrifugation (15,000 g, 10 min) or by filtration over polycarbonate membrane filters (0.2 μm pore diameter, Nuclepore, Pleasanton, CA, USA). Cells were washed and resuspended in mineral medium (pH 1.8 for *T. ferrooxidans* cultures; pH 3.0 for the other acidophilic bacteria). Oxygen uptake was measured at 30 °C with a Clark-type oxygen electrode cell (Yellow Springs Instruments Inc., Yellow Springs, OH). Oxygen uptake rates were calculated assuming an oxygen concentration in air-saturated water of 0.24 mM and were corrected for endogenous respiration.

Cells from formate-limited chemostat cultures were either assayed directly in the culture fluid or after appropriate dilution with mineral medium. Endogenous respiration rates of chemostat-grown cells were negligible.

*Preparation of cell-free extracts*

The effluent of formate-limited chemostat cultures was collected at 4 °C. Cells were harvested by centrifugation (15,000 g; 15 min) and washed with a freshly prepared buffer (pH 7.0) containing 50 mM MOPS (3-[N-morpholino]-2-hydroxypropanesulfonic acid), 2 mM L-cysteine and 0.2 mM Fe(NH₄)₂(SO₄)₂ (Fox et al. 1989). Cells were resuspended in the same buffer to a final concentration of approximately 10 mg dry weight ml⁻¹ and disrupted by sonication at 0 °C with an MSE 150 W sonifier (10 x 30 seconds with intermittent cooling). Whole cells and debris were removed by centrifugation for (45,000 g; 20 min). The clear supernatants, containing 2 to 5 mg protein ml⁻¹, were used as cell-free extracts.

Cell-free extracts for RuBPCase assays were prepared as described previously (Pronk et al. 1990a).
Table 1. Oxidation of formate (100 μM) by washed cell suspensions of acidophilic microorganisms, pregrown in shake flask cultures. Oxygen uptake rates were determined with a Clark-type oxygen electrode at 30 °C.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Growth substrate</th>
<th>formate oxidation rate (nmol O₂ (min.mg)⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>heterotrophs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Acetobacter diazotrophicus</em></td>
<td>ATCC 49037</td>
<td>56</td>
</tr>
<tr>
<td><em>Acidiphilium cryptum</em></td>
<td>ATCC 33463</td>
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<td><em>Acidiphilium angustatum</em></td>
<td>ATCC 35903</td>
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<td><em>Acidiphilium organovorum</em></td>
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<td><em>Acidiphilium</em> sp.</td>
<td>ATCC 35904</td>
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</tr>
<tr>
<td><strong>facultative autotroph</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Thiobacillus acidophilus</em></td>
<td>ATCC 27807</td>
<td>210</td>
</tr>
<tr>
<td><strong>obligate autotrophs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Thiobacillus thiooxidans</em></td>
<td>ATCC 19377</td>
<td>0</td>
</tr>
<tr>
<td><em>Thiobacillus thiooxidans</em></td>
<td>ATCC 8085</td>
<td>0</td>
</tr>
<tr>
<td><em>Thiobacillus concretivora</em></td>
<td>ATCC 19703</td>
<td>0</td>
</tr>
<tr>
<td><em>Thiobacillus ferrooxidans</em></td>
<td>ATCC 23270</td>
<td>12</td>
</tr>
<tr>
<td><em>Thiobacillus ferrooxidans</em></td>
<td>ATCC 19859</td>
<td>12</td>
</tr>
<tr>
<td><em>Thiobacillus ferrooxidans</em></td>
<td>ATCC 13728</td>
<td>15</td>
</tr>
<tr>
<td><em>Thiobacillus ferrooxidans</em></td>
<td>ATCC 13661</td>
<td>12</td>
</tr>
<tr>
<td><em>Thiobacillus ferrooxidans</em></td>
<td>ATCC 14119</td>
<td>12</td>
</tr>
<tr>
<td><em>Thiobacillus ferrooxidans</em></td>
<td>ATCC 19859</td>
<td>12</td>
</tr>
<tr>
<td><em>Thiobacillus ferrooxidans</em></td>
<td>ATCC 21834</td>
<td>55</td>
</tr>
</tbody>
</table>

Enzyme assays

Dye-linked formate dehydrogenase was assayed spectrophotometrically at 30 °C in a 1 ml assay mixture containing 100 mM potassium phosphate buffer, 10 mM MgSO₄·7H₂O, 0.2 mM 2,6-dichlorophenol-indophenol (DCPIP) and cell-free extract. The reaction was started by the addition of potassium formate to a final concentration of 40 mM. Enzyme activity was calculated from the decrease of absorbance at 522 nm, using a molar extinction coefficient for DCPIP of 8.6 mM⁻¹cm⁻¹ (Armstrong 1964). Enzyme activities were proportional to the amount of enzyme added.

NAD(P)-linked formate dehydrogenase was assayed in the same assay system, with 1 mM NAD or NADP instead of DCPIP. Reduction of NAD and NADP was monitored spectrophotometrically at 340 nm. Rates of NADH and NADPH oxidation by the extracts were negligible.

RuBPCase (Ribulose 1,5-bisphosphate carboxylase, EC 4.1.1.39) was assayed as described by Beudeker et al. (1980).

Analytical procedures

Bacterial dry weight was determined as described previously (Prönk et al. 1990a). A Beckman 915B total organic carbon analyser was used to determine the carbon content of whole cultures and culture supernatants. The carbon content of the bacteria was calculated from the difference.

Concentrations of formate in reservoir media were measured by the method of Lang and Lang (1972).

The protein content of cell suspensions was estimated as described previously (Prönk et al. 1990a). Protein concentrations in cell-free extracts were determined by the method of Bradford (1976), using bovine serum albumin as a standard.

Chemicals

Ribulose 1,5-bisphosphate was obtained from Sigma Chemical Co., [¹⁴C]-NaHCO₃ (2.1 TBq mol⁻¹) from Amersham International PLC. Formic acid (p.a. quality) was obtained from Merck, Darmstadt, FRG. All other chemicals were reagent grade and
obtained from commercial sources.

Results

Oxidation of formate by acidophilic bacteria

A variety of acidophilic bacteria, both heterotrophs and (facultative) autotrophs, were studied for their ability to oxidize formate. In view of the known toxicity of organic acids for acidophilic bacteria (Ingledew 1982, Alexander et al. 1987), formate-dependent oxygen uptake was studied at a low substrate concentration (100 μM).

The ability to oxidize formate was widespread among acidophilic bacteria (Table 1). In all cases, the stoichiometry of formate and oxygen consumption was as expected (2 : 1).

The highest rate of formate oxidation was observed with the facultative autotroph *Thiobacillus acidophilus* (Table 1). Significant rates of formate oxidation (30 - 63 nmol O₂·min⁻¹·(mg dry weight)⁻¹) were also observed in all acidophilic heterotrophs studied. The formate oxidation rates observed with obligately autotrophic bacteria were generally lower (Table 1). No oxidation of formate was observed with cells of *Thiobacillus thiooxidans* and *Thiobacillus concretivorus* (= *T. thiooxidans*) grown on elemental sulphur. Formate oxidation rates of ferrous iron-grown cells of *T. ferrooxidans* varied with the strain used (Table 1). The highest rates of formate-dependent oxygen uptake were observed with *T. ferrooxidans* ATCC 21834.

It can be expected that autotrophic organisms capable of oxidizing formate can also use this substrate as an energy source for growth. We therefore investigated whether *T. ferrooxidans* ATCC 21834 was capable of growth on formate as a sole source of energy.

Growth of *T. ferrooxidans* on evaporated formate

Attempts to grow *T. ferrooxidans* ATCC 21834 in batch cultures with 20 mM formate as a sole source of energy were unsuccessful. This was not surprising in view of the known toxicity of organic acids for this acidophile (Alexander et al. 1987). To prevent toxic effects of formate, its accumulation in the cultures had to be prevented. This was achieved by using shake flasks equipped with a central formic acid reservoir (see Methods), from which the substrate could only enter the growth medium via evaporation. Using this system, *T. ferrooxidans* could be grown on formic acid as a sole source of energy. In contrast to 'normal' growth curves, the increase of the biomass density in these cultures was linear with time (Fig. 1). This can be explained from the fact that the (constant) rate of formic acid evaporation from the reservoir was growth-limiting. The growth rate in the cultures was linearly
Table 2. Growth yields of aerobic bacteria, grown autotrophically on formate. Data were obtained from formate-limited chemostat cultures with ammonium salts as a nitrogen source.

<table>
<thead>
<tr>
<th>Organism</th>
<th>growth rate (h⁻¹)</th>
<th>growth yield (g dry weight.mol⁻¹)</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas oxalaticus</em></td>
<td>0.025-0.20</td>
<td>3.2²</td>
<td>(Dijkhuizen et al. 1977)</td>
</tr>
<tr>
<td><em>Paracoccus denitrificans</em></td>
<td></td>
<td>2.9², 2</td>
<td>(van Verseveld and Stouthamer 1978)</td>
</tr>
<tr>
<td><em>Thiobacillus versarius</em></td>
<td>0.05</td>
<td>3.0</td>
<td>J.C. Gottschal and J.G. Kuenen, unpublished</td>
</tr>
<tr>
<td></td>
<td>0.011</td>
<td>1.4²</td>
<td>(Kelly et al. 1979)</td>
</tr>
<tr>
<td><em>Xanthobacter autotrophicus</em></td>
<td>0.10</td>
<td>4.5</td>
<td>L. Dijkhuizen, pers. comm.</td>
</tr>
<tr>
<td><em>Thiobacillus acidophilus</em></td>
<td>0.05</td>
<td>2.5</td>
<td>(Prönk et al. 1990b)</td>
</tr>
<tr>
<td><em>Thiobacillus ferrooxidans</em></td>
<td>0.01</td>
<td>1.3</td>
<td>this study</td>
</tr>
</tbody>
</table>

¹ maximum growth yield, corrected for maintenance  
² data obtained from extended exponential culture

dependent on the formic acid concentration in the reservoir (Fig. 2). This was to be expected, since also the rate of formate evaporation will increase linearly with the formic acid concentration in the reservoir. Under the experimental conditions, growth rates increased up to a formic acid concentration in the reservoir of 35 % (v/v). At higher concentrations, growth did not occur. Apparently, the rate of formic acid diffusion into the latter cultures exceeded the rate of consumption by the bacteria. Obviously, the maximum concentration of formic acid that will allow growth depends on the initial cell density and the geometry of the culture flasks. Although this system may prove to be useful for screening the ability of acidophiles to utilize formate as an energy source, it is clearly not suited for more detailed physiological studies. Therefore, further studies of formate utilization were performed in formate-limited chemostat cultures.

Growth of *Thiobacillus ferrooxidans* in formate-limited chemostat cultures

*T. ferrooxidans* was pregrown as a batch culture on 180 mM ferrous iron. At the end of batch growth, the culture was switched to continuous cultivation at a dilution rate of 0.01 h⁻¹ and the pH, which was maintained at 1.6 to prevent ferric iron precipitation was raised to pH 1.8 (see below). The reservoir medium initially contained 20 mM formic acid as a sole energy source. This concentration was chosen to ensure that the formate-oxidizing capacity of the culture would be sufficient to prevent accumulation of formate in the culture. The formic acid concentration in the reservoir medium was increased stepwise. After every 20 % increase, the formate concentration was kept the same until the increase of the biomass concentration in the cultures leveled off (usually after 2 to 4 days). Increase of the influent formic acid concentration by more than 30 % at a time resulted in washout of the culture (data not shown).

The apparent molar growth yield of *T. ferrooxidans* grown under formate limitation at D = 0.01 h⁻¹ was 1.36 g dry weight.(mol formate)⁻¹ or 0.68 g dry weight.(mol electrons)⁻¹. This growth yield is higher than the growth yield of the organism on ferrous iron (0.23 g dry weight.[mol Fe²⁺]⁻¹), (Hazeu et al. 1987), but lower than the maximum growth yields reported for tetrahionate-limited chemostat cultures of this organism (0.92 g dry weight.[mol electrons]⁻¹), (Hazeu et al. 1987). The growth yield of *T. ferrooxidans* found in this study is lower than the growth yields observed with a variety of other bacteria grown autotrophically on formate (Table 2). Carbon balances gave a complete recovery of substrate carbon as biomass and carbon dioxide, with 5.44 % of the substrate carbon being converted into biomass. The carbon and protein content of formate-grown *T. ferrooxidans* were 48 % and 65 %, respectively.

Formate oxidation by intact cells of *T. ferrooxidans* was strongly inhibited at substrate concentrations above 100 μM (Fig. 3). This observation explains the inability of *T. ferrooxidans* strains to grow on formate in batch cultures, which usually contain more than 5 mM substrate. The pH optimum for formate oxidation was approximately 3.0 (Fig. 4). However, to reduce the risk of contaminations, the culture pH was maintained at 1.8. The formate-dependent oxygen uptake rate measured at this pH (53 nmol O₂.min⁻¹.mg⁻¹) was virtually the
same as the rate observed with cells from ferrous iron-grown batch cultures (Table 1). With the observed biomass yields (see below), the formate oxidation rate required for formate-limited growth at a dilution rate of 0.01 h⁻¹ is 60 nmol O₂ min⁻¹ mg⁻¹. This rate was in good agreement with the oxidation rates observed in oxygen uptake experiments.

Interestingly, cells from formate-limited chemostat cultures retained the ability to oxidize ferrous iron at high rates (Table 3). Specific iron oxidation rates remained constant after prolonged cultivation (over 3 months) in formate-limited chemostat cultures and were independent of the culture density. This is in contrast to earlier observations with *T. ferrooxidans* grown on reduced sulphur compounds, where the ability to oxidize ferrous iron was lost (Hazeu et al. 1986). *T. ferrooxidans* grown under formate limitation also oxidized sulphide and elemental sulphur. Oxygen uptake rates observed with ferrous iron, sulphide and elemental sulphur of formate- and ferrous iron-grown cells were comparable (Table 3). Oxygen uptake by formate-grown cells was not observed with thiosulphate, thiosulphonate, methanol, formaldihyde and molecular hydrogen.

Cell-free extracts prepared from formate-grown chemostat cultures contained RuBPCase activities of 10 nmol.min⁻¹.(mg protein)⁻¹. This activity is identical to the rate of carbon assimilation calculated from the observed growth yields and cellular composition (calculation not shown).

**Oxidation of formate by cell-free extracts of *T. ferrooxidans***

Cell-free extracts of formate-grown *T. ferrooxidans* LMD 81.69 catalysed the oxidation of formate with DCPIP as an artificial electron acceptor. The pH optimum of the formate dehydrogenase was close to 6 (Fig. 4), suggesting a cytoplasmic localization. At this pH the apparent $K_m$ for formate was approximately 0.1 mM, with a $V_{max}$ of 80 nmol DCPIP.min⁻¹.(mg protein)⁻¹. The formate-dependent DCPIP reduction rates were not enhanced by the addition of 0.2 mM phenazine methosulphate (PMS). In contrast to the observations made with intact cells (Fig. 1), substrate inhibition by formate did not occur up to a concentration of 80 mM. Formate dehydrogenase activities in cell-free extracts were stable at 4 °C, with a half-life of approximately 4 h. No activity was observed with the electron acceptors NAD and NADP.

**Discussion**

The present study indicates that the ability to oxidize formate is widespread among acidophilic bacteria, even in the absence of added formate. This suggests that formate oxidation may be of physiological and/or ecological significance for the organisms studied. Formate may occur in the natu-
Table 3. Oxidation of various substrates by formate- and ferrous iron grown *T. ferrooxidans*. Cell suspensions from formate-limited chemostat cultures were used directly after sampling. Cells from ferrous iron-grown batch cultures (180 mM FeSO₄, pH 1.6) were harvested by filtration, washed twice and resuspended in mineral medium (pH 1.8). Oxygen uptake rates were measured with a Clark-type electrode at 30 °C. Elemental sulphur was added as a 10 mM stock solution in acetone.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration (mM)</th>
<th>Oxygen uptake rate (nmol O₂ min⁻¹ mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fe-grown cells</td>
</tr>
<tr>
<td>Formate</td>
<td>0.1</td>
<td>55</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>4.5</td>
<td>630</td>
</tr>
<tr>
<td>Sodium sulphide</td>
<td>0.1</td>
<td>46</td>
</tr>
<tr>
<td>Elemental sulphur</td>
<td>0.1</td>
<td>19</td>
</tr>
</tbody>
</table>

The growth of *T. ferrooxidans* in formate-limited chemostat cultures grown at a dilution rate of 0.01 h⁻¹ was low compared to the growth yields of other autotrophic bacteria grown on formate (Table 2). At this low growth rate however, growth yields may be significantly influenced by maintenance requirements. For calculation of the theoretical maximum growth yield ($Y_{mm}$) of *T. ferrooxidans*, the maintenance coefficient ($m_m$) for formate-limited growth is needed. If its maintenance coefficient is assumed to be in the same order of that of *Pseudomonas aeruginosa* (Dijkhuizen et al. 1977), the $Y_{mm}$ of *T. ferrooxidans* would be approximately 2.5 g mol⁻¹. This value is similar to the growth yields on formate of other autotrophic bacteria (Table 2). Also the RubPCase activities detected in cell-free extracts suggest that *T. ferrooxidans* employs the Calvin cycle for carbon assimilation during growth on formic acid.

Growth of *T. ferrooxidans* on formic acid may be advantageous for laboratory experiments: cells can be grown at high biomass densities. We have obtained biomass concentrations exceeding 0.7 g l⁻¹ in formate-limited chemostat cultures. These biomass densities are substantially higher than those attained with conventional cultivation methods for *T. ferrooxidans* or with a method based on the electrolytic reduction of ferric iron (Blake et al. 1989). Cells grown under formate limitation retain the ability to oxidize ferrous iron at high rates. However, growth on formate does not involve the precipitation problems associated with the use of ferrous iron-grown cultures. This may be particularly useful for the preparation of cell-free extracts and for enzyme purification procedures. An additional advantage is that the high concentrations of formic acid in the reservoir media make sterilization of these media unnecessary (data not shown). Growth of *T. ferrooxidans* on mixtures of formate and reduced sulphur compounds may facilitate studies into the enzymology and bioenergetics of sulphur oxidation by this organism.

Growth of *T. ferrooxidans* at high biomass densities may also be useful for some of its industrial applications. In this respect, biomass density has been implied as a potentially critical process parameter in both the bacterial leaching of metal ores and the microbial desulphuration of coal (Atkins 1978, Kargi and Weissman 1984). Dense inocula may also be useful to confer an advantage to selected *T. ferrooxidans* strains over naturally occurring varieties, for example in heap leaching operations.

The formate dehydrogenase activity in cell-free extracts of formate-grown *T. ferrooxidans* is similar to that found in the methylotrophic bacterium *Mycobacterium gastri* (Izumi et al. 1988). The enzyme differs markedly from the *T. acidophilus* formate dehydrogenase activity which can use both NAD and artificial electron acceptors (Pronk et al. 1990b).

Acknowledgements
The authors wish to thank Mrs. Anke de Bruyn for performing the immuno-fluorescence assays and for maintaining the bacterial
strains used in this study. We are thankful to Bart Kerkdijk and Peter Kroon for help with setting up the fermentation equipment.

Literature


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CHAPTER 7

Growth of *Thiobacillus ferrooxidans* on formic acid: a patent application

Introduction

High biomass concentrations can be realized during substrate-limited growth of *T. ferrooxidans* on formic acid (Pronk *et al.* 1991). This method may be relevant both for fundamental research purposes and for the relief of biomass limitations during industrial biohydrometallurgical operations.

In view of the possible industrial application of this cultivation technique for the production of large quantities of *T. ferrooxidans*, a patent application has been written. To give the reader an idea of the contents, the abstract of the patent application and the claims are given below.

Abstract:


The invention provides a method for growing *T. ferrooxidans* with a growth-limiting supply of formate, by means of chemostat or fed batch cultures, to higher biomass densities than did the prior art. The *T. ferrooxidans* grown on formate by the method of the invention retains the ability to oxidize ferrous sulphate so that it may be used to treat refractory ores. Thus, the invention provides a more economical and abundant supply of *T. ferrooxidans* for gold ore treatment, treatment of refractory gold ore, treatment of other types of ore, the desulphurization of coal, and for supplementing the natural quantities of *T. ferrooxidans* found in ore bodies.

Claims

1. A method for the recovery of at least one metal from refractory ore which comprises the steps of:
   a. growing *T. ferrooxidans* to high biomass density by means of formate-limited culture;
   b. treating ground refractory ore with said *T. ferrooxidans* under conditions effective to oxidize the refractory components of the ore; and
   c. recovering said metal from the treated ore.

2. A method as recited in claim 1 wherein the metal is selected from a group consisting of gold, silver, copper, lead, bismuth, zinc, and uranium.

3. A method as recited in claim 1 wherein the refractory ore is selected from a group consisting of sulfidic gold ore, sulfidic silver ore, sulfidic copper ore, sulfidic lead ore, sulfidic bismuth ore, sulfidic zinc ore, sulfidic uranium ore, and sulfur containing coal.

4. A method as recited in claim 1 wherein the refractory ore is a combination of carbonaceous ore and sulfidic minerals.

5. A method as recited in claim 1 wherein the *T. ferrooxidans* is grown on a medium containing formate, with the concentration of formate in the culture not exceeding 100 μM.

6. A method as recited in claim 1 wherein the *T. ferrooxidans* is grown by the steps of:
   a. pregrowing *T. ferrooxidans* as a batch culture on a mineral-based medium;
   b. then growing *T. ferrooxidans* as a formate-limited culture; and
   c. increasing the concentration of formate in the reservoir medium at a rate which avoids accumulation of formate in the culture to a concentration above 100 μM.

7. A method as recited in claim 6 wherein the formate-limited culture is a fed batch culture.

8. A method as recited in claim 6 wherein the formate-limited culture is a continuous chemostat culture.

9. A method as recited in claim 6 wherein
the mineral-based medium includes minerals selected from a group consisting of ferrous iron and reduced sulfur compounds.

10. A method for the treatment of mineral-containing wastes which comprises contacting the wastes with *T. ferrooxidans* under conditions effective to treat the minerals of the wastes, said *T. ferrooxidans* having been grown to high density by means of formate-limited culture.

11. A method as recited in claim 10 wherein the mineral is selected from a group consisting of ferrous iron and reduced sulphur compounds.

12. A method for culturing *T. ferrooxidans* comprising culturing on formate at a concentration of less than 100 μM.

13. A method for culturing *T. ferrooxidans* comprising the steps of:
   a. pregrowing *T. ferrooxidans* as a batch culture on a mineral-based medium;
   b. then growing *T. ferrooxidans* in a formate-limited culture; and
   c. increasing the concentration of formate in the reservoir medium at a rate which avoids accumulation of formate in the culture to a concentration above 100 μM.

14. A method as recited in claim 13 wherein the mineral-based medium includes minerals selected from the group consisting of ferrous iron and reduced sulfur compounds.

15. A method as recited in claim 12 further comprising the steps of:
   a. starting the culture as a fed batch culture; and
   b. changing to a continuous chemostat culture when a desired biomass density level is attained.

16. A method as recited in claim 15 further comprising the step of recycling biomass withdrawn during chemostat culture.

17. A method as recited in claim 1 wherein the strain of *T. ferrooxidans* has the characteristics of a strain designated as LMD 81.69 (ATCC #21834).

18. A method as recited in claim 10 wherein the strain of *T. ferrooxidans* has the characteristics of a strain designated as LMD 81.69 (ATCC #21834).

19. A method as recited in claim 12 wherein the strain of *T. ferrooxidans* has the characteristics of a strain designated as LMD 81.69 (ATCC #21834).

20. A method as recited in claim 13 wherein the strain of *T. ferrooxidans* has the characteristics of a strain designated as LMD 81.69 (ATCC #21834).

**Literature**

CHAPTER 8

Energy transduction by anaerobic ferric iron respiration in Thiobacillus ferrooxidans

Abstract

Formate-grown cells of the obligately chemolithoautotrophic acidophile *Thiobacillus ferrooxidans* were capable of formate- and elemental sulphur-dependent reduction of ferric iron under anaerobic conditions. Under aerobic conditions, both oxygen and ferric iron could be simultaneously used as electron acceptors. To investigate whether anaerobic ferric iron respiration by *T. ferrooxidans* is an energy-transducing process, uptake of amino acids was studied. Under aerobic conditions, glycine uptake by starved cells did not occur in the absence of an electron donor. Uptake of glycine could be driven by formate- and ferrous iron-dependent oxygen uptake. Under anaerobic conditions, ferric iron respiration with the electron donors formate and elemental sulphur could energize glycine uptake. Glycine uptake was inhibited by the uncoupler 2,4-dinitrophenol. The results indicate that anaerobic ferric iron respiration can contribute to the energy budget of *T. ferrooxidans*.

Introduction

*Thiobacillus ferrooxidans* is an obligately autotrophic, acidophilic bacterium. Energy for autotrophic growth can be derived from the oxidation of ferrous iron and various inorganic sulphur compounds, including metal sulphides (Kelly and Harrison 1989). Molecular hydrogen (Drobner et al. 1990) and formate (Pronk et al. 1991) can also be used as energy sources for autotrophic growth.

*T. ferrooxidans* is generally considered to be an obligately aerobic organism (Kelly and Harrison 1989). However, under anaerobic conditions, the organism can use ferric iron as an alternative electron acceptor for the oxidation of elemental sulphur (Brock and Gustafson 1976). At present there is no published experimental evidence that ferric iron respiration by *T. ferrooxidans* is an energy-transducing process. *T. ferrooxidans* is an important organism for the biological leaching of metal ores (Norris and Kelly 1988). Oxidation of metal sulphides may occur by a direct biological oxidation of the sulphur moiety of the minerals. Alternatively, ferric iron formed by the bacteria during the oxidation of ferrous iron, may act as a chemical oxidant (Torma 1988). In particular during large scale, in situ bioleaching operations, *T. ferrooxidans* may often encounter environments which contain low dissolved oxygen concentrations and high ferric iron concentrations. If ferric iron respiration is an energy-transducing process, this may increase the stability of *T. ferrooxidans* populations in leaching operations. Furthermore, ferric iron-dependent oxidation of sulphur-containing minerals by the bacteria may contribute to the rate of bioleaching under such conditions.

In spite of the potential significance of the process for the industrial application of *T. ferrooxidans*, little is known about the mechanism and physiological function of ferric iron reduction. So far, ferric iron respiration by *T. ferrooxidans* has only been reported with elemental sulphur as an electron donor. We have recently found that under aerobic conditions, *T. ferrooxidans* can use formic acid as an energy source for autotrophic growth. It was therefore of interest to investigate if under anaerobic conditions, formate oxidation can be coupled to the reduction of ferric iron. The main aim of the present study was to investigate whether ferric iron reduction by *T. ferrooxidans* is an energy-transducing process.

Methods

Microorganism and maintenance

*Thiobacillus ferrooxidans* LMD 81.69 (ATCC 21834) was obtained from the culture collection of the Laboratory of Microbiology and Enzymology, Delft, the Netherlands. The organism was maintained in ferrous-iron grown shake flask cultures. Cultures were regularly checked for purity using immunofluorescence microscopy. Antisera against *T. ferrooxidans* were obtained as described previously (Muyzer et al. 1987).

Chemostat cultivation

*T. ferrooxidans* LMD 81.69 was grown in formate-limited chemostat cultures (D = 0.01 h⁻¹, pH = 1.8, T = 30 °C, Sᵣ = 100 - 500 mM formic acid).
Chapter 8

Formate-limited steady state cultures were obtained by carefully increasing the formic acid concentration in the reservoir medium of ferrous iron-grown cultures (Pronk et al. 1991). The mineral medium contained per litre of demineralized water: (NH₄)₂SO₄, 3.0 g; KH₂PO₄, 1.0 g; MgSO₄·7H₂O, 0.5 g; CaCl₂·2H₂O, 0.05 g; Na₂SO₄, 1.0 g; FeSO₄·7H₂O, 5 mg; ZnSO₄·7H₂O, 1 mg; CuSO₄·5H₂O, 2 mg; MnSO₄·H₂O, 1 mg; NaMoO₄·2H₂O, 0.5 mg; CoCl₂·6H₂O, 0.5 mg; Na₂SeO₃·10H₂O, 1 mg; NiCl₂·6H₂O, 1 mg. The mineral medium was sterilized at 120 °C. Formic acid solutions were sterilized separately at 110 °C. Continuous cultivation was performed in Applikon laboratory fermentors with a working volume of 1.5 l. The cultures were continuously sparged with water-saturated air (1.5 l.min⁻¹) and stirred at 800 rpm. The dissolved oxygen concentration in the cultures was monitored with a steam-sterilizable Clark-type electrode. Chemostat cultures were grown at a dissolved oxygen concentration of above 75 % of air saturation.

**Oxygen uptake experiments**

Cells were harvested by centrifugation (10,000 x g, 15 min) and resuspended in 50 mM K₂SO₄, 50 mM Na₂SO₄ (pH 3.0). Substrate-dependent oxygen uptake was measured with a Clark-type oxygen electrode. Oxygen uptake rates were calculated assuming an oxygen concentration of 236 μM in air-saturated cell suspensions and corrected for the (low) endogenous respiration rates. Elemental sulphur was added as stock solutions in acetone. The acetone concentration in the assays did not exceed 1 % v/v.

**Ferric iron reduction assays**

Cells were harvested by centrifugation (10,000 x g, 15 min) and resuspended in 50 mM K₂SO₄, 50 mM Na₂SO₄ (pH 3.0). Cell suspension (4 ml) was added to a thermostated (30 °C) reaction chamber. After addition of 0.5 mM Fe₂(SO₄)₃, the suspension was made anaerobic by flushing (15 min) with watersaturated argon. Norprene tubing (Cole Parmer Industries, Chicago, USA) was used to minimize oxygen diffusion. After addition of substrate to the reaction chamber, 100 μl samples were taken with a gas-tight Hamilton syringe. The samples were added to 900 μl of an Fe²⁺ assay mixture containing: 12.5 % (v/v) ammonium acetate in 25 % (v/v) acetic acid anhydride, 50 μl; 0.1 % o-phenanthroline in 10 % (v/v) ethanol, 50 μl; and demineralized water, 800 μl. After 5 min incubation, the mixture was centrifuged (15,000 x g, 5 min). The absorbance of the supernatant at 510 nm was measured with a Vitatron spectrophotometer. The ferrous iron assay was calibrated with standard solutions of FeSO₄·7H₂O in 0.05 M H₂SO₄. Rates of ferric iron reduction in the absence of an electron donor were negligible.

**Amino acid uptake experiments**

Cells were harvested by centrifugation (10,000 x g, 15 min) and resuspended in 50 mM K₂SO₄, 50 mM Na₂SO₄ (pH 3.0). To deplete endogenous energy sources, the suspension was sparged with water-saturated air for 3 h at room temperature. A cell suspension (0.5 ml) was added to a 1 ml thermostated (30 °C) reaction chamber, which was made anaerobic by a continuous flow of water-saturated argon. Ferric iron (0.5 mM Fe₂(SO₄)₃) was added to the cell suspension, 15 min prior to the addition of electron donor. ¹⁴C-labeled amino acids (20 μM; 7.77 TBq.mol⁻¹) were added 2 min prior to the addition of an electron donor. Samples were withdrawn with a gas-tight Hamilton syringe, diluted in 2 ml 0.1 M LiCl (4 °C) and immediately filtered over 0.45 μm pore size membrane filters (Schleicher and Schüll, Dassel, FRG). After washing with 2 ml 0.1 M LiCl, the filters were transferred to glass scintillation vials with 5 ml scintillation fluid (Emulsifier Scintillator 299, Packard). Radioactivity was measured in a Beckman LS 3801 bench top scintillation counter.

**Analytical procedures**

Formic acid in reservoir media was assayed by the method of Lang and Land (1972). Dry weights of cell suspensions were determined by filtering aliquots over nitrocellulose filters (0.45 μm pore diameter, Schleicher and Schüll, Dassel, FRG). The cells were washed three times with demineralized water and dried to constant weight at 70 °C.

**Chemicals**

U-[¹⁴C]-glycine (210 Ci.mol⁻¹; 7.77 TBq.mol⁻¹) was obtained from Amersham International PLC. All other chemicals were reagent grade and obtained from commercial sources.

**Results**

**Ferric iron respiration by formate-grown *Thiobacillus ferrooxidans***

Formate-grown cells of *T. ferrooxidans* exhibited substrate-dependent oxygen uptake with ferrous iron and elemental sulphur (Table 1). Even after prolonged formate-limited cultivation (over 20 volume exchanges in the chemostat), no loss of ferrous iron and sulfur-oxidizing activity was observed (Pronk et al. 1991). Under anaerobic conditions, formate-grown cells could oxidize
formate and elemental sulphur with ferric iron as an electron acceptor (Table 1). The rates of sulphur and formate-dependent ferric iron reduction were approximately 50% lower than the corresponding oxidation rates with oxygen as an electron acceptor (Table 1). As shown in Fig. 1, the observed stoichiometries of electron donor and ferric iron consumption were as expected from the complete oxidation of elemental sulphur and formate according to the equations:

\[
\text{S} + 6 \text{Fe}^{3+} + 4 \text{H}_2\text{O} \rightarrow \text{SO}_4^{2-} + 6 \text{Fe}^{2+} + 8 \text{H}^+ \\
\text{HCOOH} + 2 \text{Fe}^{3+} \rightarrow \text{CO}_2 + 2 \text{Fe}^{2+} + 2 \text{H}^+ 
\]

To investigate whether the electron acceptors oxygen and ferric iron can be simultaneously utilized by \textit{T. ferrooxidans}, formate-dependent reduction of ferric iron was studied in aerobic cell suspensions. Indeed, also under aerobic conditions, a transient accumulation of ferrous iron was observed (Fig 2).

Low concentrations of azide almost completely inhibited ferrous iron-dependent oxygen uptake, but only slightly affected the aerobic oxidation of formate and elemental sulphur (Table 2). In contrast, the rates of sulphur- and formate-dependent ferric iron reduction (under anaerobic conditions) were not reduced by azide (Table 2, Fig 2).

To investigate whether the electron acceptors oxygen and ferric iron can be simultaneously utilized by \textit{T. ferrooxidans}, formate-dependent reduction of ferric iron was studied in aerobic cell suspensions. Indeed, also under aerobic conditions, a transient accumulation of ferrous iron was observed (Fig 2). At first sight this seems peculiar, since the maximum rates of ferrous-iron dependent oxygen uptake by the cells were much higher than

---

**Table 1. Rates of substrate-dependent reduction of oxygen and ferric iron by formate-grown cells of \textit{Thiobacillus ferrooxidans}.

<table>
<thead>
<tr>
<th>Electron donor</th>
<th>Rate of electron acceptor reduction (nmol electrons.min$^{-1}$.mg$^{-1}$)</th>
<th>O$_2$</th>
<th>Fe$^{3+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td></td>
</tr>
<tr>
<td>50 μM elemental sulfur</td>
<td>200</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>100 μM formate</td>
<td>360</td>
<td>205</td>
<td></td>
</tr>
<tr>
<td>100 μM ferrous iron</td>
<td>2960</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

**Table 2. Effects of azide (10 μM) on oxidation processes catalysed by \textit{T. ferrooxidans}. Oxygen uptake and anaerobic reduction of ferric iron were assayed as described in the Methods section. The activities observed in the absence of inhibitors are given in Table 1. \textit{T. ferrooxidans} was pregrown in aerobic formate-limited chemostat cultures.**

<table>
<thead>
<tr>
<th>Electron donor</th>
<th>Electron acceptor</th>
<th>Percentage inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>elemental sulphur</td>
<td>O$_2$</td>
<td>18</td>
</tr>
<tr>
<td>elemental sulphur</td>
<td>Fe$^{3+}$</td>
<td>0</td>
</tr>
<tr>
<td>formate</td>
<td>O$_2$</td>
<td>28</td>
</tr>
<tr>
<td>formate</td>
<td>Fe$^{3+}$</td>
<td>0</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
<td>O$_2$</td>
<td>95</td>
</tr>
</tbody>
</table>

---

**Figure 1. Formate and elemental sulphur-dependent reduction of ferric iron (1 mM) under anaerobic conditions. \textit{T. ferrooxidans} was pregrown in a formate-limited chemostat culture (D = 0.01 h$^{-1}$, pH 1.8, T = 30 °C). Anaerobic ferric iron reduction was monitored by assaying ferrous iron (see Methods section). Additions: o : 100 μM formate (cell density 0.27 mg dry weight.ml$^{-1}$); • : 50 μM elemental sulphur (cell density 0.72 mg dry weight.ml$^{-1}$).**

The rate of formate-dependent ferric iron reduction (Table 1). However, the observed low rates of ferrous iron reoxidation can be explained from the relatively high $K_i$ of the cells for ferrous iron, which is approximately 1 mM (data not shown).
Low concentrations of azide almost completely inhibited ferrous iron-dependent oxygen uptake, but only slightly affected the aerobic oxidation of formate and elemental sulphur (Table 2). In contrast, the rates of sulphur and formate-dependent ferric iron reduction (under anaerobic conditions) were not reduced by azide (Table 2, Fig.2). Addition of azide to aerobic, formate-respiring cell suspensions which contained ferric iron led to an increased, non-transient accumulation of ferrous iron (Fig. 2). In effect, azide acts as a ferrous iron-trapping agent, by preventing its reoxidation by the bacteria. However, since the exact mechanism of azide inhibition in *T. ferrooxidans* is not known, the amount of ferrous iron formed in the presence of azide can not be used to quantify the significance of ferric iron respiration under aerobic conditions.

During aerobic incubation of *T. ferrooxidans* with ferric iron and elemental sulphur, the ferrous iron concentration did not increase above approximately 10 µM (data not shown). This can be explained from the rate of ferric iron-dependent sulphur oxidation, which was lower than the rate of anaerobic formate oxidation (Table 1).

Corbett and Ingledew (1987) demonstrated that the aerobic and anaerobic oxidation of elemental sulphur with ferric iron as an electron acceptor by *T. ferrooxidans* can be inhibited to a significant extent by 2-heptyl-4-hydroxyquinoline-N-oxide (HOQNO). Also both the aerobic and anaerobic oxidation of formate were inhibited by HOQNO (over 80 % inhibition by 100 µM HOQNO). As already indicated by Corbett and Ingledew (1987), this observation strongly suggests that the bc1 segment of the respiratory chain is involved in electron transport from sulphur to ferric iron. The same apparently holds for (an)aerobic formate oxidation. Since bacterial bc1 complexes are generally assumed to be proton-translocating, it seemed plausible that ferric iron respiration by *T. ferrooxidans* is an energy-transducing process. This hypothesis was tested by studying active uptake of amino acids.

**Amino acid uptake studies**

A preliminary study revealed that under aerobic conditions, formate-grown *T. ferrooxidans* cells exhibited uptake of various radio-labeled amino acids (data not shown). The highest uptake rates
were observed with glycine. This substrate was therefore used for further uptake studies.

Immediately after sampling from the chemostat cultures, aerobic glycine uptake was only slightly stimulated by the addition of electron donors (data not shown). However, after three hours of preincubation at room temperature, uptake of glycine in the absence of added electron donors was negligible (Fig. 3). Under aerobic conditions, glycine uptake could be energized by the electron donors formate and ferrous iron (Fig. 3). Uptake of glycine in the presence of ferrous iron could be completely inhibited by the uncoupler 2,4-dinitrophenol (Fig. 3). This inhibitor did not affect ferrous iron-dependent oxygen uptake by _T. ferrooxidans_, in accordance with earlier observations (Ingledew 1982).

Solute uptake studies with intact microbial cells inevitably reflect a combination of solute transport and metabolism. The possibility that during the course of the uptake experiments glycine is incorporated into protein can not be excluded. However, the uncoupler sensitivity of glycine uptake and the dependence of the process on respiration clearly indicate that glycine uptake is an energy-dependent process. Therefore, glycine uptake studies could be used to assess if energy transduction also occurs during anaerobic ferric iron respiration by _T. ferrooxidans_.

Also under anaerobic conditions, uptake of glycine was negligible when no energy sources were added (Fig. 4,5). In the absence of oxygen, addition of the electron donors formate (Fig. 4), elemental sulphur (Fig. 5), or ferrous iron (data not shown) could not supply the energy required for glycine uptake. However, when formate or elemental sulphur was added in combination with ferric iron, uptake of glycine did occur (Fig. 4,5). Glycine uptake energized by ferric iron-dependent formate oxidation was comparable to that observed under aerobic conditions (Fig. 3,4). Addition of ferric iron without an electron donor did not significantly stimulate amino acid uptake (Fig. 4,5).

When the uncoupler 2,4-dinitrophenol was added during the course of an anaerobic glycine uptake experiment, a net efflux of radioactivity was observed (Fig. 5). This observation suggests that at least part of the glycine had been accumulated in the cells without having been incorporated into proteins.
Discussion

The ability of *T. ferrooxidans* to use ferric iron as an electron acceptor for the oxidation of elemental sulphur has been known for more than a decade (Brock and Gustafson 1976). However, the mechanism and physiological function of the process have so far remained unclear. We have tried to summarize the experimental data discussed in this paper in a simple scheme (Fig. 6).

Reduction of ferric iron by *T. ferrooxidans* may be catalysed by the same oxidoreductase enzyme involved in the aerobic oxidation of ferrous iron (Fig. 6). This hypothesis is supported by the observation that both ferrous iron-dependent oxygen uptake and elemental sulphur-dependent reduction of ferric iron are repressed during growth of *T. ferrooxidans* LMD 81.68 in thiosulphate-limited chemostat cultures (W. Hazeu and J.T. Pronk, unpublished).

At low concentrations, azide is a specific inhibitor of ferrous iron-dependent oxygen uptake by *T. ferrooxidans* (Arkeseyen 1979, Table 2). At first sight, the azide insensitivity of anaerobic sulphur and formate-dependent ferric iron reduction suggests that ferric iron reduction is not catalysed by the same enzyme involved in the aerobic oxidation of ferrous iron. Alternatively, the site of azide inhibition may be a terminal oxidase rather than the ferrous iron oxidoreductase. Following this line of reasoning, the azide insensitivity of formate- and elemental sulphur-dependent oxygen uptake could then be interpreted as an indication that different terminal oxidases may be involved in oxygen consumption by *T. ferrooxidans* (Fig. 6).

Sugio *et al.* (1985) proposed that ferric iron is also the electron acceptor for sulphur oxidation under aerobic conditions. Indeed, the same group reported the purification of a periplasmic sulphur-ferric iron oxidoreductase (Sugio *et al.* 1987). Our experiments indicate that ferric iron and oxygen can be corespired (Fig. 1). However, from various considerations it is very unlikely that the mechanism proposed by Sugio and coworkers plays a quantitatively significant role in aerobic sulphur oxidation by *T. ferrooxidans* (Corbett and Ingledew 1987, Pronk *et al.* 1990). For example, the growth yields of *T. ferrooxidans* on reduced sulphur compounds are higher than the growth yields on ferrous iron (Hazeu *et al.* 1987). This would clearly not be expected if electrons derived from sulphur enter the electron transport chain at the same level as those derived from ferrous iron.

The observation that also formate can be used as an electron donor for ferric iron respiration strongly suggests that substrate oxidation and ferric iron reduction occur at different sites of the electron transport chain (Fig. 6). This seems difficult to reconcile with the involvement of a single periplasmic sulphur-ferric iron oxidoreductase enzyme.

Active uptake of amino acids by obligately autotrophic bacteria has been reported previously (Matin *et al.* 1974). It has been demonstrated that in various obligate chemolithoautotrophs, including thiobacilli, added organic compounds may be used to provide a limited amount of the cell carbon (Kuenen and Veldkamp 1973, Matin 1978).

The anaerobic amino acid uptake studies (Fig. 4 and 5) clearly indicate that in *T. ferrooxidans*, ferric iron respiration can be an energy-transducing process. In this respect, *T. ferrooxidans* resembles neutrophilic iron-reducing bacteria, in which ferric iron-dependent proton translocation has been demonstrated (Short and Blakemore 1986).
We have tried to confirm the results from the amino acid uptake studies by ATP assays, using a luciferin-luciferase assay. However, assays were hindered by inhibition of luciferase by ferric iron and we failed to obtain reproducible results.

The ability of *T. ferrooxidans* to oxidize formate with ferric iron under anaerobic conditions may be of ecological significance. Small amounts of formate may be produced in the natural habitat of *T. ferrooxidans*, either resulting from chemical reactions or from the activity of (presently unknown) acidophilic fermentative microorganisms. If formate is present, formate oxidation can provide metabolic energy. At the same time, concentrations of formate above 100 μM are toxic for *T. ferrooxidans* (Pronk et al. 1991). The ability of *T. ferrooxidans* to oxidize and detoxify formate may increase the chance of survival during spells of anaerobiosis.

Preliminary experiments indicate that at least some strains of *T. ferrooxidans* are capable of autotrophic growth under anaerobic conditions (Pronk, unpublished observations).

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References


The acidophilic ('acid-loving') thiobacilli are capable of autotrophic growth on a variety of inorganic sulphur compounds. These organisms are of considerable economic significance because of their involvement in processes such as microbial metal recovery and microbial desulphurization of coal (Chapter 1). At the Delft University of Technology, fundamental research on these bacteria focuses on the physiology and ecology of growth in acidic environments and on the enzymology of the oxidation of inorganic sulphur compounds.

So far, the obligate chemolithoautotroph Thio- bacillus ferrooxidans has been used as a model acidophile by most researchers. A major disadvantage of this organism is its very low biomass yield during autotrophic growth on sulphur compounds, which impedes enzymological research into the pathways involved in sulphur oxidation. A possible way to circumvent this problem is to use a facultatively autotrophic model organism. The rationale of this approach is that growth on mixtures of an organic carbon source and an inorganic sulphur compound may facilitate the production of large amounts of biomass with high specific activities of the enzymes involved in the metabolism of inorganic sulphur compounds. Apart from a characterization of the facultative chemolithoautotroph Thio- bacillus acidophilus and the obligate chemolithoautotroph T. ferrooxidans in view of their potential use as model organisms for laboratory studies, the fundamental physiological studies described in this thesis are aimed at understanding the role of these organisms in their natural environment and in industrial metal-leaching and coal desulphurization operations.

The pathways involved in sulphur compound oxidation by the acidophilic thiobacilli are still poorly understood. Chapter 2 gives an overview of the available literature. Although many uncertainties and unknown reaction sequences remain, the available information suggests that the pathways of sulphur compound oxidation in the facultatively autotrophic acidophile Thio- bacillus acidophilus do not differ substantially from those in the obligately autotrophic acidophilic Thio- bacillus species. In view of its potential usefulness as a model organism, studies into the physiology of T. acidophilus were initiated.

Heterotrophic growth of T. acidophilus was studied both in batch cultures and in substrate-limited chemostat cultures (Chapter 3). The range of substrates supporting heterotrophic growth in batch cultures was found to be limited to a number of sugars and some other simple organic compounds. Although pyruvate could not support growth in batch cultures, it could serve as a sole source of carbon and energy in chemostat cultures. Apparently, the low residual pyruvate concentrations in the chemostat cultures prevented substrate inhibition by this organic acid. Indeed, in oxygen uptake studies, toxic effects of pyruvate could be observed at concentrations above 150 μM. As nitrogen sources, in addition to ammonium salts and urea, a number of amino acids could be used.

The molar growth yields of T. acidophilus in heterotrophic chemostat cultures were relatively low. When the culture pH was raised from 3.0 to 4.3, neither the maximum growth yield on glucose (Y_max = 69 g mol⁻¹) nor the maintenance requirement (m = 0.10 mmol g⁻¹ h⁻¹) changed. This demonstrated that in T. acidophilus the maintenance of a large ΔpH is not a major energy-requiring process and, consequently, that this does not explain the observed low growth yields.

During adaptation of T. acidophilus to heterotrophic growth, significant activities of ribulose-1,5- bisphosphate carboxylase were retained, even under conditions of substrate excess. Moreover, in heterotrophically grown T. acidophilus, the enzymes involved in thiosulphate and tetrathionate metabolism were expressed and polyhedral inclusion bodies ('carboxysomes') could be observed (Chapter 4).

Growth of T. acidophilus on mixtures of glucose and thiosulphate or tetrathionate was studied in dual substrate-limited chemostat cultures (Chapter 4). The growth yields in these mixotrophic cultures were higher than the sum of the heterotrophic and autotrophic growth yields. Pulse experiments with thiosulphate revealed that tetrathionate is an intermediate during thiosulphate oxidation by T. acidophilus. Growth yields in mixotrophic cultures indicated that the energetic value of redox equivalents from thiosulphate and tetrathionate was approximately 50% of that of redox equivalents derived from glucose. RuBPCase activity, carboxysome abundance and rate of sulphur compound oxidation increased with increasing thiosulphate-to-glucose ratios in the influent media of the mixotrophic cultures. Highest RuBPCase activities and numbers of carboxysomes were observed in cells from autotrophic, CO₂-limited chemostat cultures. The maximum growth rate at which thiosulphate was still completely oxidized, increased when glucose was present. The mixotrophic capabilities of T. acidophilus, combined with its possession of constitutive enzymes for autotrophic metabolism indicate that the organism is adapted to
survival in environments with a fluctuating supply of organic and inorganic nutrients. Indeed, the organism may have a competitive advantage over obligate autotrophs with respect to sulphur oxidation in environments where organic compounds are available.

The experimental work described in Chapter 3 already indicated that screening for growth substrates in batch cultures may lead to an underestimation of the metabolic versatility of microorganisms. Similar observations were made concerning the ability of T. acidophilus to utilize C₁-compounds (Chapter 5). Although in batch cultures T. acidophilus is unable to grow on formate and formaldehyde, cells from glucose-limited chemostat cultures exhibited substrate-dependent oxygen uptake with these compounds. Oxidation of formate and formaldehyde, but not oxidation of tetrahionate, was uncouplersensitive. This suggests that active transport is involved in the metabolism of both C₁-compounds. Formate- and formaldehyde-dependent oxygen uptake were strongly inhibited at substrate concentrations above 150 μM and 400 μM, respectively. However, autotrophic formate-limited chemostat cultures could successfully be obtained by carefully increasing the formate-to-glucose ratio in the reservoir medium of mixotrophic chemostat cultures. The molar growth yield on formate (Y = 2.5 g mol⁻¹ at a dilution rate of 0.05 h⁻¹) and RuBPCase activities in cell-free extracts indicated that T. acidophilus employs the Calvin cycle for carbon assimilation during growth on formate. Formate-dependent oxygen uptake was expressed constitutively under a variety of growth conditions. Cell-free extracts contained both dye-linked and NAD-dependent formate dehydrogenase activities. Growth yields on mixtures of glucose and formate were higher than the sum of heterotrophic and autotrophic growth yields. A quantitative analysis of the mixotrophic growth studies revealed that formaldehyde was a more effective energy source than formate. NAD-dependent oxidation of formaldehyde by cell-free extracts required reduced glutathione. In addition, the extracts contained a dye-linked formaldehyde dehydrogenase activity. T. acidophilus was unable to utilize the C₁-compounds methanol and methylamine.

High biomass densities could be achieved in chemostat cultures grown with a growth-limiting supply of formic acid (Chapter 5). The major advantage of using formic acid as an energy source is that accumulation of salts in the cultures, as seen in sulphur- or ferrous iron-grown cultures, does not occur. Therefore, the ability of a variety of acidophilic organisms to oxidize formate was examined (Chapter 6). Most species investigated were capable of oxidizing formate. This included Thioacillus ferroxidans ATCC 21834 which, again, could not grow on formate in standard batch cultures, but could be grown in formate-limited chemostat cultures. Cell densities achieved in these cultures of T. ferroxidans (over 0.7 g dry weight.1⁻³) were higher than those reported for growth of the organism on ferrous iron or reduced sulphur compounds.

At formate concentrations above 100 μM, inhibition of formate-dependent oxygen consumption by cell suspensions of formate-grown T. ferroxidans occurred. This explains the inability of the organism to grow on high concentrations of formate in batch cultures. T. ferroxidans grown in formate-limited chemostat cultures retained the ability to oxidize ferrous iron at high rates. RuBPCase activities in cell-free extracts indicated that, like T. acidophilus, T. ferroxidans employs the Calvin cycle for carbon assimilation during growth on formate. Oxidation of formate by cell-free extracts was NAD(P)-independent. In connection with the study described in Chapter 6, a patent application has been filed for the use formate-limited growth in the production of large quantities of T. ferroxidans. This patent application is briefly summarized in Chapter 7.

Formate-grown cells of T. ferroxidans were capable of formate- and elemental sulphur-dependent reduction of ferric iron ('ferric iron respiration') under anaerobic conditions (Chapter 8). When oxygen was also present, both oxygen and ferric iron could be simultaneously used as electron acceptors. To investigate whether ferric iron respiration in T. ferroxidans can be an energy-transducing process, active uptake of amino acids was studied. Control experiments showed that under aerobic conditions, glycine uptake by energy-starved cells of T. ferroxidans did not occur in the absence of an electron donor. Uptake of glycine could be driven by formate as well as ferrous iron-dependent oxygen uptake. The energy-dependent nature of glycine uptake by T. ferroxidans was further illustrated by the fact that it could be completely inhibited by the uncoupler 2,4-dinitrophenol. Under anaerobic conditions, ferric iron respiration with the electron donors formate or elemental sulphur could energize glycine uptake. This clearly indicates that anaerobic ferric iron respiration can play an important role in the energy metabolism of T. ferroxidans. Further experimental work is required to investigate whether ferric iron respiration can support autotrophic growth under anaerobic conditions.
Samenvatting

De acidofiele (zuur-minnende) thiobacilli zijn in staat tot autotrofe groei op een verscheidenheid aan anorganische zwavelverbindingen. Deze organismen zijn van aanzienlijk economisch belang vanwege hun betrokkenheid bij processen als de microbiele winning van metalen en de microbiele onttaveling van steenkuil (Hoofdstuk 1). Aan de Technische Universiteit Delft is fundamenteel onderzoek aan deze bacteriën gericht op de fysiologie en ecologie van groei in zure milieu en op de enzymologie van de oxydatie van anorganische zwavelverbindingen.

Tot dusver hebben de meeste onderzoekers de obligaat chemolithoautotrofe bacterie *Thiobacillus ferroxidans* gebruikt als een modellsysteem voor studies aan acidofieLEN. Een belangrijk nadeel van dit organism is dat de biomassa-opbrengsten zeer laag zijn als gevolg van zijn autotrofe levenswijze. Dit bemoeilijkt enzymologisch onderzoek aan de routes die betrokken zijn bij het metabolisme van zwavelverbindingen. Een mogelijkheid om dit probleem te omzeilen is het gebruik van een facultatief autotroof modelorganisme. Het idee achter deze benadering is dat bij groei van zo'n organism op mengsels van een organische koolstofbron en een anorganische zwavelverbinding, gemakkelijk grote hoeveelheden biomassa met een hoge specifieke activiteit van zwaveloxideerdende enzymen kunnen worden geproduceerd. In dit proefschrift worden fundamentele fysiologische studies aan de facultatieve chemolithoautotroof *Thiobacillus acidophilus* en de obligate chemolithoautotroof *T. ferroxidans* beschreven. Deze studies zijn niet alleen gericht op het gebruik van deze organismen als modelorganismen in laboratoriumstudies, maar tevens op het opheffen van de rol van deze bacteriën in hun natuurlijk milieu en in industriële metaalliewinnings- en steenkoolontwavelingsprocessen.

De reacties betrokken bij het zwavelmetabolisme in de acidofiele thiobacilli zijn nog maar voor een klein gedeelte opgehelderd. In Hoofdstuk 2 wordt een overzicht gegeven van de beschikbare literatuur. Hoewel er nog veel onzeker of onbekend is, wekt de beschikbare informatie de indruk dat de wegen voor de oxydatie van zwavelverbindingen in de facultatief autotrofe acidofiel *Thiobacillus acidophilus* niet drastisch verschillen van die in de obligaat autotrofe *Thiobacilli* soorten. Gezien de mogelijke bruikbaarheid van *T. acidophilus* als modelorganisme, werd begonnen met onderzoek aan de fysiologie van dit organism.

Heterotrofe groei van *T. acidophilus* werd zowel bestudeerd in batchcultures als in substraatge- limiteerde chemostaatcultures (Hoofdstuk 3). Het spectrum van substraten waarop heterotrofe groei in batchcultures optrad, bleek beperkt tot een aantal suikers en wat andere simpele organische verbindingen. In batchcultures trad geen groei op pyruvat op. In chemostaatcultures kon pyruvat echter wel worden gebruikt als enige koolstof- en energiebron. Blijkbaar voorkwamen de lage restconcentraties pyruvat in de chemostaatcultures substratraamming door dit organism zeer. Inderdaad bleek in zuurstopname-experimenten dat pyruvat toxisch was bij concentraties hoger dan 150 μM. Als stikstofbron konden, behalve ammonium-zouten en ureum, ook een aantal aminozuren gebruikt worden.

Bij groei in heterotrofe chemostaatcultures waren de moeilijke groeiopbrengsten van *T. acidophilus* relatief laag. Wanneer de pH van de chemostaatcultures van 3 tot 4,3 werd verhoogd, veranderden noch de maximale groeiopbrengst op glucose (Y_{max} = 69 g·mol^{-1}), noch de onderhoudsenergiebehoefte (m_{e} = 0,10 mmol·g^{-1}·h^{-1}). Deze waarneming toont aan dat in *T. acidophilus* de handhaving van een grote pH-gradiënt over de cytoplasmatische membraan geen belangrijk energievergrendel proces is en dus ook niet de oorzaak kan zijn van de waargenomen lage groeiopbrengsten.

Tijdens aanpassing van *T. acidophilus* aan heterotrofe groei behield het organism significante activiteiten van het enzym ribulose-1,5-difosfaat carboxylase, zelfs in aanwezigheid van een overmaat aan organisch substraat. Ook konden in heterotroof gekweekte *T. acidophilus*-cellen veelhoekige insluitens ("carboxysomen") worden waargenomen (Hoofdstuk 4). Tijdens heterotrofe groei bracht *T. acidophilus* de enzymen betrokken bij het metabolisme van thiosulfaat en tetrahionaat tot expressie.

Groei van *T. acidophilus* op mengsels van glucose en thiosulfaat of tetrahionaat werd bestudeerd in substraatge- limiteerde chemostaatcultures (Hoofdstuk 4). De groeiopbrengsten gemeten in deze mixtirofe cultures waren hoger dan de som van de autotrofe en heterotrofe groeiopbrengsten. Pulsexperimenten met thiosulfaat toonden aan dat tetrahionaat een intermediair is in de oxydatie van thiosulfaat door *T. acidophilus*. De groeiopbrengsten van de mixtirofe cultures gaven aan dat de energetische waarde van de redoxequivanten die vrijkomen bij de oxydatie van thiosulfaat en tetrahionaat ongeveer 50 % bedroeg van die van de redoxequivanten die vrijkomen bij de oxydatie van glucose. RuBPCase activiteiten, aantallen carboxysomen per cel en de snelheden waar met zwavelreacties werden geoxideerd, namen toe met de verhouding van de thiosulfaat- en glucoseconcentraties in de voedingsmedia van de chemostaatcultures. De hoogste RuBPCase activiteit werd alsmede de hoogste aantallen carboxysomen per cel werden
waargenomen in cellen uit autotrofe, CO₂-gelimierteerde chemostaatcultures. De maximale groeisnelheid waarbij thiosulfaat nog volledig kon worden geoxideerd, nam toe wanneer glucose aanwezig was. De mixtrotrofe capaciteiten van *T. acidophilus*, gecombineerd met het feit dat het organisme constitutieve niveaus handhaft van enzymactiviteiten die betrokken zijn bij het autotrofe metabolisme, toont aan dat het organisme is aangepast aan overleving in milieus met een wisselende aanvoer van organische en anorganische substraten. Het organisme zou daarom een competitief voordeel kunnen hebben ten opzichte van obligaat autotrofe organismen met betrekking tot de oxdatie van zwavelverbindingen in milieus waar ook organische substraten beschikbaar zijn.

Het experimentele werk dat beschreven is in Hoofdstuk 3 gaf al aan dat bij inventarisatie van de substraten waarop groei optreedt in batchcultures, de metabolie veelzijdigheid van microorganismen kan worden onderschat. Vergelijkbare waarnemingen werden gedaan met betrekking tot het vermogen van *T. acidophilus* om C₄-verbindingen te gebruiken (Hoofdstuk 5). Hoewel *T. acidophilus* niet in staat is tot groei op mierzuur of formaldehyde in batchcultures, vertoonden cellen uit glucose-gelimiteerde chemostaactcultures substraatafhankelijke zuurstofopname met deze verbindingen. De oxdatie van mierzuur en formaldehyde, maar niet de oxdatie van tetrathionaat, was gevoelig voor opkoppelaars, hetgeen aannemelijk maakt dat aktief transport een rol speelt in het metabolisme van beide C₄-verbindingen. Mierzuur- en formaldehyde-afhankelijke zuurstofconsumptie werd sterk geremd bij substraataconcentraties hoger dan respectievelijk 150 μM en 400 μM. Niettegenstaande dit feit werden autotrofe mierzuurgelimiteerde chemostaatcultures verkregen door het voorzichtig verhogen van de verhouding van mierzuur en glucose in het voedingsmedium van mixtrotrofe chemostaactcultures. De molaire groeiopbrengst op mierzuur (Y = 2.5 g·mol⁻¹ bij een verdunningsnelheid van 0.05 h⁻¹) en de RuBPCase-activiteiten in celvrije extracten duiden er op dat *T. acidophilus* bij groei op mierzuur gebruik maakt van de Calvin-cyclus voor de assimilatie van koolstof. Het vermogen tot mierzuurafhankelijke zuurstofconsumptie kwam onder een aantal verschillende kweekomstandigheden constitutief tot expressie. Oxdatie van mierzuur door celvrije extracten kon gekoppeld worden aan de reductie van zowel NAD als artificiële electronacceptoren (zogenaamde "dye-linked" activiteit). De groei-opbrengsten op mengsels van glucose en mierzuur waren hoger dan de som van de corresponderende heterotrofe en autotrofe groeiopbrengsten. Een kwantitatieve analyse van de experimenten met mixtrotrofe cultures toonde aan dat formaldehyde een effectiever energieleverend substraat was dan mierzuur. Voor NAD-afhankelijke oxdatie van formaldehyde door celvrije extracten was de aanwezigheid van glutathion vereist. Tevens bevatten de extracten een "dye-linked" formaldehyde-dehydrogenase-activiteit. *T. acidophilus* was niet in staat tot groei op de C₄-verbindingen methanol en methylamine.

Hoge biomassadichtheid worden gerealiseerd in chemostaatcultures die werden gekweekt met een groeibeperkende toevor van mierzuur (Hoofdstuk 5). Een belangrijk voordeel van het gebruik van mierzuur als energiebron is dat bij gebruik van dit substraat geen ophoping van zouten in het medium optreedt, zoals dit wel gebeurt bij groei op bijvoorbeeld tweewaardig ijzer of zwavelverbindingen. Daarom werd een groot aantal acidofiele bacteriën onderzocht op hun vermogen om mierzuur te oxderen (Hoofdstuk 6). De meeste onderzochte soorten bleken hiertoe in staat, inclusief *Thiobacillus ferrooxidans*, stam ATCC 21834. Deze stam kon niet groeien op mierzuur in normale batchcultures. Het organisme groeide echter wél in mierzuurgelimiteerde chemostaatcultures. De biomassadichtheid die konden worden behaald in mierzuurgelimiteerde chemostaatcultures van *T. ferrooxidans* (meer dan 0.7 g drooggewicht)¹ waren hoger dan de biomassadichtheden die gemeld zijn voor de groei van dit organisme op tweewaardig ijzer of op gereduceerde zwavelverbindingen.

Remming van mierzuur-afhankelijke zuurstofconsumptie door celsuspensies van op mierzuur gekweekte *T. ferrooxidans* trad bij mierzuurconcentraties hoger dan 100 μM. Deze waarneming verklaart het onvermogen van de bacterie om in batchcultures te groeien op hoge concentraties mierzuur. In mierzuurgelimiteerde chemostaatcultures gekweekte *T. ferrooxidans* kon met hoge snelheid tweewaardig ijzer oxderen. De RuBPCase-activiteiten in celvrije extracten duiden er op dat *T. ferrooxidans*, net als *T. acidophilus*, tijdens groei op mierzuur gebruik maakt van de Calvin-cyclus voor de assimilatie van koolstof. Oxdatie van mierzuur door celvrije extracten was NAD(P)-onafhankelijk. In samenhang met het in Hoofdstuk 6 beschreven experimentele werk is een patentaanvraag ingediend voor toepassing van mierzuur-gelimiteerde groei voor het produceren van grote hoeveelheden *T. ferrooxidans* biomass. In hoofdstuk 7 is een korte samenvatting van deze patentaanvraag gegeven.

Onder anaërobe omstandigheden waren mierzuur-gekweekte cellen van *T. ferrooxidans* in staat tot mierzuur- en elementaire zwavel-afhankelijke reductie van driewaardig ijzer ("ijzerademhaling"; Hoofdstuk 8). Wanneer ook zuurstof aanwezig was,
Samenvatting

Chapters 2-6 and 8 of this thesis have been published or submitted for publication in refereed journals:

Chapter 2:

Chapter 3:

Chapter 4:

Chapter 5:

Chapter 6:

Chapter 8:

Other papers in refereed journals 1987-1991


