Iron Oxidation and Mineral Oxidation by Moderately Thermophilic Bacteria.

by

Simon Peter Cox.
B.Sc. (Hons.) (CNAA)

This thesis is presented for the Degree of Doctor of Philosophy in the Department of Biological Sciences, University of Warwick.

January 1992.

CONTENTS

LIST OF FIGURES

LIST OF TABLES.

LIST OF ABBREVIATIONS.

AMPS	- Ammonium persulphate
CMC	- Critical micelle concentration
DCPIP	- Dichlorophenol-indophenol.
DDGP	- Dodecyl-ß-D-glucopyranoside
$E^{o'}$	- Standard redox potential
E_2^{o}	- Redox potential at pH 2
E_a	- Energy of activation
EDTA	- Ethylene-diamine-tetraacetic acid
FIR system	- Ferric ion-reducing system
$G^{o'}$	- Standard Gibbs free energy change
G^{o}	- Gibbs free energy change
OEG	- Octyl-ethyl-gluconoride
OTG	- Octyl-ß-D-thioglucopyranoside
PEG	- Polyethylene glycol
SDS	- Sodium dodecyl sulphate
SMC	- Sodium monocaprate
t_d	- Doubling time
TEMED	- Tetramethyl-ethylene-diamine
TMPD	- Tetramethyl-p-phenylene-diamine
Tris	- Tris-(hydroxymethyl)-methylamine
YE	- Yeast extract

ACKNOWLEDGEMENTS.

Firstly, thanks to my parents for support and assistance and for their understanding. I could never have got this far without them. Thanks also to Dad for letting me monopolise his computer for a year!

Thanks to Dr. Paul Norris for his help and assistance throughout this project.

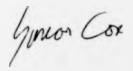
Thanks also to those in the lab and at home for keeping me sane - Alwyn, Liz, Steve, and Martin from the group and Dave, Colin, Ellen, Mik, Tom, Claire and Cate from home. Love and thanks to Alice for keeping me on the rails in the past few months and for endless patience and support.

Thank you to Jonathan and Jenny for 5 Star Bed and Breakfast on many occasions - I can recommend the evening meal!

I acknowledge the support of a SERC CASE award in collaboration with Shell UK, Sittingbourne, Kent.

DECLARATION.

I declare that this thesis has been composed by myself and has not been used in any previous application for a degree. The results presented here were obtained by myself under the sipervision of Dr. P. R. Norris. All sources of information have been specifically acknowledged by means of a reference.



Simon Peter Cox.

SUMMARY.

The microbial oxidation of minerals is a commercially important process. Until comparatively recently only one organism capable of mineral oxidation has been extensively studied - *Thiobacillus ferrooxidans*. Several new, potentially commercially important isolates were studied in comparison with *T.ferrooxidans*. This was done with regard to their iron and sulphur oxidation systems, which are vital to the process of mineral solubilisation, and their ability to solubilise the minerals pyrite or chalcopyrite. The study of the latter was undertaken with particular reference to mixed culture leaching.

The effect of growth substrate history on iron and sulphur oxidation varied between organisms. In particular, strain BC1 lost very little of its iron or sulphur oxidation capacity whether it was grown on either substrate. Conversely, the oxidation systems of *T.ferrooxidans* were directly influenced by growth substrate. SDS PAGE analysis and optical spectroscopy of *T.ferrooxidans* and strains BC1, LM2 and TH3 were used to investigate iron and sulphur oxidation systems and principally to indicate target components of these systems for further detailed study.

Further study of the iron oxidation system in strain BC1 showed that there was only one major chromophore, a membrane bound cytochrome $\underline{aa_3}$. This had absorption peaks at 443 nm, 560 nm and 604.5 nm. Spectra run at 77°K indicated a shoulder on the Soret peak at approximately 450 nm. Extensive investigation into the effect on the cytochrome $\underline{aa_3}$ of various detergents culminated in the solubilisation from the membrane of the terminal oxidase in an intact form. This allowed the mid-point redox potential of this cytochrome to be determined as +524 mV. The mid-point redox potentials of *T.ferrooxidans* cytochrome $\underline{c}$ and $\underline{a_1}$ were determined as +317 mV and +497 mV respectively. SDS PAGE analysis indicated two proteins that could be involved in growth on ferrous iron, at 27 kD and 31 kD. One or both of these proteins appeared to contain very high levels of iron. High levels of iron were also found in fractions from column chromatography which contained cytochrome $\underline{aa_3}$.

Mineral oxidation studies showed that there was a slight increase in the rate of leaching of chalcopyrite in mixed cultures of strain BC1 and strain BC13 when compared to a pure culture of strain BC1, when the organisms where grown in shake flasks. In airlift reactor leaching of chalcopyrite the addition of strain BC13 to strain BC1 had several major effects. The pH in the mixed culture reactor was kept far more constant. Despite this, the leaching of copper from the mineral was not significantly altered in the mixed culture. However, a notable phenomenon in this mixed culture of strain BC1 and strain BC13 was the apparent reduction of the specific iron oxidation capacity of strain BC1 in comparison with its activity in pure culture.

INTRODUCTION

INTRODUCTION.

The leaching of metals from mineral sulphides is a phenomenon that has been known for centuries, but it is only comparatively recently that this process has been shown to be a chiefly microbially mediated process, not, as had been thought, a purely chemical process. Colmer and Hinkle (1947) first demonstrated that the production of ferric ions in acid mine water drainage was due to the presence of a bacterium similar to *Thiobacillus thiooxidans*. This organism was later named *Thiobacillus ferrooxidans* (Temple and Colmer, 1951) and is now considered to be the main agent of mineral solubilisation.

1.0. THE ORGANISMS.

T.ferrooxidans is an obligate chemolithotroph, obtaining energy by the oxidation of ferrous iron and reduced sulphur compounds, including elemental sulphur, although when the organism was named the authors claimed that it was incapable of oxidising elemental sulphur (Temple and Colmer, 1951). The organism is a mesophilic acidophile, with a temperature optimum for growth of approximately 30^{0}C (Colmer *et al.*, 1950). The optimum pH for growth varies, depending upon the substrate. For example, in this study the organism was routinely cultured at pH 1.7 for growth on

ferrous iron, and pH 3.0 for growth on elemental sulphur. It is Gram-negative, flagellated, motile, non-spore-forming and is generally considered to be an obligate autotroph. The rod shaped cells are approximately 0.1 x 1.5 μm and usually occur singly, although they occasionally form pairs. Ultrastructural study has shown the presence of carboxysomes, and in the resting cell glycogen like inclusion bodies. There are no intracytoplasmic membranes and the organism does not appear to store poly ß-hydroxy-butyrate (Lütters and Hanert, 1989). If stored on a suitable mineral such as chalcopyrite or pyrite the organism can remain viable for several months, especially if kept at a low temperature (Gupta and Agate, 1986).

T.thiooxidans is commonly isolated from the same environments as *T.ferrooxidans*. This too is an acidophilic, Gram-negative, motile organism. The major physiological difference between it and *T.ferrooxidans* is its inability to oxidise ferrous iron, relying on reduced sulphur compounds and elemental sulphur for its energy source. Unlike *T.ferrooxidans* this organism is capable of using organic nitrogen sources in the form of purines, although ammonia is always utilised preferentially (Metzdorf and Kaltwasser 1988).

Another thiobacillus recently isolated from leaching environments is *Thiobacillus cuprinus*. This is a mesophilic, acidophilic, aerobic, Gram-negative rod. It is facultatively organotrophic; able to use either elemental sulphur and sulphidic ores or organic substrates such as yeast extract

or peptone. Although this organism is not capable of ferrous iron oxidation it is apparently able to solubilise mineral sulphides and is reported to show a pronounced preference for copper leaching from some chalcopyrite ores (Huber and Stetter, 1990).

Thiobacillus acidophilus was first described in 1975 (Guay and Silver, 1975). This Gram-negative, motile, non-sporulating bacterium is a facultative autotroph, has a temperature optimum between 25 and 30^{0}C and is an acidophile, with an optimum pH of 3.0. It is capable of growth on elemental sulphur and several organic substrates but not on ferrous iron. It has been shown to be able to utilise a number of amino acids as organic nitrogen sources (Pronk *et al.*, 1990).

T.acidophilus was initially isolated from a supposedly pure culture of *T.ferrooxidans*, which highlights a problem of working with the latter organism. It is difficult to plate, has a very low growth yield when iron-grown and it is difficult to obtain pure cultures. Early claims of heterotrophically-grown *T.ferrooxidans* (eg. Shafia and Wilkinson, 1969) were misleading due to heterotrophs contaminating cultures. One such organism isolated from a supposedly pure *T.ferrooxidans* culture is *Acidiphilium cryptum*. This organism is incapable of oxidising elemental or reduced sulphur compounds or ferrous iron, but utilises the lysis products or the organic products excreted from *T.ferrooxidans* (Harrison, 1981). It was the lack of agreement on the capabilities of *T.ferrooxidans* and the

apparent disparity of different workers' results that prompted Harrison (1982) to characterise a wide range of strains supplied from several different groups. These strains, twenty three in all, proved to be quite diverse, both physiologically and genomically. Twenty five per-cent of the strains contained contaminants, some of which were observable by microscopic examination. They could be grouped into seven DNA homology groups, the mol % guanosine + cytosine content of which varied from 55 - 65 mol %.

Most mesophilic, acidophilic, iron-oxidising autotrophs isolated from mineral environments were considered to be *T.ferrooxidans* and so it was not until 1974 that another species of iron-oxidising bacteria, *Leptospirillum ferrooxidans,* was described (Balashova *et al.*, 1974). This species too is a mesophilic, acidophilic, autotrophic chemolithotroph. The Gram-negative organism is curved and forms chains of vibrioid cells. Unlike *T.ferrooxidans* it is incapable of oxidising reduced sulphur compounds and was initially thought incapable of mineral dissolution in pure culture, although it is now known to be able to break down at least some mineral types.

The oxidation of mineral sulphides is an exothermic process. Because the size of commercial leach dumps is large, involving millions of tons of rock, the dissipation of heat through the dump is slow. In this way, temperatures in excess of $50^{o}C$ can occur. This temperature is inhibitory to mesophilic growth. It is therefore no surprise that recently several thermophilic iron- and sulphur-oxidising

bacteria have been isolated from these environments. They are of obvious commercial interest, since such elevated temperatures mean elevated rates of mineral oxidation and, potentially, a reduction of the costs incurred in cooling reactors.

Several of these isolates were available for study. Table 1 compares the nutritional versatility of these organisms with the mesophiles *T.ferrooxidans*, *T.thiooxidans* and *L. ferrooxidans*.

Of particular interest to this study is strain BC1, an organism capable of both iron and sulphur oxidation (Brierley *et al.*, 1978). This organism may be the same as *Sulfobacillus thermosulfidooxidans* (Karavaiko *et al.*, 1988), but this has not been confirmed. The organism is a Gram-positive, usually non-motile, rod with a growth temperature optimum of approximately 45^{o}C. It is now considered to be the same species as an earlier isolate, strain TH1, due to physiological considerations (Norris and Barr, 1985) and a DNA:DNA homology of over 95% between the two strains (Harrison, personal communication). It was initially considered to require organic carbon for growth on iron (Brierley *et al.*, 1978), but has since been shown to be capable of autotrophic growth (Marsh and Norris, 1983a). During heterotrophic growth on yeast extract, the organism undergoes a morphological change, becoming swollen and forming chains (Norris *et al.*, 1980). Although the organism is thus more nutritionally versatile than *T.ferrooxidans*, it has been shown to have an absolute requirement for a reduced

ORGANISM	SUBSTRATE					
	Fe^{2+}	S^{o}	'Thio-salts'	YE	FeS_2	$CuFeS_2$
T.ferrooxidans	++	++	++	-	+++	++
T.thiooxidans	-	+++	+++	-	-	-
L.ferrooxidans	+++	-	-	-	++	+
Strain BC1	+++	+	-	+	+++	+++
Strain BC13	-	+++	+++	-	(+)[a]	(+)[a]
Strain ALV	+++	++	-	+++	+	++
Strain LM2	+++	++	-	++	-	++
Strain TH3[b]	+++	++	-	++	+++	++

Table 1. Nutritional versatility of several strains of iron and/or sulphur oxidising bacteria. All organisms grown autotrophically (except see (b)) and at optimum conditions for growth. The plus symbols indicate the readiness with which the organism will grow on the substrate, from very poor growth (+), to excellent growth (++++). A minus symbol indicates no growth.
(a) BC13 is capable of growth on the elemental sulphur present in the mineral. It does not solubilise the mineral itself.
(b) TH3 will not grow autotrophically and therefore all results refer to lithoheterotrophically grown cells.

sulphur source (Norris *et al.*, 1980). Although considered asporogenous, there is a recent report of spores being found in this organism (Ghauri and Johnson, 1991).

Strain ALV is another Gram-positive organism capable of both iron and sulphur oxidation. It was first described in an investigation into its mixotrophic growth (Wood and Kelly, 1983), in which it was described as an obligate mixotroph, only being capable of growth on an organic substrate if ferrous iron was present. This is now known to be incorrect. Unlike the other moderate thermophiles used in this study, this organism is capable of using sulphate as a sulphur source, and so requires no reduced sulphur source (Norris and Barr, 1985). It is capable of autotrophic growth on chalcopyrite (Marsh and Norris, 1983b), although growth on pyrite is very poor (Norris *et al.*, 1986).

A facultatively autotrophic iron- and sulphur-oxidising organism was isolated from Lake Myvam, Iceland (Marsh and Norris, 1983a) and is currently designated strain LM2. The need for a reduced sulphur source has been demonstrated (Norris and Barr, 1985). The organism is Gram-positive, non-motile and asporogenous.

Unlike other strains in this study, strain TH3 requires an organic carbon source. As with all the Gram-positive moderate thermophiles, it is capable of growth on both ferrous iron (Brierley, *et al.*, 1978) and sulphur (Norris *et al.*, 1986), as well as pyrite and CuS (Norris *et al.*, 1980).

The only Gram-negative moderate thermophile used in this study was also the only organism incapable of iron or

mineral oxidation. Strain BC13 is a motile, asporogenous, autotrophic sulphur oxidiser that shows growth over a wider range of temperatures than either the moderate thermophiles or the mesophiles, showing considerable activity at 30^{0}C and at 55^{0}C, although 45^{0}C is the optimum. The organism will not utilise glucose as a sole substrate, but will in the presence of sulphur. As well as elemental sulphur, growth on tetrathionate has been demonstrated (Norris *et al.*, 1986).

Recently a moderately thermophilic *Thiobacillus* species was isolated (Wood and Kelly, 1988). *Thiobacillus aquaesulis* oxidises reduced sulphur compounds with an optimum temperature of 43^{0}C and was isolated from a thermal sulphur spring. Although this would appear to be one of the few Gram-negative moderately thermophilic sulphur oxidisers, it is not an acidophile and therefore cannot be considered to be a true mineral leaching organism.

Ghauri and Johnson (1991) have recently isolated three strains of moderately thermophilic bacteria. All isolates were Gram-positive and non-motile and occurred as single cells except strain HPTH, which tended to form filaments. All three strains were able to grow heterotrophically on yeast extract. Only one strain, strain HPTH, was capable of sustaining autotrophic growth on ferrous iron, although all three strains were able to grow heterotrophically on this substrate, and all three were apparently capable of carbon dioxide fixation. However strains YTF1 and THWX were far better elemental sulphur oxidisers than strain HPTH. The (G+C) mol % varied between 43% for strain THWX and 63 and

68% for strains YTF and HPTH respectively. There was some variation also in the metal tolerances of the isolates, with strain HPTH being overall the most tolerant. For example, the copper concentration required for complete inhibition of iron oxidation by strains THWX and YTF1 was 10 mM, whereas with strain HPTH it was 50 mM. These three recent isolates further demonstrate the diversity of the moderate thermophiles isolated from leaching environments.

Although no extreme thermophiles were used in this study it is worth noting that there are several genera of thermophilic iron- and sulphur-oxidising archaebacteria. These include *Sulfolobus*, *Acidianus* and *Metallosphaera*.

2.0. IRON OXIDATION.

2.1. *Chemical and Physical Aspects.*

The oxidation of ferrous iron to ferric iron is considered to involve the following stoichiometry:-

$$4FeSO_4 + 2H_2SO_4 + O_2 \longrightarrow 2Fe_2(SO_4)_3 + 2H_2O \qquad (I)$$

Thus under conditions where Fe^{2+} is stable, in particular in acidic conditions, the production of Fe^{3+} can occur, with the concurrent removal of H^+ and a consequent increase in pH. As has been mentioned *T.ferrooxidans* is

probably the main agent of production of ferric ions in this way in acid mine waters. It is the production of ferric sulphate that is vitally important in the solubilisation of sulphidic minerals and it is therefore of great importance to understand the mechanisms which *T.ferrooxidans* and other iron-oxidising organisms use in this production.

The above overall reaction consists of the two following redox reactions:-

$$2Fe^{2+} \longrightarrow 2Fe^{3+} + 2e^{-} \qquad E^{o\prime} = +771 \text{ mV} \quad \text{(II)}$$

$$2e^{-} + \tfrac{1}{2}O_2 + 2H^{+} \longrightarrow H_2O \qquad E^{o\prime} = +820 \text{ mV} \quad \text{(III)}$$

where $E^{o\prime}$ is the mid-point redox potential of the couple under standard conditions at pH 7.0. From the redox potentials the overall Gibbs free energy change ($\Delta G^{o\prime}$) for the reaction can be calculated as -9.65 kJ/mol. This is a low level of available energy. For example this can be compared with the complete oxidation of ß-D-glucose, which has a value of -915.79 kJ/mol (Chang, 1981). The presence of a high concentration of sulphate ions in the growth media of *T.ferrooxidans* at pH 2.0 has been shown to lower the mid-point potential of the Fe^{2+}/Fe^{3+} couple, such that E^{o}_{2} (redox potential at pH 2) = +650 mV (Ingledew *et al.*, 1977). The O_2/H_2O couple is a proton-dependent reaction and so it is no surprise that the potential is altered by acidity. The E^{o}_{2} of this couple is +1120 mV (Ingledew, 1982). With these values we get a ΔG^{o} of -90.71 kJ/mol, an improvement on

standard conditions at pH 7.

The rate equations for the oxidation of Fe^{2+} are also altered by pH. Pesic *et al.* (1989) have noted that the rate equation for the oxidation of ferrous iron in very acidic conditions is different from that for oxidation in more neutral or basic conditions. According to the equations derived, the autooxidation of Fe^{2+} is particularly sensitive at pH values above 3.5, such that at pH 5.5, 50% oxidation of 1mM Fe^{2+} is ten times faster than at pH 4.5. At lower pH, autooxidation is slower and less pH dependent. At pH 1.45, the derived rate equation gives a figure of 3740 days for 1mM Fe^{2+} to be 50% oxidised.

2.2. Biological Aspects.

Ferrous iron can be seen therefore to be a poor substrate energetically. It has been suggested that two Fe^{2+} ions need to be oxidised to promote the conversion of one ADP to ATP (Tuovinen and Kelly, 1972). *T.ferrooxidans* is an obligate autotroph and a requirement for 22.4 Fe^{2+} oxidised per CO_2 fixed has been calculated (Ingledew, 1982). These considerations help explain why *T.ferrooxidans* has a low dry weight growth yield. Both Beck (1960) and Barnes and Metcalf (personal communication) found that when *T.ferrooxidans* is grown autotrophically on ferrous iron the yield is 0.26g dry weight per mole Fe^{2+} oxidised. Factors such as these are responsible for the relatively high concentration of electron transport chain components found within this

organism, i.e. a high rate of iron turnover is required to supply sufficient energy for growth. Molar growth yields of autotrophically-grown moderate thermophiles show similar values for strains ALV and K, being 0.31 and 0.38g dry weight per mole Fe^{2+} respectively. (Strain K is an iron and sulphur-oxidising, Gram-positive moderate thermophile first described by Marsh and Norris. (1983a)) Strain BC1 showed a much lower value, the molar growth yield being only 0.14g dry weight per mole Fe^{2+} (Wood and Kelly, 1983).

The pH and temperature optima for iron oxidation by various organisms have already been mentioned. However the situation is slightly more complex than it appears. The temperature optimum for *T.ferrooxidans* appears to be strain dependent and therefore the existence of diverse strains leads to a range of values. These values tend to fall between 25°C and 35°C. It also appears that the temperature optimum is linked to the pH. MacDonald and Clark (1970) reported that *T.ferrooxidans* had a temperature optimum of 33°C at pH 2.5 and 30°C at pH 1.5.

This interaction between various parameters affecting *T.ferrooxidans* iron oxidation was further studied by Okereke and Stevens (1991). By use of a mathematical model they investigated the statistical relationships between four parameters that could effect iron oxidation rates (ferrous iron concentration, temperature, cell concentration and salt concentration). The cell concentration had the most significant effect, although levels above 0.6 mg protein/ml produced possible inhibition of iron oxidation. When

interaction between all four parameters was studied it was deduced that optimum iron oxidation would be achieved at -6°C, 0.62 mg dry weight/ml cells, 73 mM ferrous iron and 29.6 mM sodium ion concentration. If the sodium ion concentration was left out of the model, then the optima became 2.11°C, 0.43 mg protein/ml cells and 233.07 mM ferrous iron concentration. Such a low temperature optimum led the authors to suggest that in a leaching environment, leaching may still occur in certain situations at low winter temperatures as long as the other parameters were optimum.

A more complete reaction for bacterial ferrous iron oxidation was suggested by Lees *et al.* (1969) and can be represented thus:-

$$Fe^{2+} \longrightarrow Fe^{3+} + e^{-} \qquad \text{(IV)}$$

$$4H^{+} + O_2 + 4e^{-} \longrightarrow 2H_2O \qquad \text{(V)}$$

$$Fe^{3+} + 3H_2O \longrightarrow Fe(OH)_3 + 3H^{+} \qquad \text{(VI)}$$

According to these equations the product is ferric hydroxide. Ferrous iron is oxidised with the production of one electron. This reaction is coupled to the consumption of protons within the cytoplasm, thereby setting up a proton gradient across the membrane. Partial maintenance of the environmental pH may result from the precipitation of Fe^{3+} as the hydroxide. However, Barnes and Metcalf (personal communication) found this scheme to be unrepresentative of

the complex iron chemistry occurring in a biologically active solution of iron sulphates at low pH. They suggested the following reaction steps:-

$$4[Fe^{II}(H_2O)_6]^{2+} \longrightarrow 4[Fe^{III}(OH)(H_2O)_5]^{2+} + 4H^+ + 4e^- \quad \text{(VII)}$$

$$4H^+ + O_2 + 4e^- \longrightarrow 2H_2O \quad \text{(VIII)}$$

$$3[Fe^{III}(OH)(H_2O)_5]^{2+} \rightleftharpoons 2[Fe(H_2O)_6]^{3+} + FeO(OH) + 4H_2O \quad \text{(IX)}$$

The scheme allows for the hydration shells of iron compounds in solution. Hydrated Fe^{2+} is oxidised to iron hydroxide with the production of protons and electrons. Protons are consumed within the cell to provide the necessary proton gradient. The hydrated iron hydroxide reaches an equilibrium with hydrated Fe^{3+} and the solid FeO(OH). The production and consumption of protons is balanced and therefore using this scheme it would be predicted that the system is largely self-buffering (depending upon the equilibrium constant for reaction IX), an observation that was largely responsible for the derivation of this scheme.

Pesic *et al.* (1989) derived two rate equations for the oxidation of ferrous iron in the presence of *T.ferrooxidans*; for pH values greater and lesser than 2.2. At a pH below 2.2 it was reported that the oxidation rate of low concentrations of ferrous iron was independent of pH. With 1.5 mg dry weight cells/ml at pH 1.45, 1mM ferrous iron

would take 31 minutes to be 50% oxidised. Although this figure is probably extremely conservative, when compared to the time taken in the absence of *T.ferrooxidans* (3740 days, see above), derived in the same manner, the significance of the organism to iron oxidation in leaching environments can be clearly seen.

The energy of activation (E_a) for Fe^{2+} oxidation has been variously reported to be 83 kJ/mol, (Ahonen and Tuovinen, 1989a), and 95kJ/mol, (Ferroni *et al.*, 1986), for growing cultures, and 42 (Lundgren, 1975), 50 (Guay *et al.*, 1975) and 48 kJ/mol (Okereke and Stevens, 1991) for resting cell suspensions. The figures for the growing cultures indicate that oxidation is likely to be biochemically-limited rather than diffusion-controlled, particularly at lower temperatures. Indeed Ahonen and Tuovinen (1989a) found that normal *T.ferrooxidans* growth kinetics applied at temperatures as low as 4°C, although obviously the growth rate was significantly lower than for *T.ferrooxidans* grown at 28°C, an average t_d of 62.5 h rather than 6.9 h.

The rate of Fe^{2+} oxidation will also depend upon the affinity of the oxidising system for the substrate. For *T.ferrooxidans* a range of K_m values has been reported for iron oxidation, including 0.11 mM (Suzuki *et al.*, 1989), 0.7 mM (Kelly and Jones, 1978) and up to 1.34 mM (Norris *et al.*, 1988). In the latter paper K_m values were given for other organisms as follows; *L.ferrooxidans* 0.25 mM, strain TH1 1.04 mM, strain ALV 2.96 mM and strain TH3 0.47 mM.

Another aspect of growth on iron that has not needed to be considered for *T.ferrooxidans* and *L.ferrooxidans* is the influence of the carbon source. It appears that for the moderate thermophile strains, chemolithoheterotrophic growth might produce higher growth yields per unit iron oxidised and faster growth rates than does chemolitho-autotrophic growth. For example, when grown in ferrous iron media containing yeast extract, both strains BC1 and LM2 exhibited growth and oxidation rates approximately five times faster than in the presence of CO_2 as the sole carbon source. (Marsh and Norris, 1983a). Strain BC1 has been shown to be a relatively inefficient CO_2 fixer (Wood and Kelly, 1983) and this could explain the relatively poor growth.

2.3. Inhibition of Iron Oxidation.

The inhibition of Fe^{2+} oxidation by *T.ferrooxidans* by various metal ions, including the product Fe^{3+}, has been investigated by many workers. Considering the potentially elevated levels of metals in commercial leach solutions the sensitivity of any organism involved in a commercial process is important. Table 2 gives inhibitory concentrations for a range of metals from several sources. What is immediately apparent is that the organism has a high tolerance for base metals, particularly zinc and copper, but that some other metal ions are toxic in low concentrations. Oxy-anions of several metals are certainly toxic in low concentrations.

The competitive inhibition of Fe^{2+} oxidation by Fe^{3+} is

TOXIC ELEMENT	INHIBITORY CONCENTRATION (mM)			
	Complete Inhib^{n}		50% Inhib^{n}	
	Tuovinen[A]	Huber[B]	Paknikar[C]	Torma[D]
Zn^{2+}	>153	1500		1835
Ni^{2+}	>170	850	1021	1226
Cu^{2+}	15	790	944	866
Co^{2+}	170	85	187	
Mn^{2+}	182			
Al^{3+}	371		445	
UO^{2+}	2.9	4		
Ag^{+}	0.46			
AsO_4^{2-}	2.6[oa]	13		
Se^{4+}	1.3			
TeO_3^{2-}	0.78[oa]			
MoO_4^{2-}	0.52[oa]	1[oa]		
Cd		0.9		

Table 2. The toxicity of various metals to *T.ferrooxidans* according to different authors. Figures for complete and fifty per-cent inhibition of iron oxidation are given. Metals present as oxy-anions are marked oa. References are as follows:-

A - Tuovinen *et al.*, 1971.

B - Huber and Stetter, 1990.

C - Paknikar and Agate, 1988.

D - Torma, 1977.

complicated by the insolubility of the latter species. Bacterial production of Fe^{3+} leads to the formation of $Fe(OH)_3$. The solubility product of this is approximately 10^{-36}, which implies that at pH 2.0 1 M Fe^{3+} can be supported in solution, but at pH 4.0, only 1 μM Fe^{3+} can be dissolved. The abiotic autooxidation of iron has already been shown to be greatly influenced by pH, so that at high pH the rate is markedly increased. Altogether this means that as the pH increases, not only is the rate of production of iron compounds increased, but the solubility of these compounds decreases. This perhaps provides a more convincing explanation of why iron-oxidising organisms are obligate acidophiles with such low pH optima. The influence of pH is such that only a small increase could cause the loss of a large proportion of the organism's growth substrate. Bioenergetically the influence of a low pH only gives a relatively small advantage.

The ferric iron K_i values for several strains of iron-oxidising bacteria have been calculated as follows - *T.ferrooxidans* 3.1 mM, *L.ferrooxidans* 42.8 mM, strain TH1 2.74 mM, strain ALV 1.13 mM and strain TH3 1.89 mM (Norris *et al.*, 1988). These values indicate that the moderate thermophiles are more sensitive than the mesophiles to Fe^{3+} inhibition.

It has been suggested that the cells themselves may inhibit the oxidation of ferrous iron by competition for the ferrous iron binding site. The unusual affinity for Fe^{3+} of the cell surface of *T.ferrooxidans* may be responsible

(Suzuki *et al.*, 1989). This inhibition could apparently occur synergistically with Fe^{3+} inhibition, i.e. both inhibitors could bind at the same time, although binding of the second inhibitor was affected by the presence of the first (Lizama and Suzuki, 1989). These results were however strain dependent, only being valid for two fresh isolates from mine waters and not for two established culture collection strains.

The effect of elemental sulphur on iron oxidation has also been investigated. Within 24 hours of elemental sulphur being added to an active culture of iron-grown cells the rate of iron oxidation had been reduced by 52%, despite the fact that there was still a substantial amount of oxidisable iron available (Oliver and Van Slyke, 1988).

Experiments with *T.ferrooxidans* biofilms have shown that the influences of several parameters on iron oxidation are altered. Karamanev and Nikolov (1988), using an 80 μm biofilm in an airlift reactor, found that the oxidation rate was almost unaffected by temperatures in the range 13 to 38^{o}C, by pH between 1.3 and 2.2, 70 to 230 mM ferrous iron and 250 mM ferric iron. This last figure is a large improvement on, for example, the figure of 100 mM Fe^{3+} that caused 50% inhibition in batch cultures in investigations by Barnes and Metcalf (personal communication).

3.0. COMPONENTS OF THE IRON OXIDATION SYSTEM.

The only iron oxidation system studied in detail is that of *T.ferrooxidans*, which is largely a reflection of the fact that other organisms capable of iron oxidation have only comparatively recently been identified. Despite the fact that much investigation has been done on this organism there is as yet no definitive hypothesis on its oxidation system. There is general agreement on the composition of the electron transport chain, it is the sequence and location of these components for which there is only a limited concensus. The organism contains c-type and b-type cytochromes, quinones, cytochrome a_1, the blue copper protein rusticyanin and an 'iron oxidase' (although some authors have identified this as rusticyanin or cytochrome c). The concentration of these components within the cell have been calculated. Cytochrome a_1 is present at 1.06 µmol/mg total cell protein, c cytochromes at 1.43 µmol/mg total cell protein, rusticyanin at 3 µmol/mg total cell protein and cytochrome b at 0.44 µmol/mg total cell protein (Tikhonova *et al.*, 1967). Rusticyanin accounts for 5% of total cell protein (Tikhonova *et al.*, 1967) and cytochrome c has been estimated, in a different investigation, to account for 9% of cell protein (Vernon *et al.*, 1960). This relatively high concentration of electron transport chain components is typical of chemolithotrophs, which require a high through put of electrons to compensate for the low

energy availability from substrates with high redox potentials (Aleem *et al.*, 1963).

3.1. The 'Iron Oxidase.'

The term iron oxidase is a general term applied to the initial electron acceptor from the Fe^{2+}/Fe^{3+} couple. As yet there is no agreement on the nature of this first component of the chain and there are claims for iron-sulphur proteins, glycoproteins, cytochromes, rusticyanin and an Fe^{3+} coat surrounding the cell being involved.

The ferrous iron must be oxidised outside the cytosol, since at the nearly neutral internal pH the Fe^{2+} would rapidly autooxidise to Fe^{3+}, and the cell would become filled with an insoluble precipitate of $Fe(OH)_3$. Such precipitation would cause the cytosolic pH to lower (Ingledew *et al.*, 1977).

An iron-oxidising enzyme has been purified from *T.ferrooxidans* by Fukumori *et al.* (1988). This 63kD protein showed absorbance maxima at 282 nm and 382 nm and contained an iron-sulphur cluster. Since this enzyme reduced *T.ferrooxidans* cytochrome c_{552} in the presence of Fe^{2+} but not rusticyanin it was concluded that the primary electron acceptor for this enzyme was cytochrome c_{552}. Rusticyanin could only be reduced if both the enzyme and cytochrome c_{552} were present.

An outer membrane protein containing an iron-sulphur cluster has been partially purified by Mjoli and Kulpa

(1988). This protein was induced by the presence of iron. Prevention of the glycosylation by the addition of 2-deoxy-D-glucose prevented the formation of the protein, with a consequent loss of iron oxidation capability. The location of the glycoprotein in the outer membrane suggests that it might be involved in an initial interaction with iron. The authors noted that the protein discovered by Fry *et al.* (1986) was also an outer membrane protein that, in this case, was reduced by ferrous iron in the presence of sulphate ions. This too was an iron-sulphur protein.

It has also been proposed that the initial electron acceptor could be a polynuclear Fe^{3+} coat surrounding the cell (Dugan and Lundgren, 1965). Fe^{2+} would be autooxidised by O_2 in the coat and the superoxide produced would then be capable of reducing electron transport chain components, which include a flavoprotein, coenzyme Q and cytochrome c. This theory was modified by Ingledew (1982), who suggested that at low pH's the chelate coat itself was capable of transferring the electrons through a series of Fe^{2+}/Fe^{3+} redox reactions and that therefore superoxides were not necessary.

In an early attempt to isolate a protein responsible for the iron oxidation Din *et al.* (1967) purified an enzyme capable of oxidising ferrous iron and reducing cytochrome c. This membrane bound 100 - 110 kD protein consisted of two subunits, a 27 - 30 kD protein and an RNA subunit and had a pH optimum between 5.7 and 6.0. In kinetic studies, Din and Suzuki (1967) determined the K_m values for the substrates as

0.59 mM for ferrous iron and 0.085 mM for cytochrome c. The inhibition constant for ferric iron was 0.137 mM. The enzyme was proposed as the initial electron acceptor, with cytochrome c then passing the electron to the oxidase, cytochrome a. At this time the existance of rusticyanin was unknown.

A summary of the proposed initial electron acceptors of *T.ferrooxidans* is given in Table 3. Three of these proposals are Fe-S proteins.

3.2. Rusticyanin.

The presence of rusticyanin in *T.ferrooxidans* was reported in 1975 (Cobley and Haddock, 1975), when the involvement of this blue Cu^{2+}-containing protein in iron oxidation was first suggested. Upon purification the protein was shown to be a single acid-stable polypeptide chain of molecular weight 16.5 kD. It has absorbance maxima at 287 nm, 450 nm, and 750 nm and a particularly distinctive peak at 590 nm. The fact that the protein could be reduced directly by Fe^{2+} with the loss of the 287, 450 and 750 nm peaks led the authors to suggest that rusticyanin was the initial electron acceptor for the electron transport chain (Cox and Boxer, 1978). The mid-point potential at pH 3.2 of +680 mV, close to the Fe^{2+}/Fe^{3+} couple value of +771 mV could support a direct role of this protein in the initial stage of iron oxidation (Ingledew and Cobley, 1980). However, kinetic studies by Blake *et al.* (1988) suggest

AUTHOR	'IRON OXIDASE'
Cox and Boxer (1978).	Rusticyanin.
Ingledew (1982).	Polynuclear Fe^{3+} chelate coat.
Fry *et al.* (1986).	Outer Membrane Fe-S protein.
Blake and Shute (1987).	Cytochrome c.
Fukumori *et al.* (1988).	63 kD Fe-S protein.
Mjoli and Kulpa (1988).	90 kD Fe-S protein.

Table 3. Various claims for the nature of the initial electron acceptor (the 'iron oxidase') in *T.ferrooxidans*. More details are given in the text.

otherwise. They demonstrated that the flow of electrons from Fe^{2+} to rusticyanin was two orders of magnitude too slow to account for the observed rate of oxygen consumption if rusticyanin accounts for 5% of cell protein.

3.3. *The c-Cytochromes.*

In Gram-negative bacteria the role of soluble c-cytochromes in mediating the transfer of electrons from periplasmic or outer membrane proteins to the membrane bound electron transport chain components is well understood. In *Paracoccus denitrificans,* for example, cytochrome c_{550} has been shown to transfer electrons from periplasmic cytochromes c_{551} and c_{553} to the membrane (Davidson and Kumar, 1989). In *Pseudomonas stutzeri* cytochrome c_4 exists in two forms, soluble and membrane bound (Hunter *et al.*, 1989), an arrangement that has also been hypothesised for *T.ferrooxidans* (Ingledew *et al.*, 1977).

There are probably two c-cytochromes and one cytochrome c_1 in *T.ferrooxidans* (Tikhonova *et al.*, 1967), and several attempts have been made to purify and characterise them.

Vernon *et al.* (1960) were able to characterise a partially purified cytochrome c from iron-grown *T.ferrooxidans*. This had absorption maxima at 552 nm, 523 nm and 417nm. Mid-point redox potential at pH 2.9 was +380 mV and +310 mV at pH 7.0. This gives some agreement with the cytochrome c_{552} that was purified from iron-grown *T.ferrooxidans* by Sato *et al.* (1989). This 14 kD protein

otherwise. They demonstrated that the flow of electrons from Fe^{2+} to rusticyanin was two orders of magnitude too slow to account for the observed rate of oxygen consumption if rusticyanin accounts for 5% of cell protein.

3.3. *The c-Cytochromes.*

In Gram-negative bacteria the role of soluble c-cytochromes in mediating the transfer of electrons from periplasmic or outer membrane proteins to the membrane bound electron transport chain components is well understood. In *Paracoccus denitrificans*, for example, cytochrome c_{550} has been shown to transfer electrons from periplasmic cytochromes c_{551} and c_{553} to the membrane (Davidson and Kumar, 1989). In *Pseudomonas stutzeri* cytochrome c_4 exists in two forms, soluble and membrane bound (Hunter *et al.*, 1989), an arrangement that has also been hypothesised for *T.ferrooxidans* (Ingledew *et al.*, 1977).

There are probably two c-cytochromes and one cytochrome c_1 in *T.ferrooxidans* (Tikhonova *et al.*, 1967), and several attempts have been made to purify and characterise them.

Vernon *et al.* (1960) were able to characterise a partially purified cytochrome c from iron-grown *T.ferrooxidans*. This had absorption maxima at 552 nm, 523 nm and 417nm. Mid-point redox potential at pH 2.9 was +380 mV and +310 mV at pH 7.0. This gives some agreement with the cytochrome c_{552} that was purified from iron-grown *T.ferrooxidans* by Sato *et al.* (1989). This 14 kD protein

also showed absorption maxima at 417 nm, 523 nm and 552 nm, although the mid-point redox potential at pH 7.0 was higher, at +360 mV. This cytochrome was unusual in that it showed a pH optimum of 3.5, and was not autooxidisable at pH as low as 2.5. This is what would be expected from a protein isolated from the periplasm. It was suggested from kinetic data that this cytochrome is responsible for mediating the transfer of electrons from the iron oxidase of Fukumori *et al.* (1988) to the terminal oxidase. No mention was made of rusticyanin.

A partially purified cytochrome c from *T.ferrooxidans* has been shown by Blake and Shute (1987) to catalyse the transfer of electrons from Fe^{2+} to rusticyanin. The kinetic properties of this reaction show similarities to the oxidation of iron by whole cells. The suggested sequence of electron transfer is $Fe^{2+} \longrightarrow$ cyt c $\longrightarrow$ rusticyanin.

3.4. *The Terminal Oxidase.*

The terminal oxidase of *T.ferrooxidans* is a cytochrome a_1, located in the inner membrane. Kai *et al.* (1989) suggested that the cytochrome oxidase is an aa_3, despite the fact that their preparation had reduced absorption peaks at 436 and 595 nm, far more typical of an a_1. They suggest that, as they found a pH optimum of 3.5 for the transfer of electrons between cytochrome c_{552} and their cytochrome oxidase, the reduction of molecular oxygen must be periplasmic. However, the preparation they used contained

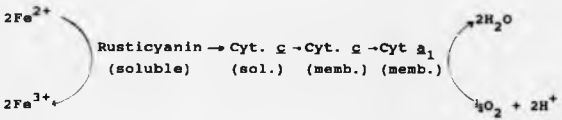
cytochrome c contamination and they did not explain the inhibition studies carried out by Ingledew *et al.* (1977) that indicated that the site of oxygen reduction was on the cytosolic side of the inner membrane. The cytosol of *T.ferrooxidans* has been shown to be about pH 6.5, and to vary little over a wide range of external pH values (Cox *et al.*, 1979).

The mid-point redox potentials of *T.ferrooxidans* cytochrome a_1 have been reported as 500 mV and 420 mV at pH 7 and 725 mV and 610 mV at pH 3.2 . The reduced cytochrome shows absorbance peaks at 597 and 440 nm (Ingledew and Cobley, 1980).

3.5. The Arrangement of the Electron Transport Chain in T.ferrooxidans

Several possible arrangements of the electron transport chain components in *T.ferrooxidans* have been suggested. The variety of suggestions emphasises the lack of agreement in this area. A summary of some of these proposals can be found in Figure 1. There is general agreement on the proposal of Ingledew *et al.* (1977) that the consumption of protons in the cytosol is responsible for the proton gradient that drives ATP production, therefore most of the differences between these proposals are in the initial steps.

Ingledew *et al.* (1977).



Sato *et al.* (1989).

Fe^{2+} ⟶ 'Iron Oxidase' ⟶ Cytochrome c_{552} ⟶ Cytochrome a_1

Blake *et al.* (1988).

Fe^{2+} ⟶ Cytochrome c_{552} ⟶ Rusticyanin ⟶ ?

Dugan and Lundgren (1965).

Fe^{2+} ⟶ Fe^{3+} 'coat' ⟶ Flavoprotein ⟶ Coenzyme Q ⟶ Cyt c ⟶ Cyt a_1

Figure 1. Some proposed arrangements for the electron transport components involved in ferrous iron oxidation in *T.ferrooxidans*.

3.6. The Components of the Electron Transport Chains of Other Iron-Oxidising Bacteria.

Although the majority of work on iron-oxidising bacteria has been carried out on *T.ferrooxidans*, there have been some advances made on other bacteria.

Barr *et al.* (1990) showed the presence of a cytochrome in whole cells of *L.ferrooxidans* with reduced absorbance peaks at 442 nm and 579 nm. This was the only cytochrome detectable by optical spectroscopy. Low temperature ($77^{o}K$) spectra further split the 579 nm peak into two peaks, at 573 nm and 585 nm. This protein was purified and further characterized by Hart *et al.* (1991). It is an acid-stable cytochrome with a molecular weight of 18 kD, and although most occurs in the soluble fractions, some of the protein is bound to the membrane. The mid-point potential at pH 3.5 is 680 mV, similar to that of rusticyanin. It is red in colour and is abundant enough to give cell-free extracts a deep red/brown colour. The fact that it is apparently the only major cytochrome in this organism, plus the fact that it has a mid-point potential close to the Fe^{2+}/Fe^{3+} couple suggests that it has a major role in iron oxidation in this organism.

Examination by optical spectroscopy of two of the moderate thermophiles showed similar spectra for strains TH1 and ALV (Barr *et al.*, 1990) which were quite distinct from those of *T.ferrooxidans* and *L.ferrooxidans*. Absorption peaks occurred at 442 nm, 528-530 nm, 564 nm and 604-606 nm. The presence of cytochrome $\underline{aa}_3$ and $\underline{b}$-type cytochromes was

suggested.

3.7. *Biochemical Interaction Between Iron and Sulphur Metabolism.*

There is one well-studied system that provides an example of an interaction between iron and sulphur metabolism in *T.ferrooxidans*. This interaction occurs between sulphur oxidation and ferric iron reduction. The ability to reduce ferric iron has been demonstrated in several of the species that occur in leaching environments. Both *T.ferrooxidans* and *T.thiooxidans* have this ability (Brock and Gustafson, 1976, Kino and Usami, 1982), as does the thermophile *S.acidocaldarius*, (Brock and Gustafson, 1976). Johnson and McGinness (1991) found several mesophilic heterotrophic bacteria from these environments capable of ferric iron reduction, although none of their moderately thermophilic strains had this ability.

Sugio *et al.* (1985) demonstrated the existence in *T.ferrooxidans* AP19-3 of a ferric ion-reducing (FIR) system that had a role in sulphur oxidation. In whole cells this system reduced six moles Fe^{3+} with one mole of elemental sulphur to give six moles Fe^{2+} and one mole of sulphate. This reduction of ferrous iron could only be shown under anaerobic conditions or when the iron oxidation system had been inhibited. The system had a pH optimum between 2.0 and 3.8. They concluded that the FIR system played a major role in sulphur oxidation in this strain. By inhibition studies

they were able to demonstrate that, during aerobic sulphur oxidation the organism relied not just on the FIR system but also the iron oxidation system in order to oxidise the sulphur (Sugio *et al.*, 1986). They found that both these systems were apparently induced by growth on elemental sulphur. Three days after inoculation into sulphur salts medium they found that the specific activity of the iron oxidation system had increased by approximately eight-fold, whilst that of the FIR system had more than doubled (Sugio *et al.*, 1988a).

A purified sulphur:ferric ion oxidoreductase purified from *T.ferrooxidans* AP19-3 was claimed to be the FIR system (Sugio *et al.*, 1987). Under anaerobic conditions it could be shown to reduce four moles Fe^{3+} with one mole of elemental sulphur to give four moles of Fe^{2+} and one mole of sulphite. The enzyme comprised of two identical 23 kD units, had a pH optimum of 6.5 and was probably located in the periplasmic space. It had an absolute requirement for reduced glutathione.

A sulphite oxidase was then identified. This was located in the inner membrane and had a pH optimum of 6.0. The rate of sulphite oxidation was markedly increased by the addition of Fe^{3+}, which also resulted in the production of Fe^{2+}, suggesting that ferric iron could be the electron acceptor for the oxidase (Sugio *et al.*, 1988b).

The absolute requirement for glutathione was explained when it was revealed that the actual substrate for sulphur:ferric ion oxidoreductase was hydrogen sulphide.

They were able to demonstrate that in the presence of glutathione, elemental sulphur becomes reduced to hydrogen sulphide, and that the hydrogen sulphide is a substrate for the sulphur:ferric ion oxidoreductase (Sugio *et al.*, 1989). The oxidation of hydrogen sulphide has also been shown for another *Thiobacillus* species, *T.denitrificans*, and has been used in a removal process (Ongcharit *et al.*, 1991) for sewage effluent.

Sugio *et al.* (1985, 1989) suggested the following scheme for the oxidation of sulphur and the concomitant reduction of ferric iron (Fig. 2).

4.0. PYRITIC MINERAL OXIDATION.

Despite the fact that the biology of the iron- and sulphur-oxidising bacteria is worthy of study in its own right, perhaps the main driving force behind such study is the probable commercial benefits. Although bacterial populations have been used for many years for metal extraction, the processes have been largely uncontrolled. By understanding better the mechanisms involved and refining the methods it will become easier to control and consequently improve yields and rates. Already these benefits are becoming apparent, for example, at Denison Mines in Canada, which has recently produced 70,000 lbs of uranium oxide per month during an underground leaching

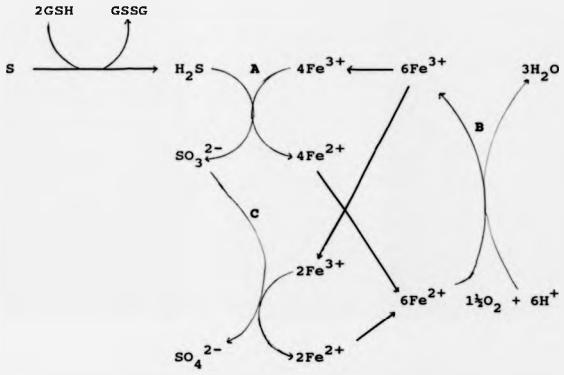


Figure 2. Sulphur oxidation in *T.ferrooxidans* AP19-3, after Sugio *et al.* (1985, 1889), showing the dependance on the ferric ion-reducing system and iron oxidation system. (GSG - reduced glutathione, GSSG - oxidised glutathione). For details see text.

A - Sulphur:ferric-ion oxidoreductase

B - Iron oxidation system

C - Sulphite oxidase

process (Norris, 1988). In Chile, thin layer leaching is used to increase the copper extraction from recalcitrant ores such as chalcopyrite (Norris, 1988). There are now several successfully operating pilot plants for the extraction of gold from refractory mineral sulphides (Van Aswegan *et al.*, 1988).

The activity of iron- and sulphur-oxidising bacteria in uncontrolled and largely man-made environments often has a deleterious effect on the surrounding environment. Although the effects of acid mine water have been recognised for many years, it was not until the initial paper describing *T.ferrooxidans* that it was recognised that bacteria were responsible for its formation (Colmer and Hinkle, 1947). An investigation into the effect of acid water on the heterotrophic population of a normal stream concluded that most Gram-positive aerobic and anaerobic bacteria died out upon the addition of acid mine water, and that the effect on the microbial population as a whole could be very serious (Tuttle *et al.*, 1968). Although the effects of leaching and the consequent production of acid mine water have been known for decades the production of acid mine water continues to be a major environmental problem. In 1982 it was estimated that the formation of acid mine water was responsible for the degradation of 10,000 miles of rivers and streams in the U.S. (Kim *et al.*, 1982). Therefore for both environmental and commercial considerations it is necessary to further understand the behaviour of such bacteria on mineral sulphides.

4.1. Factors Affecting the Rate of Leaching.

There are several factors that can affect the efficiency of the leaching process, and much work has been done on understanding these effects. There has been particular interest in recent years in the use of mineral oxidisers in removing pyritic sulphur from coal. Removal of sulphur from coal would cut down acid rain, and the use of a microbial method may provide a relatively cheap, low-technology method.

For reasons already discussed, the pH of environments where iron oxidation is occurring must be low. A pH between 1.5 and 2.0 is typical in these environments, although mineral solubilisation can occur at a greater range of pH values than this. The pH tolerance of the bacteria found in these environments can have on an effect on the leaching. Norris (1983) noted the greater acid tolerance of *L.ferrooxidans*, which was not inhibited in pure culture at pH 1.3, over *T.ferrooxidans*, which is inhibited in pure culture at pH 1.5. He suggested that the continued activity of a mixed culture on pyrite at pH 1.5 was due to the presence of *L.ferrooxidans* in the culture. This greater pH tolerance may help explain the gradual dominance of *L.ferrooxidans* over *T.ferrooxidans* in mixed serial cultures.

The activity of bacteria in these environments may cause changes in the pH. Tillet and Myerson (1987) noted that, using a mixed culture in coal desulphurisation experiments in packed bed reactors, the pH continually rose

from its initial level of pH 2.5 and had to be adjusted several times by the addition of sulphuric acid. This contrasts with the results of Radway *et al.* (1987) who found that in coal columns the pH dropped. This drop in pH is more typical, and several authors have demonstrated that as it does so the numbers of heterotrophic bacteria within a leaching environment decrease to be replaced by the iron- and sulphur oxidisers (Tuttle *et al.*, 1968, Harrison, 1978, Radway *et al.*, 1987).

It is worth noting here that in experiments with pyrite-oxidising *Acidianus brierleyi* in air-lift fermentors, when the pH was kept at a constant 2.0 by the addition of NaOH, the formation of jarosite precipitation showed a marked increase (Larsson *et al.*, 1990). This illustrates the necessity of ensuring a balance in pH during leaching; too high will probably cause precipitation, whereas too low will inhibit the bacteria.

Sensitivity of the bacteria to toxic substances, particularly metals, will also affect the leaching process. The reduced sensitivity of *L.ferrooxidans* to ferric iron, coupled with its greater affinity for ferrous iron when compared to *T.ferrooxidans*, (Norris *et al.*, 1988, Eccleston *et al.*, 1985), is probably another reason for this organism's gradual dominance in serial cultures. In comparison, moderate thermophile strain ALV, which is relatively sensitive to ferric iron and has a low affinity for ferrous iron, (Norris *et al.*, 1988), grows very poorly on pyrite (Norris *et al.*, 1986). The sensitivity of various

organisms to other metals has been noted earlier, but it is interesting to note that the sensitivity to copper of *L.ferrooxidans* may be altered by growth on chalcopyrite. When grown on ferrous iron copper concentrations as low as 1 mM may inhibit growth (Norris, 1988) but mixed cultures grown on chalcopyrite in the presence of 25 g/l (approx 400 mM) copper were observed to contain numerous *L.ferrooxidans*-like bacteria (Harrison and Norris, 1985). The situation here is a complex one, involving a comparatively undefined substrate and unknown bacterial population, but it does illustrate well the effect such a situation can have on the behaviour of individual species in these environments.

A study into the influence of temperature on leaching by *T.ferrooxidans* of a mixed sulphide ore in percolaters showed a lag phase of ten days at 20°C, whereas at 4°C the lag phase was 5 months (Ahonen and Tuovinen, 1989b). Although this is a more extreme difference than usually reported, there is no doubt that, in general, elevated temperatures increase leaching rates. For example, at 30°C the copper release from chalcopyrite by the moderate thermophile strains TH1 and TH3 has been shown to be slightly lower in rate and yield than that by *T.ferrooxidans* at the same temperature. However, at 50°C the rate and yield were approximately double that of *T.ferrooxidans* grown at its optimum temperature (Norris *et al.*, 1980). At 70°C, a five-fold increase in the rate of extraction of iron has been reported for a *Sulfolobus* species over the maximum

attainable by *T.ferrooxidans* (Norris and Barr, 1988). A temperature effect has been claimed for strain TH1 whereby pyrite dissolution at 50°C is slightly reduced by autotrophic growth as compared to heterotrophic growth (Marsh and Norris, 1983b).

Ahonen and Tuovinen (1991) carried out experiments on the effect of temperature on the leaching of several sulphidic minerals by mixed culture in shake flasks. As well as showing that the leaching of chalcopyrite, sphalerite, pyrrhotite and pyrite was increased as temperature increased between 4°C and 37°C, they also measured several other parameters. The redox potential in all flasks reached approximately +600 mV, although in the lower temperature flasks this took several times longer to be reached. At 4°C and 7°C the pH increased, whereas at 10, 13, and 19°C it remained fairly constant at approximately 2.0. It was only at 19, 28 and 37°C that the activity of the bacteria was high enough to cause a drop in the pH. The E_a for the minerals were calculated as follows; chalcopyrite 77 kJ/mol, sphalerite 45 kJ/mol, pyrrhotite 40 kJ/mol and pyrite 95.5 kJ/mol. These values led the authors to suggest that the leaching of chalcopyrite and pyrite is biochemically or chemically controlled whereas the lower values for pyrrhotite and sphalerite indicated a diffusion and chemical/biochemical controlled reaction.

Norris (1988) has noted that some unusually recalcitrant pyritic minerals which cannot be solubilised by mesophilic organisms may become susceptible to attack at

higher temperatures. The higher temperature may prevent the formation of a passivation layer on the surface of the mineral particles. This layer of elemental sulphur and precipitates which builds up as the mineral is oxidised can prevent further oxidation. Another suggestion for prevention of the development of a passivation layer was made by Grishin and Tuovinen (1988), who showed that in packed bed reactors with activated charcoal as the support matrix, iron oxidation could still occur at rapid rates at a pH as low as 1.3, but that low pH prevented the build up of ferric iron precipitates on the support matrix.

4.2. The Microbial Population in Leaching Environments.

It is a general observation that a mixed population isolated in entirety from a leaching environment will often out-perform a pure-strain mineral oxidiser in laboratory experiments. The interactions that may occur within such populations, which can be very diverse, are likely to be complex but in order to optimise leaching processes, knowledge of such interactions will be a distinct advantage.

There appears to have been comparatively little done in the way of ecological studies into these microbial populations. As well as the expected *T.ferrooxidans*, Ehrlich (1963) found yeasts, flagellates and amoebae in the acid mine water of a copper mine. A study with an artificial coal spoil showed that the development of the population began with heterotrophic bacteria, which were followed by sulphur

oxidisers and finally *T.ferrooxidans*. The final population contained moulds, algae, protozoa, an arthropod and a moss (Harrison, 1978).

The iron and sulphur oxidisers in these populations are known to be sensitive to many organic compounds, often the products of metabolism. For example, low molecular weight organic acids such as acetic, formic, fumaric and oxaloacetic acids will completely inhibit the growth of *T.ferrooxidans* in concentrations between 10^{-2} and 10^{-4} M. At the same concentrations, other acids that have the some inhibitory effect include lactic, oxalic and succinic acids (Tuttle *et al.*, 1981). This toxicity can be alleviated by the heterotrophic bacteria present in a population (Dugan, 1987a). Andrews *et al.* (1988) found that during bacterial removal of pyrite from coal slurries, the heterotrophic bacteria that established themselves increased the process rate by the removal of such inhibitors.

The principle of toxicity removal by heterotrophs was used by Dugan (1987a,b) in inhibition studies. He proposed the use of organic inhibitors of iron and sulphur oxidisers in the treatment of spoil heaps to prevent the formation of acid water drainage. These inhibitors could be slowly broken down by the heterotrophic population to prevent leakage into the environment.

A commonly quoted example of microbial interaction in a leaching environment is that demonstrated between *Beijerinckia lacticogenes* and *T.ferrooxidans* (Tsuchiya *et al.*, 1974). In a nitrogen- and carbon-free

media, *B.lacticogenes*, a nitrogen fixer, allegedly supplied a nitrogen source, whilst *T.ferrooxidans*, an autotroph, supplied a carbon source, enabling the organisms to grow in mixed culture. At the time it was assumed that *T.ferrooxidans* was incapable of fixing nitrogen, despite the fact that the authors noted growth in the pure culture until the finish of the experiment. It is now known that *T.ferrooxidans* can fix nitrogen (Mackintosh, 1978) but it would appear that it was still responsible for enabling the growth of *B.lacticogenes*. It has also been shown that moderate thermophile strain TH3 can grow in bacteria-free filtrates from cultures of *T.ferrooxidans* when these are supplemented with ferrous iron, indicating that breakdown products from *T.ferrooxidans* can support the growth of this heterotroph (Norris *et al.*, 1980).

A commonly investigated interaction is the effect of adding sulphur oxidisers to cultures of iron oxidisers. Balashova *et al.* (1974) reported extensive degradation of chalcopyrite concentrates by *L.ferrooxidans*, but only in the presence of the sulphur-oxidising *Thiobacillus organoparus* (now considered to be the same species as *T.acidophilus* (Kelly and Harrison, 1989)). A similar result has been reported with *T.thiooxidans* as the sulphur oxidiser (Norris, 1983) and also with the moderate thermophile strain BC13 (Norris, 1988). The latter case is particularly interesting, since use of the thermophile allowed the experiment to be carried out at a temperature inhibitory to *T.thiooxidans*, and consequently increased the effectiveness of the leaching

by *L.ferrooxidans*. Although *T.ferrooxidans* is capable of sulphur oxidation there is much strain variation as to its effectiveness (Groudev, 1985) and since it would appear that, in general, the more effective sulphur oxidisers are better chalcopyrite degraders, there may be a role for using sulphur oxidisers to 'supplement' the sulphur oxidation of poorer *T.ferrooxidans* strains (Norris, 1988). The probable importance of sulphur oxidation in chalcopyrite oxidation is also suggested by the observation that the activities of sulphite oxidase and rhodanese, two enzymes involved in sulphur oxidation, are significantly increased by growth on chalcopyrite (Cwalina *et al.*, 1990).

The improvement in leaching capacity that can be caused by sulphur oxidisers is probably due to their removal of elemental sulphur layers that can build up around the mineral particles. This passivation layer builds up as the iron oxidisers solubilise the mineral, and can block further attack. The sulphur oxidisers prevent the formation of this layer and thereby increase the effectiveness of the culture in solubilising the mineral.

4.3. The Mechanism Of Pyrite Oxidation.

In an initial paper on the mechanism of pyrite oxidation by *T.ferrooxidans* by Duncan *et al.* (1967), the four possible mechanisms first suggested by Silverman and Ehrlich (1964) were discussed. These mechanisms were grouped into two types of attack; `direct attack' and `indirect

attack'. In direct attack the bacteria directly attack either the iron or sulphide moieties or both. In indirect attack the bacterial oxidation of ferrous iron produces ferric iron. It is this ferric iron that causes mineral dissolution by chemically oxidising the mineral. This chemical oxidation of the mineral reduces the ferric iron to ferrous iron which is then re-oxidised in a cyclic process by the bacteria. Duncan *et al.* (1967) stated that the role of ferric iron was of minor importance. They attempted to show that *T.ferrooxidans* attacked both moieties directly, and reported that the relative per-centages of iron and sulphide oxidation depended on the mineral type and the growth history of the bacteria. Further evidence for the role of the direct attack came from CO_2 fixation studies, which showed that both moieties were attacked during pyrite oxidation by *T.ferrooxidans* (Beck and Brown, 1968).

Silverman (1967) concluded that, in actual fact, both direct and indirect attack occurred simultaneously. He demonstrated that pyrite could be chemically oxidised by ferric sulphate in the absence of bacteria and oxygen. In the presence of bacteria, removal of acid-soluble iron from pyrite samples slowed the oxygen uptake and altered it from a linear to a non-linear rate. Addition of ferric sulphate partially restored the linear oxidation rate, indicating that ferric iron had a greater role than Duncan *et al.* (1967) had thought. As early as the turn of the century, the following scheme for the chemical oxidation of pyrite had been suggested (Stokes, 1901):-

$$FeS_2 + Fe_2(SO_4)_3 \longrightarrow 3FeSO_4 + 2S \qquad (X)$$

$$2S + 6Fe_2(SO_4)_3 + 8H_2O \longrightarrow 12FeSO_4 + 8H_2SO_4 \qquad (XI)$$

Overall:-

$$FeS_2 + 7Fe_2(SO_4)_3 + 8H_2O \longrightarrow 15FeSO_4 + 8H_2SO_4 \qquad (XII)$$

Silverman stated that although the recovery of iron species from his chemical oxidation of pyrite was in good agreement with reaction X, he found little evidence of elemental sulphur. However he was using *Ferrobacillus ferrooxidans*, an organism then believed to be incapable of oxidising elemental sulphur. It is now generally accepted that this organism is actually *T.ferrooxidans*, and consequently does oxidise sulphur. This may go some way to explaining the lack of elemental sulphur observed. The presence of very small quantities of ferric iron can as much as double the rate of the direct mechanism, and since iron is ubiquitous in leaching environments this is further evidence that both mechanisms occur simultaneously (Hutchins *et al.*, 1986). In fermentor experiments with the thermophile *Acidianus brierleyi*, where precipitation of nearly all the soluble iron occurred, the rate of pyrite oxidation was significantly lowered. This may indicate a greater influence of the indirect mechanism (Larsson *et al.*, 1990). However, iron precipitates can cause the build up of passivation

layers and therefore also inhibit 'direct' attack so this is by no means conclusive. Evidence from work with the moderate thermophiles suggests that, as might be expected, different species rely on different mechanisms. In copper sulphide oxidation work, strain TH1 required no ferric iron (above levels necessary for a micronutrient) to effect copper release, whereas strain TH3 required relatively high concentrations of added ferric iron. This suggests that copper sulphide solubilisation by strain TH3 is effected by indirect leaching (Norris *et al.*, 1980).

It is now generally accepted that both mechanisms do occur simultaneously. The indirect leaching probably occurs as follows. Initially ferrous iron is oxidised by bacteria to ferric iron:-

$$2FeSO_4 + \tfrac{1}{2}O_2 + H_2SO_4 \longrightarrow Fe_2(SO_4)_3 + H_2O \qquad \text{(XIII)}$$

The ferric sulphate then oxidises the mineral sulphide, and is reduced to the ferrous form, eg:-

$$CuFeS_2 + 2Fe_2(SO_4)_3 \longrightarrow CuSO_4 + 5FeSO_4 + 2S \qquad \text{(XIV)}$$

$$Cu_2S + 2Fe_2(SO_4)_3 \longrightarrow 2CuSO_4 + 4FeSO_4 + S \qquad \text{(XV)}$$

The ferrous iron is then re-oxidised to ferric iron by the bacteria (eqn. XIII).

The elemental sulphur can then be oxidised to sulphuric acid by sulphur-oxidising bacteria.

The direct mechanism is likely to follow the simple reaction:-

$$MS + 2O_2 \longrightarrow MSO_4$$

where M is the metal moiety of the sulphide.

It is worth noting that although indirect leaching appears to rely on soluble ferrous iron oxidation, it has been reported several times that, at least for the moderate thermophiles, there is no correlation between their iron oxidation rates and pyrite solubilisation rates (e.g. Ghauri and Johnson, 1991). Given the fact that the situation in leaching experiments is more complicated than that in ferrous iron oxidation experiments, this is perhaps not surprising.

Ahonen and Tuovinen (1990) investigated the kinetics of leaching of a mixed sulphidic mineral by a mixed culture in shake flasks. They proposed two models. In the first, a shrinking particle model, it was assumed that the rate of leaching is directly proportional to the surface area of the mineral. In the second, a simplified shrinking core model, it was assumed that the rate was inversely proportional to the dissolution of the mineral and thus to the concentration of the dissolved metal. It was found that the sphalerite in the mineral exhibited shrinking particle kinetics, but the pyrrhotite followed the shrinking core model, as, possibly, did the chalcopyrite.

4.4. The Role of Attachment in Mineral Oxidation.

The adsorption of *T.ferrooxidans* to its substrate may be vitally important to the mechanism of mineral oxidation. Several workers have demonstrated that attachment to suspended solids occurs within minutes of the introduction of bacteria (Takakuwa *et al.*, 1979, DiSpirito *et al.*, 1983, Myerson and Kline, 1983). These solids do not have to be substrates, for instance, attachment to glass beads (DiSpirito *et al.*, 1983) and activated charcoal (Grishin and Tuovinen, 1988) has been shown.

The adsorption is not random and it has been shown to preferentially occur around dislocations and other non-uniform sites in the pyrite crystal structure (Bagdigian and Myerson, 1986). Bärtels *et al.* (1989) have produced a photomicrograph of *T.ferrooxidans* growing along a fracture in an ultrathin section of pyrite. This predilection for fracture sites may provide an advantage for *T.ferrooxidans*. It is known that during coal desulphurisation the oxidation of pyrite does not follow the expected stoichiometric ratio - the sulphur is apparently preferentially oxidised (Andrews 1988). It is this preference for sulphur oxidation during pyrite oxidation that may explain the congregation of *T.ferrooxidans* around the dislocation sites on the pyrite crystal. According to Andrews (1988) the amount of available sulphur is greater at these dislocation sites. This is because although the amount of diffusion in a crystal is very low, around dislocation sites the concentration of

diffusitives is many orders of magnitude higher (Shewmon, 1963). Because the oxidation of sulphur is energetically more favourable than iron, the increased availability at the dislocation sites makes them energetically preferable. In other words, because sulphur can diffuse through the crystal, and is found at much higher concentrations at the dislocation sites, and because sulphur is a better substrate for growth than iron, attachment to these sites is energetically preferable for *T.ferrooxidans*. Such attachment helps explain the non-stoichiometric oxidation of coal pyrite that has been observed. It would appear, however, that this theory allows only a small role for indirect oxidation.

The attachment of *T.ferrooxidans* to coal has been studied by Bagdigian and Myerson (1986), who found that not only does *T.ferrooxidans* selectively adsorb to dislocation sites, it also selectively adsorbs to the pyrite crystals within the coal. This attachment has two stages, an initial reversible stage, where the cell remains in close proximity to the surface but may still dissociate, and a second irreversible stage, where the cell produces the biopolymers necessary for adsorption and so becomes bound to the surface site.

The behaviour of *T.ferrooxidans* on a pyrite surface was studied by Bärtels *et al.* (1989). They synthesised ultrathin layers of pyrite on glass slides and then observed the growth of the bacteria by light microscopy. After attachment, most bacteria show a steadily increasing halo,

caused by the formation of a pit as the pyrite is broken down. The presence of, in many cases, a cell with little or no halo adjacent to a cell with a clearly pronounced halo suggested to the authors that the bacteria replicated *in situ*, and that one of the bacteria then starts a separate pit. As growth continues the pits merge to form large craters. In these craters the bacteria were congregated around the edges of the pit, which are, in effect, dislocation sites.

The preference for dislocation sites has also been noted by Grishin and Tuovinen (1989), who found that when ion-exchange resin particles were used as a support matrix, colonization was centred around cracks commonly found in these particles. However they point out that this is more likely to be due to the fact that the particles were used in a fluidised bed reactor, and the cracks would provide protection against the shear effects found in such an environment.

There has been very little study on the role of attachment in organisms other than *T.ferrooxidans*. One study comparing the attachment of *T.ferrooxidans* and *L.ferrooxidans* to pyrite found that whilst only 7% of *T.ferrooxidans* cell protein was associated with the mineral, 87% of *L.ferrooxidans* was so associated (Norris *et al.*, 1988). Only 7% association of protein with the mineral is a little difficult to reconcile with the alleged importance of attachment to pyrite oxidation and would seem to suggest a larger role for indirect attack. The large difference

between the two organisms may, however, suggest a different method of attack.

5.0. THE AIMS OF THE PROJECT

Several recently isolated iron- and sulphur-oxidising bacteria were available for study. Little was known of their mineral solubilisation capacity and less was known about their iron oxidation systems. These isolates were moderate thermophiles and therefore, as has been explained, a better commercial prospect than the mesophilic *T.ferrooxidans*. Further understanding of these bacteria would be expected to benefit their commercial application.

Iron oxidation is obviously of key importance in mineral solubilisation and it was therefore the aim of this project to examine the iron oxidation systems of these bacteria, and to investigate the system in more detail in one or more chosen isolates. Similarly understanding of the leaching mechanisms was also to be investigated. Previous work had suggested that mixed culture leaching was more effective than pure culture, and so the benefits of mixed culture leaching by these organisms was to be studied in tandem with pure culture leaching.

MATERIALS AND METHODS

CHAPTER TWO.

M E T H O D S .

The following gives details of all the methods used throughout this project. They are divided into two parts. The first deals with methods used throughout, i.e. methods or techniques that were commonly used, such as growth conditions or iron titrations. The second details those methods that were only used for specific experiments.

NON-SPECIFIC METHODS

1.0. The Organisms.

1.1. The Strains.

The strains of *T.ferrooxidans* and *L.ferrooxidans* used throughout this study were *T.ferrooxidans* DSM583 and *L.ferrooxidans* DSM2705.

Five moderate thermophile strains were used. Strain TH3 was first described by Brierley *et al.*, (1978). Strain BC1 was isolated from a drainage channel at the Birch Coppice Mine, Warwickshire, and LM2 from a hot spring in Lake Myvam, Iceland by Marsh and Norris (1983a). Strain BC13 was also isolated from the Birch Coppice Mine by Norris *et al.*

(1986). Strain ALV was isolated from a coal spoil tip at the Alvecote Mine, Warwickshire by Norris (unpublished data). The first publication to mention this organism was Wood and Kelly (1983).

1.2. Storage of Cultures.

The organisms were stored as pyrite-grown cultures at 4^{o}C. After approximately 5 - 6 months the organisms were subcultured into fresh pyrite basal salts. The organisms were also stored on sulphur basal salts, with subculturing every 3 - 4 months. When required for experimental procedures an organism was subcultured from the relevant mineral- or iron-grown culture, or, if an iron-grown culture was required, from the mineral-grown culture. In all cases at least two subcultures were carried out before experiments were undertaken. For iron-grown strains, at least six subculturings were carried out.

1.3. Large Scale Growth of Organisms.

Large quantities of biomass (approximately 5 - 10 g wet weight) of *T.ferrooxidans* and strain BC1 were required for protein purification processes. The organism was grown in batch culture in the appropriate medium and conditions (see section 2.0), but in 2 - 4 20 litre carbouys. Cells were harvested using a Westphalia continuous flow centrifuge.

2.0. Growth Medium and Conditions.

2.1. Basal Salts Media.

This was the basal mix to which the required substrate was added. It consisted of 0.4 g $MgSO_4.7H_2O$, 0.2 g $(NH_4)_2SO_4$, 0.1 g K_2HPO_4 and 0.1 g KCl per litre of distilled water.

For ferrous iron media, the basal salts were acidified with H_2SO_4 to pH 1.7 and sterilised at 121°C for fifteen minutes. Stock $FeSO_4.7H_2O$ solution was made up to 1M strength and acidified to pH 1.3 - 1.4. This stock solution was sterilised at 112°C for ten minutes. Just before inoculation of the media, 50 ml of stock $FeSO_4.7H_2O$ were added per litre of salts, to give a final concentration of 50 mM iron in the medium. For the autotrophically-grown moderate thermophiles on ferrous iron, 0.2 ml of a sterile potassium tetrathionate solution were added per 100 ml of growth media to provide a reduced sulphur source. The tetrathionate stock was made up to a concentration of 30 grams per litre distilled water and sterilised at 121°C for fifteen minutes.

For elemental sulphur media, the basal salts were acidified to pH 3.0. 5 g elemental sulphur flowers were then added per litre, along with 10 mg $FeSO_4.7H_2O$ per litre, for an iron source. The medium was sterilised in the flasks intended for use, at 105°C for five minutes. The flasks were left overnight and then further sterilised under the same

conditions. A final sterilisation was carried out on the third day.

For mineral media, the basal salts were acidified to pH 2.0. Crushed mineral, particle size < 50 μm, was added to 1% w/v. Sterilisation was carried out at 121°C for fifteen minutes.

For yeast extract-grown cells, the basal salts were acidified to pH 2.0 and sterilised at 121°C for fifteen minutes. A stock solution of 1% w/v yeast extract was sterilised under the same conditions. Just prior to inoculation 20 ml yeast extract per litre was added to the growth medium, giving a final concentration of 0.02% yeast extract. For chemoheterotrophically-grown cultures the same concentration of yeast extract was used in conjunction with the ferrous iron or sulphur.

2.2. Growth Conditions.

Moderate thermophiles were incubated in rotary incubators, at 45°C. Shaking was at approximately 80 - 100 revolutions per minute. Autotrophically-grown cultures were gassed with 5% (v/v) CO_2 in air, so that the gas was just trickling through.

Mesophiles were incubated at 30°C, in rotary incubators at the same speed as the moderate thermophiles.

3.0. Atomic Absorption Spectrophotometry.

All atomic absorption spectrophotometry was undertaken using a Varian 1200 spectrophotometer, fitted with an automatic sampler. In all cases an air-acetylene flame was used. Standard atomic absorption spectrophotometry procedures were used throughout.

3.1. Detection of Iron and Copper from Leaching Samples.

Approximately 0.5 ml of solution was removed from the culture and placed in a bench top centrifuge tube. The sample was spun at high speed for five minutes. 200 µl of the sample supernatant was removed and added to 9.8 ml 1% HCl (v/v). If samples were being taken from a culture over a period of time they were then refrigerated until all the samples had been collected.

For calibration curves, solutions of 10 mM $FeSO_4.7H_2O$ or $CuSO_4.5H_2O$ were made up in 1% HCl (v/v), and then further diluted in 1% HCl to give a range of concentrations from 20 to 100 µM. For a blank, 1% HCl was used.

The iron or copper in samples was then measured after dilution so that sample values were always between standard concentrations.

3.2. Detection of Sulphate in Leaching Samples.

Sulphate detection was used in conjunction with mineral

leaching experiments and therefore the first step was to remove particles of mineral from the sample. Approximately 1.5 ml of solution was removed from the culture and placed in a bench top centrifuge tube. The sample was spun at high speed for five minutes. 1 ml of the solution was removed and added to 4 ml of 1% HCl (v/v). If the samples were being taken from a culture over a period of time, they were then refrigerated until all the samples had been collected.

A stock solution of 1 g $BaCl_2$ per litre distilled water was made up. This was then further diluted 1:5 and 5 ml of the resulting solution added to the samples. The samples were well mixed and then left overnight to allow the precipitate to form.

For the calibration curve a stock solution of KH_2SO_4 was made up to give a final concentration of 1 g sulphate per litre distilled water. This was diluted with 1% HCl (v/v) to give a range of final concentrations between 0 and 175 mg sulphate per litre. The resulting samples were well mixed and then left overnight to form a precipitate.

The samples were read on the atomic absorption spectrophotometer using standard conditions for barium detection. Any samples whose values lay outside the calibration curve had to be repeated with a greater dilution factor.

3.3. *Detection of Iron in SDS Polyacrylamide Gel Samples.*

Whole cell lysates of strain BC1 grown

chemoheterotrophically on ferrous iron/yeast extract or heterotrophically on yeast extract were run on an SDS polyacrylamide gel. The gel was 13% polyacrylamide and the running conditions were 35 mA for approximately four hours. The resulting gel tracks were each removed and cut into seven pieces. Each piece corresponded to the area above and below a molecular weight marker. The gel pieces were placed in 2 ml conc. HNO_3 and then placed in a boiling water bath for ten minutes. The resulting solutions were diluted 1:5 with distilled water. The samples were then assayed for iron in the spectrophotometer using the method already outlined.

3.4. Detection of Iron in Fractions from Column Experiments.

200 µl of the fraction were removed and placed in 5 ml of 1% HCl (v/v). The resulting samples were then assayed for iron in the usual manner, after appropriate dilutions.

3.5. Detection of Iron in Cell Fractions.

In order to determine the concentration of iron in the membrane and soluble fractions resulting from ultracentrifugation, 100 µl of sample were removed and added to 100 µl conc. HNO_3. The sample was placed in a boiling water bath for five minutes. The resulting solutions were added to 4.8 ml of 1% HCl (v/v). The samples were then assayed for iron.

4.0. Ceric Sulphate Assay for Ferrous Iron.

Ferrous iron was frequently assayed using this method. Pre-measured ceric sulphate (Volucon) was diluted as per the instructions supplied to give a solution of such concentration that every 1 ml of $Ce(SO_4)_2$ added indicated the presence of 5 mM $FeSO_4$ when the following assay procedure was followed.

1 ml of the sample to be assayed was removed and added to 1 ml of 5% H_2SO_4. One drop of 1,10-phenanthroline ferrous sulphate indicator (BDH Laboratory Supplies) was added to the sample. The sample was then titrated with the $CeSO_4$. The end point was taken to be the appearance of a blue colour.

Ferrous iron growth curves had their 'best fit' lines approximated by eye.

5.0. Tiron Assay for Ferrous Iron.

Ferrous iron was occasionally assayed using Tiron (1,2-Dihydroxy- benzene-3,5-disulphonic acid). Two methods have been proposed, using either acidic buffers or alkaline buffers (Johnson, 1964). The method used here was adapted from the acid preparation .

The tiron was made up to 100 mM concentration. A 500 mM solution of sodium acetate was made up in 400 mM HCl. This buffer solution gave a final pH of 4.0.

In order to assay a solution, a 150 µl sample was taken

and added to 1320 µl buffer in an Eppendorf tube. The solution was mixed and then 30 µl of the tiron added. The solution was mixed again and then left at room temperature for 30 minutes. A calibration curve consisting of concentrations between 0 and 1000 µM $FeSO_4.7H_2O$ was set up using the same method. The absorbance of the samples was then measured spectrophotometrically at 620nm.

6.0. Warburg Respirometry.

In most cases the conditions for the use of this apparatus remained constant throughout. Any deviation from these conditions will be noted when the results are discussed.

The volume in the flask was 2 ml. This was acidified water; the pH depended on the substrate to be used. If ferrous iron was the substrate the pH was 1.7, for elemental sulphur the pH was 3.0 and for mineral the pH was 2.0. The thermobarometer was also acidified to the relevant pH.

The final concentration of ferrous iron in the flask was 20 mM. 100 mg of elemental sulphur or mineral were added to the flasks requiring such solids. 200 µl of cells with an O.D. of 10 at 440nm were added to the flask. This gave a final protein concentration in the flask of 0.1 - 0.2 mg/ml, depending upon the organism. For accurate protein determination a sample of the cells was kept.

The temperature of the water bath was either 30^0C for the mesophilic organisms or 45^0C in the case of the moderate

thermophiles.

The oxygen uptake graphs in this project had their 'best fit' lines fitted by eye.

7.0. Mineral Leaching Experiments.

Batch culture leaching experiments were undertaken using 250 ml conical flasks, which contained 100 ml of medium. The media used for these experiments have already been detailed, as have the growth conditions.

The flasks were inoculated with organisms that were actively growing on the mineral in question. Inoculum size was 5%, i.e. 5 ml. The flasks were weighed and then incubated at the correct temperature. Samples were removed for iron and/or copper determination in the manner already described. The flasks were again weighed. Weighing the flasks allowed the extent of evaporation to be determined. This meant that the increased concentration of the metal in solution could be calculated and if necessary sterile distilled water added.

If necessary, pH and ferrous iron levels could be determined. To determine the ferrous iron levels by ceric sulphate assay, a little over 1 ml of the medium was removed, placed in a bench top centrifuge tube and spun at high speed for five minutes. This removed particles of mineral and allowed 1 ml of the supernatant to be assayed as already detailed.

The mineral leaching graphs in this work had their

'best fit' lines approximated by eye.

8.0. Protein Assays.

The method used throughout this project was adapted from the Lowry method (Lowry *et al.*, 1951).

Three stock solutions were made up. These were 2% Na_2CO_3 in 100 mM NaOH (w/v), 1% $CuSO_4$ and 1% sodium potassium tartrate. Just before use these were mixed together in the ratio 98:1:1 respectively, to give reagent C.

The sample size was 100 µl. If it was considered that this would give too much protein then a smaller sample was used and made up to 100 µl with distilled water. If the sample required lysing then 100 µl of 100 mM NaOH was added and the sample incubated at 65^0C for fifteen minutes.

After the sample had been allowed to cool to room temperature 1000 µl of reagent C was added and the sample immediately well mixed. The samples were left for 10 minutes at room temperature. Folins reagent (Fisons Laboratory Supplies) was diluted; 2 parts to 3 parts distilled water. After ten minutes, 100 µl were added to the samples. The solution was thoroughly mixed and left in the dark at room temperature for 30 minutes.

The absorbance of the samples was then read at 700nm and the results compared to the calibration curve prepared in the same way, using concentrations of bovine serum albumin ranging between 0 and 500 µg/ml. This calibration

curve was always a straight line between these two protein concentrations.

9.0. SDS Polyacrylamide Gel Electrophoresis.

The majority of electrophoresis undertaken was adapted from the discontinuous buffer method of Laemmli (1970) and used the LKB 2001 Vertical Electrophoresis Unit. The molecular weight markers used throughout were from the Pharmacia Low Molecular Weight kit.

9.1. The Solutions.

The running buffer consisted of 3.3 g Tris/l, 14.4 g glycine/l and 1 g sodium dodecyl sulphate (SDS)/l. The pH was adjusted to pH 8.3 by the addition of HCl.

The buffer solution for the resolving gel was made up as follows. 30.27. g Tris/l and 2 g SDS/l. The pH of the solution was adjusted to 8.8 with HCl.

The stacking gel buffer consisted of 90.8 g Tris/l and 2 g SDS/l. The buffer was adjusted to pH 6.8 by the addition of HCl.

The sample buffer was made up of 25 ml of the stacking gel buffer to which were added 2 g SDS, 10 ml glycerol, 5 ml 2-mercaptoethanol (Fisons) and 500 µl of 1% bromophenol blue (BDH) (w/v). Finally, 60 ml of distilled water was added.

The acrylamide stock consisted of 300 g acrylamide (BDH)/l and 8 g bis-acrylamide (BDH)/l in distilled water.

The solution was stored in the dark at $4^{0}C$.

A fresh solution of 1% ammonium persulphate (AMPS) (w/v) in distilled water was made up every time it was needed.

9.2. The Gel.

The gel was cast using the plates supplied, with the 2 mm spacers. The resolving gel was normally in the range 10 - 13% acrylamide, depending upon the degree of separation obtained in previous attempts.

The following is the make up used for a 10% resolving gel. 10 ml acrylamide stock, 15 ml resolving gel buffer, 1.5 ml AMPS, 3.5 ml water and 10 µl Tetramethyl-ethylene-diamine (TEMED) (BDH). The stacking gel consisted of 1.3 ml acrylamide stock, 5 ml stacking gel buffer, 1 ml AMPS, 2.7 ml of water and 5 µl of TEMED.

Approximately 25 ml of the resolving gel was poured and then overlayed with water. The water was removed after the gel had set and the stacking gel was then added.

9.3. The Samples.

If necessary the samples were lysed first (see section 11.1). The sample was then added to an equal volume of sample buffer and placed in a boiling water bath for five minutes. After the sample had cooled it was added to the wells in the stacking gel.

9.4. The Running Conditions.

The running conditions varied considerably and so they have been noted whenever a gel is discussed. However, in general, gels were run at 30 - 45 mA for between 3 and 5 hours.

9.5. Fixing and Staining.

Two types of fixing and staining methods were used, Coomassie staining and silver staining.

9.5.1. Coomassie Staining.

The gel was placed in approximately 300 ml of Brilliant Blue R Coomassie stain and left on a rotary shaker overnight. The stain was removed and the gel was added to approximately 500 ml of destain. The destain consisted of 400 ml water, 50 ml acetic acid and 50 ml ethanol. The gel was left in destain until the protein bands could be clearly distinguished against the gel. If necessary the gel was kept stored in 10% acetic acid (v/v).

9.5.2. Silver Staining.

The gel was fixed by immersion in a 50% methanol (v/v) solution for approximately 16 - 18 hours. The fixative was changed three times during this period, but not necessarily at regular intervals. The gel was then washed once in distilled water before being placed in the stain.

The stain was made up in the following manner. To 21 ml of 0.36% NaOH (w/v) was added approximately 180 ml distilled water. To this was added 1.25 ml NH_3. Finally 5 ml of 16% $AgNO_3$ (w/v) were added slowly whilst the flask was shaken in order to dissolve the precipitate. The gel was left in this stain for 15 minutes. After the stain was removed the gel was washed very thoroughly; initially in running de-ionised water until the precipitate cleared and then in distilled water.

The developer consisted of 2.5 ml 1% citric acid and 0.8 ml formaldehyde added to 500 ml of water. After the water was removed this developer was added. The gel was soaked in developer until the required band intensity was apparent.

As soon as this point was achieved the developer was quickly removed and the stop solution added. This consisted of 225 ml methanol, 50 ml acetic acid and 250 ml water. If required the gel could be stored in this solution.

10.0. Scanning Spectrometry.

For all this work the same instrument was used, a Beckman DU70 scanning spectrophotometer.

10.1. Room Temperature Difference Spectra.

All the spectra in this work were reduced minus oxidised. Standard operating procedures were followed

throughout.

The sample, in a 1 ml cuvette, was oxidised by one of two oxidants. Initially the oxidant was AMPS, of which two or three crystals were added to the sample. In later experiments it was found that sodium hexachloroiridate (Sigma Chemical Company) gave clearer results. 200 - 400 µl of a 5 mM solution was added to the sample.

The sample was reduced in all cases by the addition of sodium dithionite. A few crystals were added to the sample.

In most cases a small amount of the sample was collected for a protein determination.

10.2. Liquid Nitrogen Difference Spectra.

The use of special temperature control equipment allowed the use of liquid nitrogen to obtain low temperature spectra. The sample was first oxidised with sodium hexachloroiridate and then frozen to -170^{o}C by the addition of liquid nitrogen. The background scan was then carried out and the sample thawed. The sample was then reduced by the addition of sodium dithionite, refrozen to the same temperature and the final reduced minus oxidised difference spectra taken.

11.0. Redox Potentials.

Several redox potentials were determined. The Beckman DU 70 spectrophotometer was used for all experiments. A

reaction chamber was designed especially for these experiments (Figure 3). The redox electrode was supplied by Russell pH Ltd (Fife, Scotland), as was the meter.

11.1. Preparation of Sample.

The redox electrode was calibrated to +440 mV with an equal volume mix of 10 mM potassium ferricyanide (Fisons) and 10 mM potassium ferrocyanide (Fisons). The electrode was then placed in the chamber.

Approximately 25 ml of sample was prepared and placed in the chamber. The chamber's cuvette was placed in the fitting in the spectrophotometer. The whole apparatus was sealed to exclude light. The sample was oxidised by the addition of 400 µl of 5 mM sodium hexachloroiridate and the background reading taken.

11.2. Addition of the Mediator.

The mediator was then added. The mediator added depended upon the region in which the redox potential change was expected to occur. For higher potentials, around +350 - 500 mV, the potassium ferri/ferrocyanide couple was used again, at a final concentration of 100 µM. NNN'N'tetramethyl-p-phenylene-diamine (TMPD) (BDH) has a potential of +260 mV and was used as the mediator for samples with a potential between +240 mV and +350 mV. The concentration used was 40 µM. It was rarely necessary to go

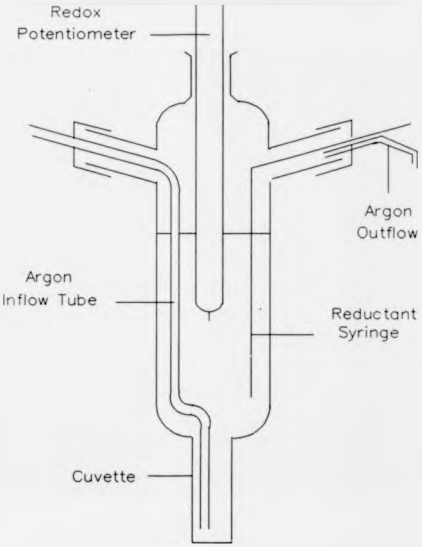


Figure 1. The apparatus used to determine the mid-point redox potentials of cytochromes. Argon was bubbled through a crude preparation of the cytochrome in order to mix the sample and ensure the vessel was oxygen free. Reductant was introduced via a long syringe needle. The E_h of the sample was measured with the redox potentiometer.

below these values in this work but occasionally 40 μM dichlorophenol-indophenol (DCPIP) (BDH) was used. This has a potential of +217 mV.

11.3. Determination of the Redox Potential.

After the mediator had been added the reduction of the sample was begun. Solutions of sodium dithionite were made up at 100 mM, 50 mM and 10 mM concentrations. The argon was bubbled through the sample. This had the effect of both mixing the sample and removing the air from the chamber. The dithionite was then added. The concentration and volume added depended entirely on the extent of the drop in potential of the sample. As the sample reached its mid-point redox potential the potential would drop more quickly. Therefore small volumes of weaker concentration would be added. Most of the additions were however with the 10 mM dithionite and were in the region of 10 to 20 μl.

After each addition the potential of the sample was noted and then a spectrum was run. In this way a large number of spectra were collected, some showing an increase in peak height as the sample was reduced. The experiment was run until it was certain that the sample was fully reduced, i.e. there was no more increase in the peak height.

It was then possible to correlate the increase in peak height with the potential of the sample and in this way calculate the redox potential.

12.0. Cell Lysis.

Cell lysis was necessary prior to electrophoresis and as the initial step in purification processes. Several methods were examined. It was found that different methods were required for gel samples and samples to be fractionated.

12.1. Lysis of Cells in Preparation for PAGE.

The cell sample was collected by centrifugation and cleaned thoroughly. Enough sample was required to cover the bottom of an Eppendorf tube. To the sample was added 50 µl of 50mM Tris - EDTA at pH 8.5. A stock solution of 500 µg lysozyme/ml was made up and 10 µl added to the sample. The sample was then incubated at 37^{0}C for 20 minutes. After 60 µl of sample had been added the sample was placed in a boiling water bath for fifteen minutes. After the sample had been cooled it was ready for PAGE.

12.2. Lysis of Cells in Preparation for Cell Fractionation.

For these experiments approximately 5 - 10 g wet weight of cells were used. The pellet was repeatedly resuspended in 20 ml of 50 mM Tris-chloride at pH 8.0, and then repelleted by centrifugation at 12,000 rpm for 15 minutes, until the pH reached 7.0 - 7.5. To the final sample volume of approximately 25 ml was added 6.25 mg of lysozyme, giving a

final approximate concentration of 250 µg/ml. The sample was incubated at 37^{0}C for 45 minutes, during which time it was shaken several times. The sample was then passed three times through a French press.

After lysis the sample was spun at 14,000 rpm for 20 minutes. The resulting supernatant was spun at 45,000 rpm for 90 minutes. After the centrifugation the soluble fraction was carefully removed and the membrane fraction resuspended in 50 mM Tris-chloride, pH 7.0.

SPECIFIC METHODS.

13.0. Mineral Leaching with Strain BC1 and Strain BC13 in Airlift Fermentors.

The effect of mixed culture leaching of the mineral chalcopyrite was investigated in airlift fermentors. These fermentors were designed specifically for mineral leaching (Figure 4).

13.1. The Cultures and the Medium.

Two cultures were set up, a pure culture of strain BC1 and a mixed culture of strain BC1 and strain BC13. The cultures were both grown autotrophically on chalcopyrite under standard shake flask conditions for two subcultures.

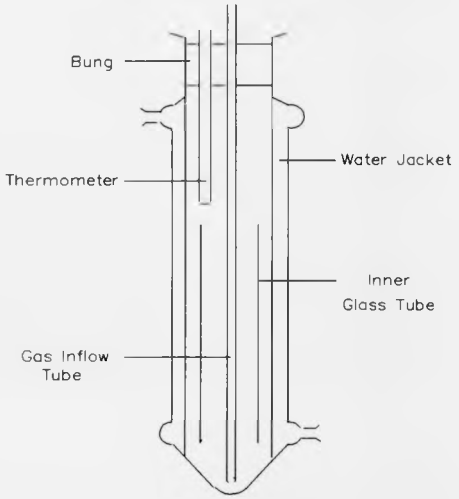


Figure 4. An airlift reactor. 1% CO_2 (v/v) in air was introduced via the gas inflow tube. This flow of gas kept the mineral in suspension, which circulated around the inner glass tube. The water jacket was used to maintain the required temperature.

Although strain BC13 cannot oxidise chalcopyrite, it can oxidise the elemental sulphur present in the mineral.

The medium used in the airlift fermentors consisted of (g/l) 0.4 $(NH_4)_2SO_4$, 0.5 $MgSO_4.7H_2O$ and 0.2 K_2HPO_4. It was acidified to pH 2.0 by the addition of HCl.

13.2. Running the Airlift Fermentors.

Two fermentors were set up, each containing 40 ml of medium. The water flowing through the water jacket was heated to 48^{0}C. The gas flow, 1% CO_2 in air, was then started and set at 400 ml/min. Initially the mineral concentration was 1% chalcopyrite (w/v), i.e. 4.4 g of mineral. To one fermentor was added 40 ml of the strain BC1 culture, to the other 40 ml of the strain BC1 / strain BC13 mixed culture.

Whilst the mineral concentration was only 1%, only iron and copper were assayed, using the atomic absorption spectrophotometer.

When iron and copper readings and examination of the culture by microscopy indicated that growth was well established, a further 17.6g of chalcopyrite were added, taking the added mineral concentration to 5%. After this point more frequent readings were taken of solubilised total iron and copper levels, as well as ferrous iron levels and pH.

Although the fermentors were fitted with condensers it was occasionally necessary to add sterile distilled water to

the fermentors, in order to keep the liquid level constant.

The experiment was continued until the assays indicated that the leaching of copper and iron had finished in both fermentors.

14.0. Preparation of Rusticyanin from Thiobacillus ferrooxidans.

The method of Cox and Boxer (1978) was adapted for this work. The cells were grown up in double strength salts in 100 mM ferrous sulphate. In all other respects the growth conditions were as already detailed.

14.1. Lysis of the Cells.

The lysis of the cells differed from the method previously described in that the preparation was undertaken at pH 2.0, rather than pH 7.0. After spinning down, the cells were resuspended in water acidified to pH 2.0 with sulphuric acid. The cells were passed through a French press and then spun down in exactly the same manner as previously described.

14.2. Ammonium Sulphate Fractionation.

Enough $(NH_4)_2SO_4$ was added to the supernatant from the lysed cells to give a final concentration of 65% (w/v). The resulting precipitate was removed by centrifugation at

15,000 rpm for 20 minutes. The pellet was discarded and the concentration in the supernatant increased to 95% (w/v). The precipitate was again spun out. The resulting pellet was kept and resuspended in 10 mM sodium acetate buffer at pH 5.5.

14.3. Dialysis of Rusticyanin Fraction.

The fraction was dialysed against 10mM sodium acetate buffer at $4^{0}C$ for approximately 18 hours. The buffer was changed twice during this period, but not at regular intervals.

14.4. Ion Exchange Chromatography.

A 10 ml CM Sephadex column was set up and was equilibrated with the sodium acetate pH 5.5 buffer. Approximately 1.5 ml of the dialysate was added to the column. The column was then washed through with two volumes of the buffer. The column was run at approximately 8 ml/hour throughout the experiment. After the column had been washed in the buffer, 100 mM NaCl in buffer was added to the column and the resulting fractions collected.

14.5. Spectrophotometric Analysis.

The fractions were read in a spectrophotometer at 280 nm and 590 nm to determine protein and rusticyanin content

respectively. Those fractions that showed high absorbance at both wavelengths were pooled and dialysed against water acidified to pH 2.0 with H_2SO_4 for approximately 16 - 18 hours. The water was changed twice during this period, but not at regular intervals. A spectrum was run of the resulting dialysate.

15.0. Strain BC1 Membrane Preparation and Solubilisation.

The cells were lysed and the membrane prepared in the same manner as has already been described. After adequate lysis had been achieved an investigation into a method for membrane solubilisation was begun.

15.1. Investigation into Membrane Solubilisation with Detergents.

A series of detergents and other membrane disruption methods such as pH and temperature were used, often in conjunction. Details of each treatment follows. After incubation the membrane was spun down and the supernatant removed. The membrane was then resuspended. Both the supernatant and the membrane were then assayed for iron using the tiron assay method. Absorbances at 280nm and 604.5nm were measured and then reduced minus oxidised spectra run.

15.1.1. Dodecyl β-D-Glucopyranoside (DDGP).

The sample of membrane was incubated at room temperature in 1% (w/v) DDGP (Sigma) in 50 mM Tris-chloride at pH 8.0.

15.1.2. Cholate / Deoxycholate.

The solution the membrane was resuspended in contained 2% sodium cholate (BDH) (w/v), 1% sodium deoxycholate (BDH) (w/v) and 200 mM NaCl in 30 mM Tris-sulphate at pH 8.0. The membranes were incubated on ice for 30 minutes.

15.1.3. Propan-2-ol.

The membrane was resuspended in a solution of 30% propan-2-ol 10 mM sodium phosphate buffer at pH 7.0. It was incubated at room temperature for 30 minutes.

15.1.4. Octyl-β-D-Thioglucopyranoside (OTG).

Membranes were resuspended in a 1% OTG (Sigma) (w/v) solution in 50 mM Tris-chloride at pH 8.0 for 30 minutes.

15.1.5. Sarkosyl.

The solution the membrane was resuspended in contained 1% sarkosyl (Sigma) (w/v) in 50 mM Tris-chloride at pH 9.0. Incubation was at room temperature for 30 minutes.

15.1.6. Octyl-ethyl-Gluconoride (OEG) / Sodium Monocaprate (SMC).

The membrane was first resuspended in a 1% OEG (Sigma)

(w/v) solution in 20 mM sodium phosphate buffer at pH 9.0. After incubating for 30 minutes at room temperature the membrane was spun down and resuspended in 1% SMC (Sigma) (w/v) in the same buffer. The sample was then incubated again for 30 minutes at room temperature.

15.1.7. Triton X-100.

The membranes were resuspended in a 1% Triton X-100 (Sigma) solution (v/v) in 50 mM Tris-chloride at pH 8.0. The sample was then incubated for 30 minutes at room temperature.

15.2.Investigation into Membrane Solubilisation with Cholate and pH, Temperature and Sonication.

The effects of pH, temperature and sonication were investigated in slightly more detail. The previous work suggested that cholate would be an effective detergent so the membrane sample was resuspended in conjunction with the following methods. After incubation the membrane was spun down and the supernatant removed. The membrane was then resuspended. Both the supernatant and the membrane were then assayed for iron using the tiron assay method. Absorbances at 280nm and 604.5nm were measured and then reduced minus oxidised spectra run.

15.2.1. The effect of Sonication.

A 2 ml sample was sonicated on ice for 30 seconds at

twelve cycles. It was then incubated at room temperature for 30 minutes.

15.2.2. The effect of Temperature.

The resuspended membrane was incubated at 50°C for 30 minutes.

15.2.3. The effect of pH.

The membrane was resuspended in the cholate mix at pH 9.0, allowing comparison with the previous attempt at pH 8.0. Incubation was for 30 minutes at room temperature.

15.3. Combination of Detergents and Temperature and pH.

The next step was to combine other detergents with temperature and pH. After incubation the membrane was spun down and the supernatant removed. The membrane was then resuspended. Both the supernatant and the membrane were then assayed for iron using the tiron assay method. Absorbances at 280nm and 604.5nm were measured and then reduced minus oxidised spectra run.

15.3.1. Nonidet.

A 1% solution of nonidet (Sigma) (v/v) in 100 mM Tris-chloride was used to resuspend the membrane. For investigation of pH the buffer was at pH 9.0. For investigation of the effect of temperature the pH was 8.0. Incubation was for thirty minutes at room temperature for

the pH investigation and at 60°C for the temperature experiment.

15.3.2. Taurine / Betaine.

The membrane was resuspended in a 1% taurine (Sigma) (w/v) and 1% betaine (Sigma) (w/v) solution in 100 mM Tris-chloride. For investigation of pH the buffer was at pH 9.0. For investigation of the effect of temperature the pH was 8.0. Incubation was for thirty minutes at room temperature for the pH investigation and at 60°C for the temperature experiment.

15.3.3. Octyl-β-D-Thioglucopyranoside (OTG).

For the temperature experiment the membrane was resuspended in a 1% solution (w/v) in 100 mM Tris-chloride at pH 8.0 and incubated at 60°C for 30 minutes.

For the pH experiment the membrane was resuspended in 100 mM Tris-chloride. Sufficient OTG was then added to give a 1% solution and the sample agitated at room temperature as the OTG dissolved. After a total of 30 minutes had passed since the addition of the OTG the membrane was spun down. (This was always sufficient time for the OTG to dissolve completely).

16.0. BC1 Membrane Protein Purification Processes.

In order to purify proteins from the solubilised membrane several processes were utilised, including column

chromatography and ammonium sulphate fractionation. The membrane was first solubilised using the OTG / pH method detailed above. Membranes could be stored at -20°C in either solubilised form or as the pellet with little loss of cytochrome activity.

16.1. Column Chromatography.

Five different column matrices were used in attempts to isolate the terminal oxidase and/or constituents of the iron oxidising systems. Before addition to the appropriate column, the solubilised membrane was dialysed for 16 - 18 hours against 10 mM Tris-chloride buffer, which was changed three times during this process, but not at regular intervals.

16.1.1. Ion Exchange Chromatography.

5 ml columns of DEAE Sepharose and CM Sephadex were equilibrated with at least four volumes of 10 mM Tris-chloride at pH 7.0. Approximately 2 ml of sample were added to the columns which were run at 15 ml/hour. 2 ml fractions were collected. After the sample, 1 volume of buffer was run through the columns. The pH of the column was then raised to 8.0 by the addition of approximately two volumes of the same buffer at pH 8.0. A NaCl gradient was then run through the columns, up to a 2 M concentration. The columns were then washed through with 10 mM Tris-chloride at pH 4.0.

chromatography and ammonium sulphate fractionation. The membrane was first solubilised using the OTG / pH method detailed above. Membranes could be stored at -20°C in either solubilised form or as the pellet with little loss of cytochrome activity.

16.1. Column Chromatography.

Five different column matrices were used in attempts to isolate the terminal oxidase and/or constituents of the iron oxidising systems. Before addition to the appropriate column, the solubilised membrane was dialysed for 16 - 18 hours against 10 mM Tris-chloride buffer, which was changed three times during this process, but not at regular intervals.

16.1.1. Ion Exchange Chromatography.

5 ml columns of DEAE Sepharose and CM Sephadex were equilibrated with at least four volumes of 10 mM Tris-chloride at pH 7.0. Approximately 2 ml of sample were added to the columns which were run at 15 ml/hour. 2 ml fractions were collected. After the sample, 1 volume of buffer was run through the columns. The pH of the column was then raised to 8.0 by the addition of approximately two volumes of the same buffer at pH 8.0. A NaCl gradient was then run through the columns, up to a 2 M concentration. The columns were then washed through with 10 mM Tris-chloride at pH 4.0.

16.1.2. Gel Filtration Chromatography.

The gel filtration matrices were of different volumes. The Sephadex G-75 column was 35 ml, the Sephacryl S300 was 50 ml and the Sepharose CL-6B was 350 ml. All columns were equilibrated with at least four volumes of 10 mM Tris-chloride at pH 7.0. In all cases the sample was eluted with the same buffer. The sample size varied with the size of the column, as did the running speed. 2 ml of sample was added to the Sephadex G-75 column, which was then run at 10 ml/hour. 3 ml was added to the Sephacryl S300 column, which was run at 6 ml/hour. The Sepharose CL-6B column was run at 5 ml/hour, after the addition of 25 ml of sample. 2 ml fractions were collected from all columns.

16.1.3. Analysis of the Column Fractions.

The absorbance of the 2 ml fractions at 280nm, 420nm and 604.5nm were read. Reduced minus oxidised spectra were run of those fractions that showed elevated readings at 420nm and 604.5nm. Protein assays were run of those fractions whose spectra were analysed.

16.2. Concentration of Samples.

In those cases where samples from the columns had to be concentrated in order to carry out further purification, this was achieved by dialysis. The samples were dialysed against 30% PEG (w/v) at 4^{o}C until appreciable loss of volume occurred. This was in the region of 4-5 hours. In

order to ascertain the effect of this treatment on the activity of the sample, protein assays and reduced minus oxidised spectra were carried out before and after treatment.

16.3. Ammonium Sulphate Fractionation.

A 5 ml sample of solubilised membrane was used. The initial protein concentration and wavelength absorbances at 420nm and 604.5nm were determined and then sufficient $(NH_4)_2SO_4$ added to give 20% saturation. The precipitate was spun down and discarded and the supernatant's protein concentration and wavelengh absorbances at 420nm and 604.5nm determined. The supernatant was then treated to give a final $(NH_4)_2SO_4$ concentration of 30% (w/v) and the determinations repeated. This was carried out at 40%, 50% and 60% $(NH_4)_2SO_4$ concentrations (w/v).

17.0. Duplication and Reproducibility of Experiments.

All the growth curves, mineral leaching, sulphate production and oxygen uptake rates were done in duplicate. The graphs were plotted from averages.

The redox titrations were also done in duplicate, and again the average of the two runs were plotted.

The gel work did prove to be reproducible. The results as shown are those that gave the clearest illustration of the results as described. The experiment to determine the

iron content of tracks (chap. 6, 2.3) in an SDS polyacrylamide gel was done in duplicate and the average of the results plotted.

The spectra results were also reproducible. As with the gel results, the spectra given in the results section were chosen as they gave the clearest illustration of the result.

RESULTS AND DISCUSSION

CHAPTER THREE.

GROWTH AND OXIDATION DATA FOR MESOPHILES AND MODERATE THERMOPHILES.

1.0. GROWTH RATES AND DOUBLING TIMES OR SOLUBILISATION RATES ON FERROUS IRON, PYRITE AND SULPHUR.

These experiments were carried out to obtain data, on a very basic level, about the organisms involved in this study. A comparison between the better studied mesophiles and the less well known moderate thermophiles could then highlight major differences between these two groups. Although the intention of this study was primarily to investigate the oxidation of ferrous iron, the sulphur-oxidising systems were also considered. To this end growth of the organisms on sulphur was also investigated.

1.1. Mesophile Growth Curves.

Growth curve and solubilisation rate experiments were carried out and ferrous iron doubling times calculated for two mesophiles, *T.ferrooxidans* and *L.ferrooxidans*.

1.1.1. Thiobacillus ferrooxidans.

The growth curve of *T.ferrooxidans* on ferrous iron gave a doubling time of 8.5 hours (Figure 5). The graph also showed the characteristic 'tailing off' of the oxidation rate as the levels of ferrous iron dropped to 50% of the initial concentration.

This 'tailing off' effect is suggestive of nutrient limitation. In order to investigate this hypothesis a simple experiment was undertaken whereby four cultures of *T.ferrooxidans* were grown in the presence of varying concentrations of a trace element solution. The addition of such trace elements had no discernible effect on the growth of this organism (Figure 5). In all cases the 'tailing off' could still be seen. Furthermore the doubling times in each flask were not significantly altered. Because there was no significant difference only one such growth curve is shown in comparison to the trace element-free growth curve.

The rate of iron solubilisation from pyrite was calculated from the leaching graph (Figure 6). This rate reached 7.6 mg/l/hour at its fastest.

In order to calculate the oxidation of sulphur, the production of sulphate ions was measured. The results of these measurements showed the fastest rate of sulphate production to be 121.2 mg/l/hour (Figure 7).

1.1.2. Leptospirillum ferrooxidans.

This organism is less well known than *T.ferrooxidans*. It is therefore not surprising that fewer values have been

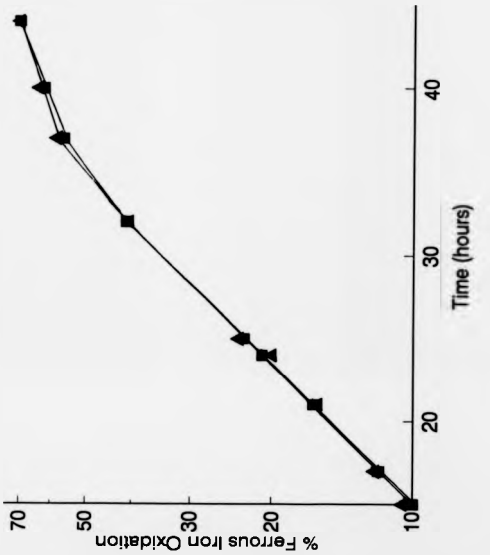


Figure 5. Growth curve of *T.ferrooxidans* on 50 mM ferrous iron, grown at 30°C. The organism was grown in the presence (■) and absence (▲) of a trace element solution, but this made no difference to the growth curve. The production of ferric iron was measured by the ceric sulphate assay.

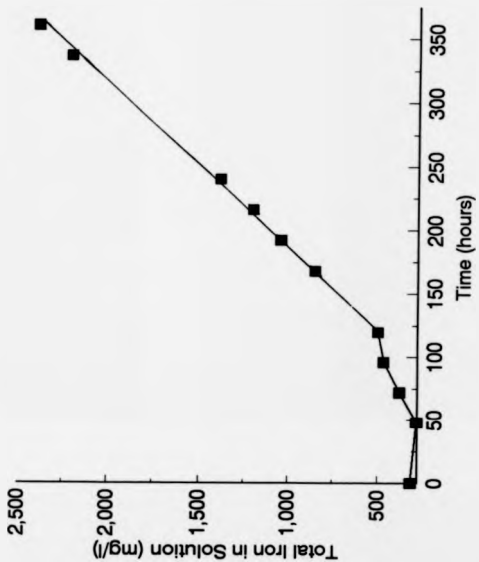


Figure 6. Iron solubilisation by *T.ferrooxidans* on 1% pyrite (w/v), grown at 30°C. The production of soluble iron was measured by atomic absorption spectrophotometry.

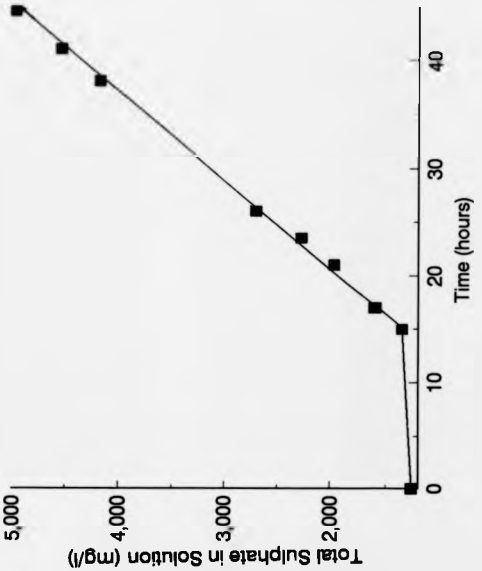


Figure 7. Sulphate production during *T.ferrooxidans* growth on 0.5% elemental sulphur (w/v), at 30°C. The production of sulphate was measured by atomic absorption spectrophotometry.

published for doubling times on iron and solubilisation rates of pyrite. The organism was on the whole slower growing than *T.ferrooxidans*.

From the growth curve for ferrous iron-oxidising *L.ferrooxidans* a doubling time of eleven hours could be calculated (Figure 8). This particular growth curve did not exhibit the 'tailing off' of oxidation rate when ferrous iron levels became low.

The solubilisation rate of pyrite, 6.1 mg/l/hour, also proved to be slower than the rate for *T.ferrooxidans* (Figure 9).

1.2. Moderate Thermophile Growth Curves.

Growth and solubilisation curves were obtained and the appropriate doubling times and solubilisation rates calculated for three moderate thermophiles, BC1, BC13 and ALV.

1.2.1. Strain BC1.

The growth rate of strain BC1 when oxidising ferrous iron was slightly faster than that of *T.ferrooxidans* and much faster than that of *L.ferrooxidans*. The growth curve , (Figure 10), demonstrated a doubling time of 6.3 hours, compared to 8.5 hours for *T.ferrooxidans* and 11 for *L.ferrooxidans*. The 'tailing off' effect could quite clearly be seen in this curve, indicating that this problem is not peculiar to the mesophilic organisms.

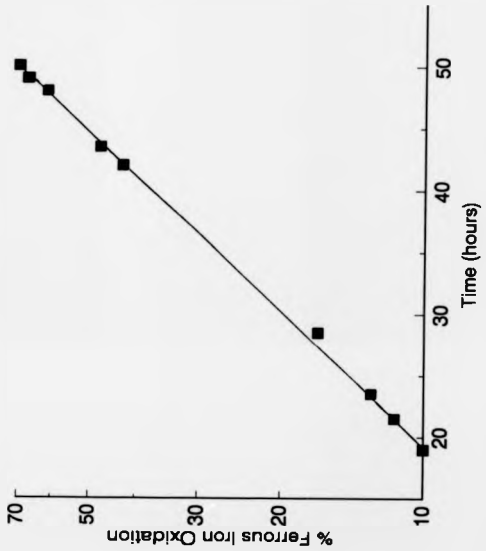


Figure 8. Growth curve of *L.ferrooxidans* on 50 mM ferrous iron, grown at 30°C. The production of ferric iron was measured by the ceric sulphate assay.

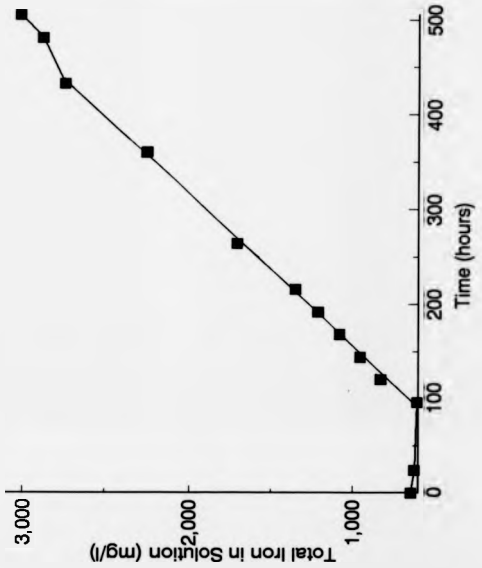


Figure 9. Iron solubilisation by *L.ferrooxidans* on 1% pyrite (w/v), grown at 30°C. The production of soluble iron was measured by atomic absorption spectrophotometry.

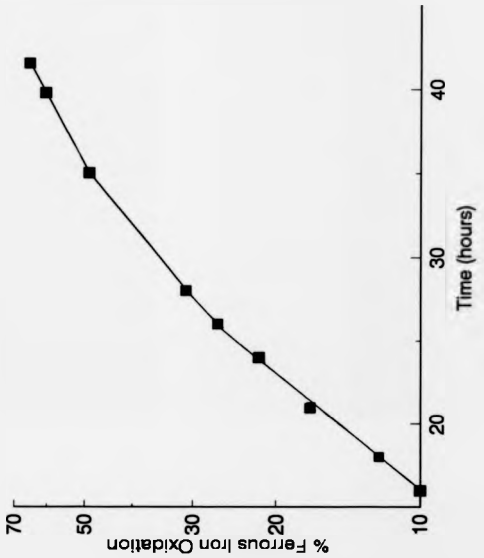


Figure 10. Growth curve of strain BC1 grown autotrophically on 50 mM ferrous iron, at 45^{o}C. The production of ferric iron was measured by the ceric sulphate assay.

Strain BC1 grows readily on pyrite, as the leaching curve demonstrated (Figure 11). The solubilisation rate obtained from this graph was much faster than that of the two mesophiles, being 14.3 mg/l/hour, compared to *T.ferrooxidans* 7.6 mg/l/hour and *L.ferrooxidans* 6.1 mg/l/hour.

Strain BC1 was an extremely poor autotrophic sulphur oxidiser. In order for comparisons to be made with the obligately autotrophic mesophiles, attempts were made to obtain an autotrophic sulphur oxidation curve. The organism could be grown in the standard sulphur flasks with the addition of 0.02% yeast extract but it was only after several subcultures that the organism was capable of growth autotrophically on sulphur. However this growth was slow and the cells always looked unhealthy. The graph gave a sulphate production rate of approximately 5.8 mg/l/hour (Figure 12). This was far slower than the 121.2 mg/l/hour of *T.ferrooxidans*.

1.2.2. Strain ALV.

Strain ALV is also capable of oxidising ferrous iron and sulphur, although its pyrite oxidation rate is extremely poor. For this reason only the ferrous iron and elemental sulphur rates of growth were calculated.

The doubling time during iron oxidation of strain ALV was similar to that of strain BC1, being 7 hours as opposed to the latter's 6.3 hours (Figure 13). Once again the growth rate for this moderate thermophile was faster than for the

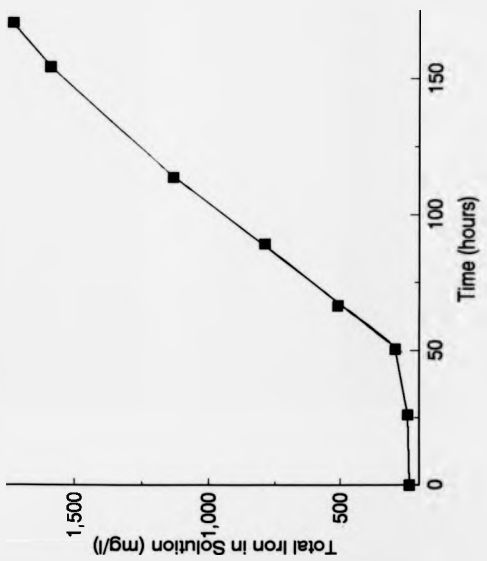


Figure 11. Iron solubilisation by strain BC1 grown autotrophically on 1% pyrite (w/v), at 45°C. The production of soluble iron was measured by atomic absorption spectrophotometry.

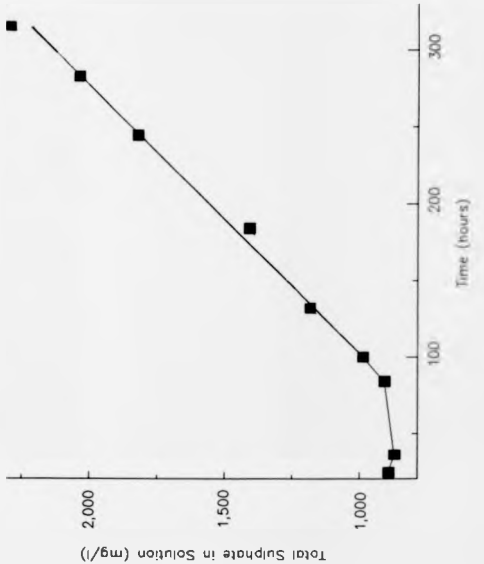


Figure 12. Sulphate production during strain BC1 autotrophic growth on 0.5% elemental sulphur (w/v), at 45°C. The production of sulphate was measured by atomic absorption spectrophotometry.

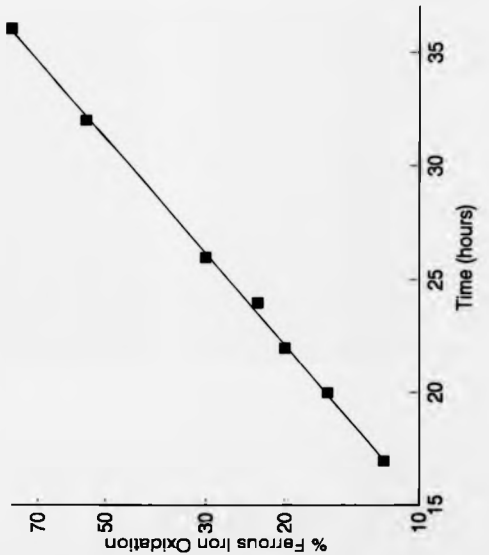


Figure 11. Growth curve of strain ALV grown autotrophically on 50 mM ferrous iron, at 45°C. The production of ferric iron was measured by the ceric sulphate assay.

mesophiles.

Unlike strain BC1, strain ALV will readily oxidise elemental sulphur. The parameter for the curve is once again the production of sulphate ions (Figure 14). This curve gave a sulphate production rate of 91.4 mg/l/hour, which was a little slower than the rate of *T.ferrooxidans*.

1.2.3. Strain BC13.

Strain BC13 is incapable of oxidising either ferrous iron or pyrite but is a rapid oxidiser of elemental sulphur. The sulphate production rate of 245.8 mg/l/hour was the fastest rate of all the organisms looked at in this study (Figure 15).

DISCUSSION.

The doubling times and solubilisation rates given here show good agreement with those already published, particularly for *T.ferrooxidans* for which there are many values published. However, as has already been discussed, the growth rate of *T.ferrooxidans* will depend on the interactions of many of the parameters affecting the oxidation of the substrate in question, as well as there being strain dependent factors. Ahonen and Tuovinen's (1989a) study on the effect of temperature on iron oxidation by a mixed culture gave a doubling time of 6.9 hours at 28^{o}C, reasonably close to the value given here for

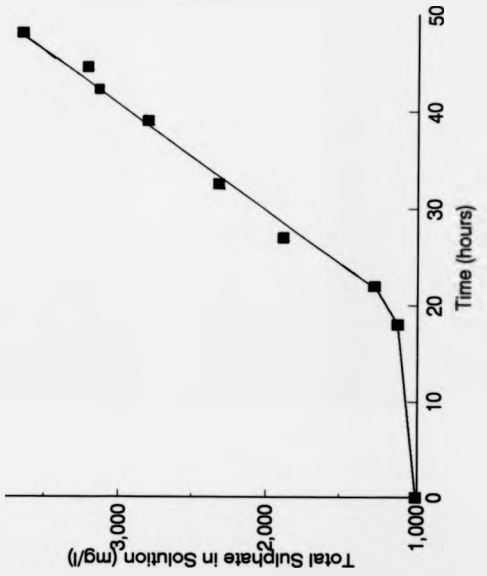


Figure 14. Sulphate production during strain ALV autotrophic growth on 0.5% elemental sulphur (w/v), at 45°C. The production of sulphate was measured by atomic absorption spectrophotometry.

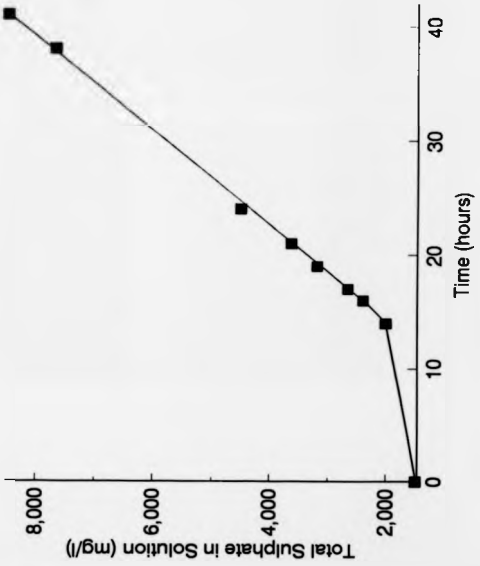


Figure 15. Sulphate production during strain BC13 growth on 0.5% elemental sulphur, at 45°C. The production of sulphate was measured by atomic absorption spectrophotometry.

T.ferrooxidans of 8.5 hours. Their value at 28°C was the optimum. At 4°C, the average doubling time was 63.9 hours, whereas at 37°C the rate had dropped slightly to 9.1 hours. The authors suggested that the majority of the organisms in their cultures were *T.ferrooxidans*. Huber and Stetter (1990) gave a doubling time for *T.ferrooxidans* of 4.5 hours at 36°C, twice the rate of Ahonen and Tuovinen's mixed culture at the same temperature. However, the fact that Huber and Stetter's organism was in pure culture is unlikely to be the explanation, since the strain used in the present study could not reach this rate of growth at this temperature. It is more likely to be a strain effect.

The doubling time on ferrous iron for *L.ferrooxidans* at 30°C, 11 hours, was different from that demonstrated by Norris *et al.* (1988) of just under fourteen hours.

The doubling time of the moderate thermophiles on ferrous iron is complicated by their nutritional diversity. The effect of different carbon sources has already been mentioned, but Norris and Barr (1985) have also demonstrated an effect due to the concentration of the tetrathionate, particularly with strain BC1. Under conditions otherwise identical to those in this study, the doubling time of strain BC1 supplemented with 100 μM tetrathionate was approximately 14 hours, whereas with 500 μM it was a little over 7 hours. In the present study, where the concentration of tetrathionate routinely used was 200 μM, the doubling time achieved was 6.3 hours, slightly faster than that achieved by Norris and Barr. Wood and Kelly (1983) have

published a figure of 12 hours for strain BC1 under the same conditions except that sodium hydrogen carbonate was used to supply the CO_2.

The rate of production of sulphate during growth on elemental sulphur gave an indication of the relative ability of the organisms to grow on sulphur. It highlighted the fact that strain BC1 had great difficulty growing autotrophically on sulphur and the fact that BC13 grew more readily than the other organisms tested. Perhaps a better method of determining the relative ease with which these organisms will grow on elemental sulphur is to examine their doubling times. The doubling time of *T.ferrooxidans* on elemental sulphur has been published as 10 hours (Norris *et al.*, 1986). Data on the moderate thermophile's growth on sulphur was given in the same paper and showed a doubling time of approximately 13 hours for strain ALV and 8 hours for strain BC13, again demonstrating that strain BC13 is capable of the quickest growth on elemental sulphur. There are no previously published figures for the autotrophic growth of strain BC1 on elemental sulphur, presumably because of the difficulty in growing the organism in this way, and its very slow growth rate.

T.ferrooxidans demonstrated an iron solubilisation rate on pyrite of 7.6 mg/l/hour, which is considerably faster than that obtained from data given by Vuorinen and Tuovinen (1987) of approximately 4 mg/l/hour. Since the organism was grown under similar conditions to those used in the present study it may be that this difference is again due to strain

differences. In a recent interlaboratory comparison (Olsen, 1991) it was demonstrated that where leaching conditions are constant and the same strain of *T.ferrooxidans* used, different laboratories obtain results closer together than these two results. Although a direct comparison between the solubilisation rates obtained in Olsen's work and that obtained in this study would be invalid due to the different conditions used, it is interesting to note that the majority of the rates fell between 10 and 18 mg/l/hour.

The iron solubilisation rate for *L.ferrooxidans* on pyrite proved to be slower, at 6.1 mg/l/hour, than the other organisms, although it must be added that in later experiments the organism did appear to grow at a faster rate, although accurate readings were not taken in these later cases. There is a large difference between *L.ferrooxidans* and *T.ferrooxidans* in the proportions of bacteria that become attached to mineral during pyrite leaching - apparently 87% of *L.ferrooxidans* becomes attached to the mineral, compared with only 7% of the *T.ferrooxidans* (Norris *et al.*, 1988). If the rate of attachment by *L.ferrooxidans* was slower than *T.ferrooxidans* this may account for some of the difference in the rate of leaching. However, there is no evidence to suggest that the rate of attachment is in any way significantly different to that of *T.ferrooxidans*.

The iron solubilisation rate of BC1, at 14.3 mg/l/hour, was very quick and demonstrates that at least one of the moderate thermophiles was indeed a quicker solubiliser of

pyrite than the mesophiles.

Because the solubilisation of minerals is a complex process involving an undefined, solid substrate, dual energy source, potentially high levels of inhibition and other important factors, it is worth noting that the rate of iron solubilisation may not reflect the rate of growth of the organisms.

The 'tail-off' of iron oxidation seen with some of the organisms is suggestive of some nutrient requirement but this is not likely to be a common trace element. The effect of tetrathionate on the doubling time of strain BC1 has already been mentioned, but at low levels this can also lead to a 'tailing-off' effect (Norris and Barr, 1985). However the levels added to the cultures are not likely to be limiting to growth. *T.ferrooxidans* has no requirement for tetrathionate anyway and so this cannot be the explanation for this organism. Another likely explanation for this effect is that the ferric iron being produced is inhibiting the oxidation of the ferrous form. The situation is complicated by the fact that it has proved possible to grow the organisms in the past, under the same conditions, without this effect.

2.0. OXYGEN UPTAKE RATES OF MESOPHILES AND MODERATE THERMOPHILES ON FERROUS IRON AND SULPHUR.

A comparison of the oxidation capabilities of several organisms was undertaken, by investigating the effect of growth substrate history on the abilities of the organisms to oxidise ferrous iron or sulphur. To this end the organism in question was grown on ferrous iron or elemental sulphur and then harvested. These cells were then washed and used in a Warburg Respirometer, where their ability to oxidise ferrous iron or elemental sulphur could be evaluated. Specific rates of oxidation were then worked out after the protein content of the cells added to the respirometer had been calculated.

2.1. Mesophilic Oxygen Uptake Rates.

T.ferrooxidans was the only mesophilic organism capable of oxidising both ferrous iron and sulphur. Because of this it was the only mesophile studied in this way.

2.1.1. Thiobacillus ferrooxidans.

When grown with iron as the only available energy source, the ferrous iron oxidation rate was faster than the oxidation rate of elemental sulphur (Figure 16).

The oxygen uptake on ferrous iron ceased after 55 minutes, at about 160 μl oxygen, but this was not presumably

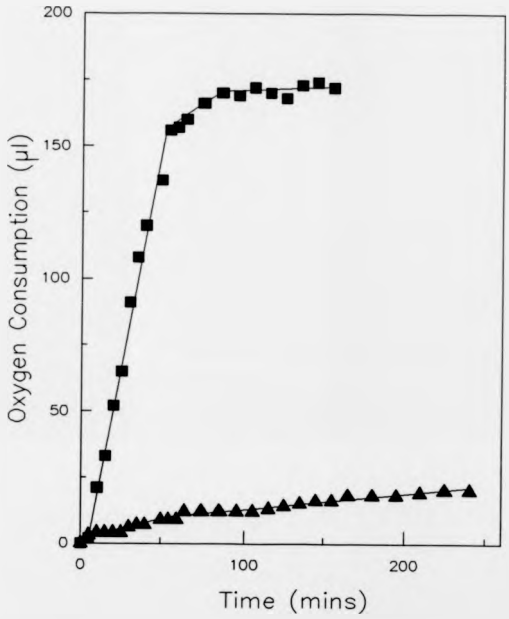


Figure 16. The oxygen uptake of a washed cell suspension of ferrous iron-grown *T.ferrooxidans* at 30°C on 25 mM ferrous iron (■) and 5% elemental sulphur (w/v) (▲), as determined by Warburg respirometry.

due to the exhaustion of the substrate, since the theoretical oxygen uptake required to fully oxidise 20mM ferrous iron is 243.2 µl.

The oxygen uptake on elemental sulphur was still rising at the end of the experiment, and had only reached 20 µl.

When the organism was grown on elemental sulphur the iron oxidation rate was comparatively much slower, but the elemental sulphur rate was much improved (Figure 17).

The ferrous iron oxygen uptake levels off after 170 minutes. As before this oxidation ceased after about 160 µl of oxygen had been consumed.

The increase in the ability to oxidise elemental sulphur when grown on elemental sulphur was clearly demonstrated. At the end of the experiment 130 µl of oxygen had been consumed.

2.2. Moderate Thermophile Oxygen Uptake Rates.

Several of the available strains were investigated in this way. They showed varying patterns in their response to the presence of ferrous iron and sulphur.

2.2.1. Strain BC1.

When the cells were grown on ferrous iron, the oxygen uptake rate in the presence of ferrous iron was rapid but the elemental sulphur oxygen uptake rate was extremely low (Figure 18).

On ferrous iron the uptake was beginning to slow after

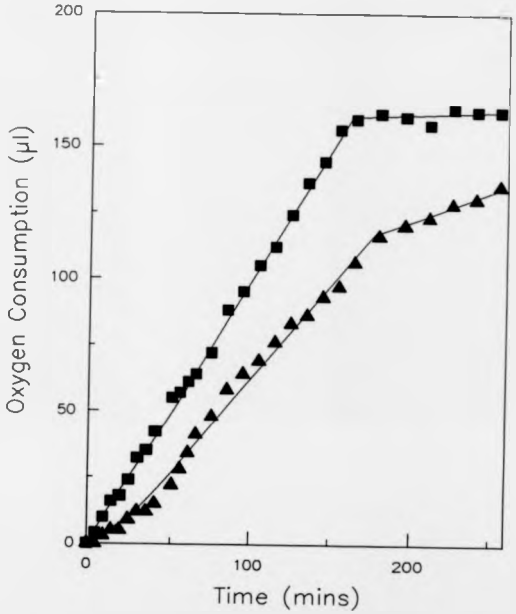


Figure 17. The oxygen uptake of a washed cell suspension of elemental sulphur-grown *T.ferrooxidans* at 30°C on 25 mM ferrous iron (■) and 5% elemental sulphur (w/v) (▲), as determined by Warburg respirometry.

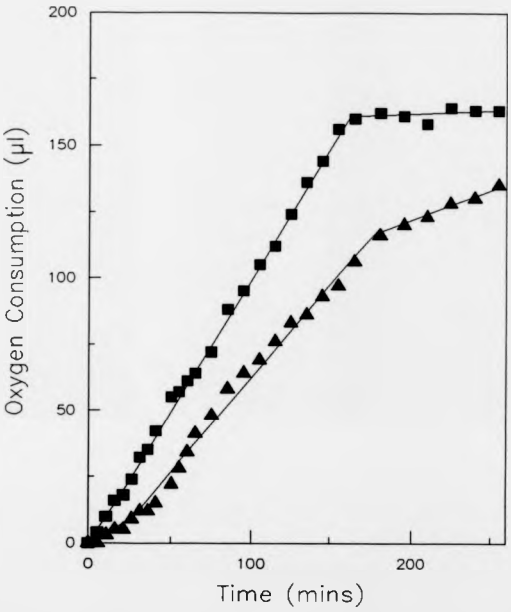


Figure 17. The oxygen uptake of a washed cell suspension of elemental sulphur-grown *T.ferrooxidans* at 30°C on 25 mM ferrous iron (■) and 5% elemental sulphur (w/v) (▲), as determined by Warburg respirometry.

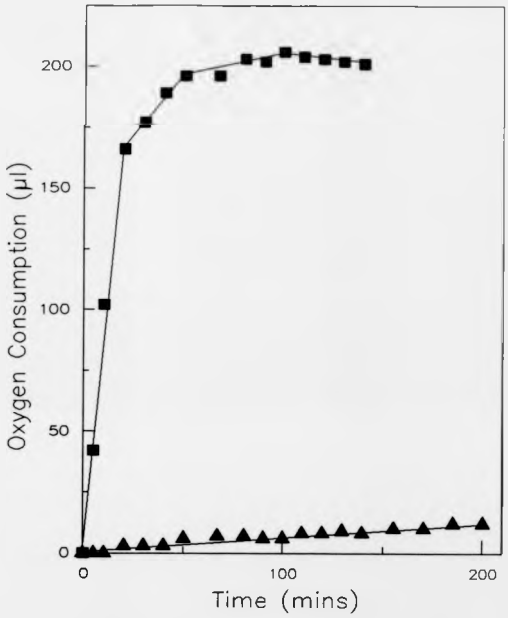


Figure 18. The oxygen uptake of a washed cell suspension of ferrous iron-grown strain BC1 at 45°C on 25 mM ferrous iron (■) and 5% elemental sulphur (w/v) (▲), as determined by Warburg respirometry.

only twenty minutes, and by fifty minutes the uptake had completely ceased. Although the level of oxygen consumption was lower again than the theoretical maximum, it was higher than that observed in *T.ferrooxidans*.

The uptake on elemental sulphur was very low, much lower than for *T.ferrooxidans* in the same situation. After three hours only 12 µl of oxygen had been consumed.

When this organism was grown on elemental sulphur, it remained a very efficient ferrous iron oxidiser although its elemental sulphur oxidation rate was still very poor (Figure 19).

As with the iron-grown cells the oxygen uptake on ferrous iron was over after only forty minutes and there was little difference in the initial rate. This was the opposite to the pattern observed in *T.ferrooxidans*, where growth on elemental sulphur reduced the oxygen uptake on ferrous iron significantly.

The oxygen uptake on elemental sulphur was relatively unchanged compared to to the oxygen uptake on sulphur by cells grown on ferrous iron. At the end of the experiment, only 15 µl of oxygen had been consumed, compared to 12 µl when the organism was grown on ferrous iron. Again this was different to the pattern observed in *T.ferrooxidans*, where growth on elemental sulphur increased the oxygen uptake on sulphur by a significant degree, but lowered the uptake on ferrous iron.

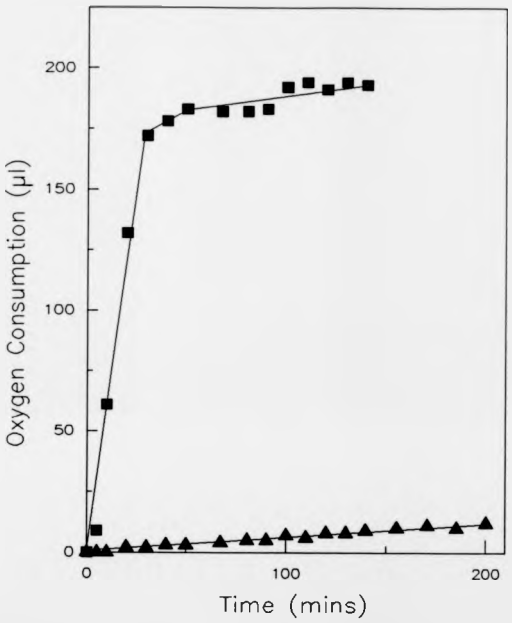


Figure 19. The oxygen uptake of a washed cell suspension of elemental sulphur-grown strain BC1 at 45°C on 25 mM ferrous iron (■) and 5% elemental sulphur (w/v) (▲), as determined by Warburg respirometry.

2.2.2. Strain ALV.

When grown on ferrous iron this strain showed a rapid oxygen uptake on ferrous and the highest oxygen uptake on elemental sulphur of any of the iron-grown organisms studied (Figure 20).

The oxygen consumption on ferrous iron ceased within 45 minutes, and reached a level of 210 µl. This level of consumption was the nearest to the theoretical maximum observed with any of the organisms.

At the end of the experimental period, the oxygen consumption on elemental sulphur was the highest of any of the iron-oxidising organisms studied, having reached 35 µl of oxygen.

Growth on elemental sulphur affected both the oxygen uptake on elemental sulphur and ferrous iron, although not as much as the effects seen in *T.ferrooxidans* (Figure 21).

The final oxygen consumption on ferrous iron was virtually unchanged. However the time taken to reach this level was increased to nearly 100 minutes. This slowing down was not as great as the reduction in *T.ferrooxidans*, but was very different to the pattern seen in strain BC1.

The oxygen consumption in the presence of elemental sulphur was increased by nearly three times, to 90 µl at the end of the experiment. This increase was greater than that seen in strain BC1 but not as high as the increase seen in *T.ferrooxidans*.

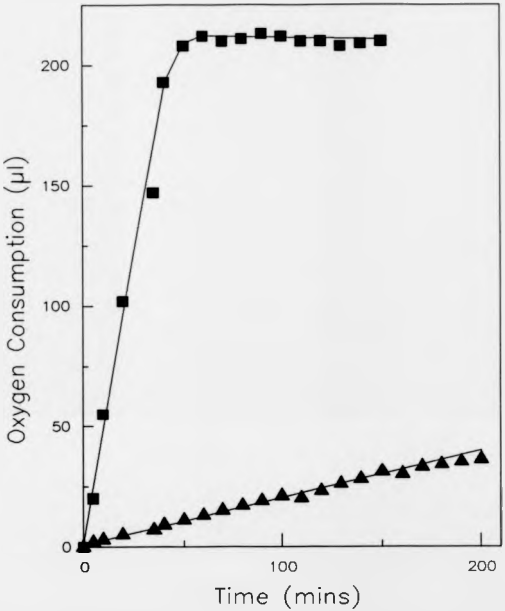


Figure 20. The oxygen uptake of a washed cell suspension of ferrous iron-grown strain ALV at 45°C on 25 mM ferrous iron (■) and 5% elemental sulphur (w/v) (▲), as determined by Warburg respirometry.

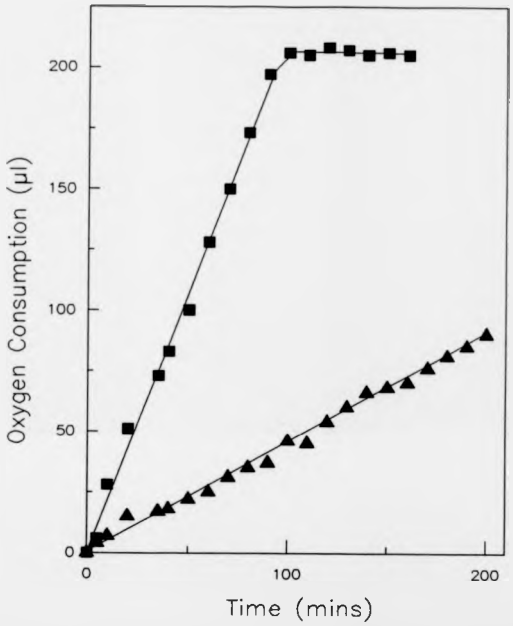


Figure 21. The oxygen uptake of a washed cell suspension of elemental sulphur-grown strain ALV at 45°C on 25 mM ferrous iron (■) and 5% elemental sulphur (w/v) (▲), as determined by Warburg respirometry.

2.2.3. Strain LM2.

When this strain was grown with ferrous iron as the energy source the oxygen uptake on ferrous iron reached its maximum within 35 minutes, this maximum being 195 µl oxygen (Figure 22). Its uptake on elemental sulphur was, however, very low, only 12 µl at the experiment's end.

This response to the substrates after growth on iron was very similar to strain BC1 and *T.ferrooxidans*, but the response after growth on elemental sulphur was somewhat different (Figure 23). The oxygen consumption on ferrous iron was much lowered. It was the only organism in which oxygen consumption never reached a maximum in the period of the experiment, having only reached 140 µl after three hours. The oxygen consumption on sulphur showed a slight improvement, from 12 µl to 39 µl, although nowhere near the increase demonstrated by *T.ferrooxidans*.

2.2.4. Strain BC13.

This strain is an very fast growing organism on elemental sulphur compared to the other sulphur oxidisers used in this study, and the oxygen uptake values reflected this (Figure 24). Within 180 minutes this organism had consumed over 500 µl of oxygen. This was far higher than any of the other organisms considered.

2.3. Specific Oxygen Uptake Rates.

Rates of oxidation were calculated from the Warburg

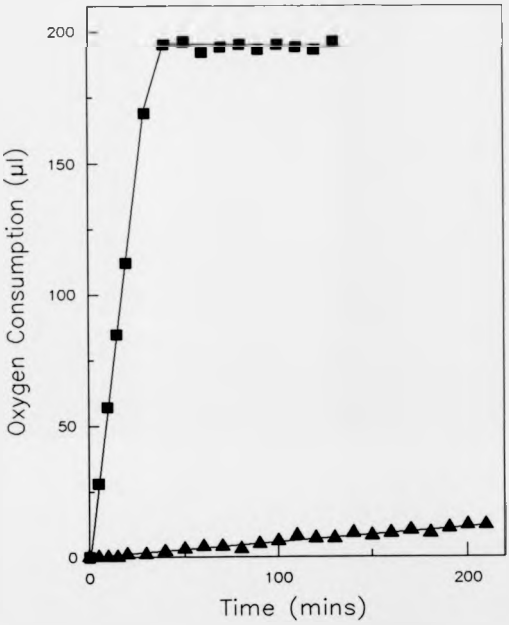


Figure 22. The oxygen uptake of a washed cell suspension of ferrous iron-grown strain LM2 at 45°C on 25 mM ferrous iron (■) and 5% elemental sulphur (w/v) (▲), as determined by Warburg respirometry.

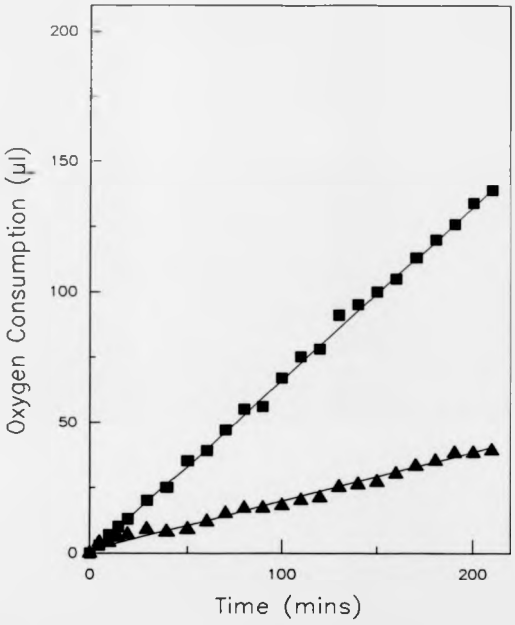


Figure 23. The oxygen uptake of a washed cell suspension of elemental sulphur-grown strain LM2 at 45°C on 25 mM ferrous iron (■) and 5% elemental sulphur (w/v) (▲), as determined by Warburg respirometry.

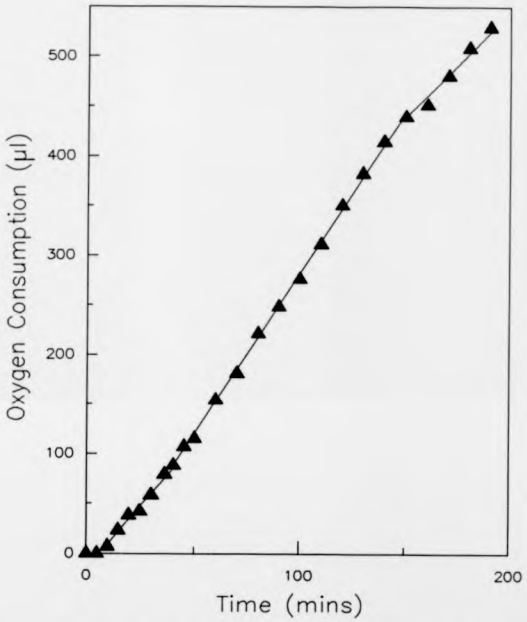


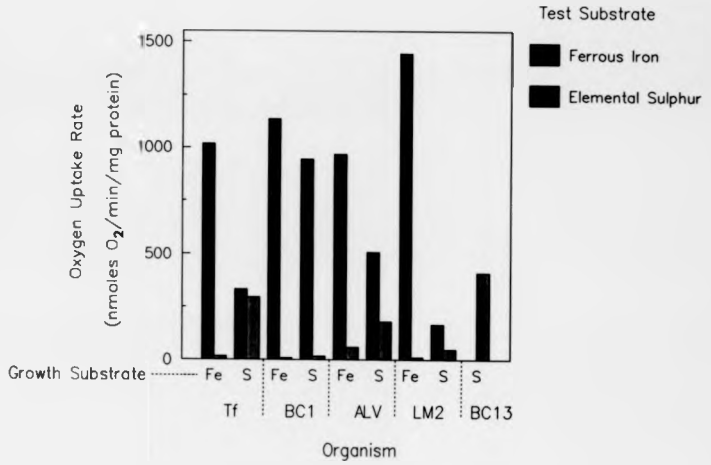
Figure 24. The oxygen uptake of a washed cell suspension of strain BC13 at 45°C on 5% elemental sulphur (w/v), as determined by Warburg respirometry.

results and the protein concentration in the flasks calculated. From this information the specific rates of oxygen consumption were calculated and expressed in a histogram (Figure 25). This chart clearly demonstrates some large differences in the response of the organisms to growth on elemental sulphur.

The response of the mesophile *T.ferrooxidans* to growth on iron or sulphur was to enhance its oxidation capabilities of the growth substrate at the expense of its oxidation capabilities of the other substrate. So, for example, when the organism was grown on ferrous iron its rate of iron oxidation was three times greater but its sulphur oxidation rate was 18 times slower compared to the organism grown on sulphur. Whilst it was always capable of oxidising both substrates its rates of ferrous iron and elemental sulphur oxidation were both affected the growth substrates. Overall its maximum ferrous iron oxidation rate compared favourably with the maximum rates of strains BC1 and ALV. Strain LM2 did have a 40% higher rate than *T.ferrooxidans*. (1442 nmoles O_2/min/mg protein for strain LM2, compared to 1011 nmoles O_2/min/mg protein in *T.ferrooxidans*' case.) Of those organisms capable of oxidising both substrates, *T.ferrooxidans* had the best sulphur oxidation rate, although this was slower than the obligate sulphur oxidiser, strain BC13.

The pattern of response for strain BC1 was different. There was little reduction in the iron oxidation rate whether the organism was grown on ferrous iron or elemental

Figure 25. The specific oxygen uptake rates of *T.ferrooxidans*, and strains BC1, ALV, LM2 and BC13. The oxygen uptake rates of washed cell suspensions of iron- or sulphur-grown cells were determined in the presence of 25 mM ferrous iron and 5% (w/v) elemental sulphur by Warburg respirometry.


Test Substrate
Ferrous Iron
Elemental Sulphur
Oxygen Uptake Rate
(nmoles O_2/min/mg protein)
1500
1000
500
0
Growth Substrate
Fe S Fe S Fe S Fe S S
Tf BC1 ALV LM2 BC13
Organism

sulphur. The drop between the two rates of iron oxidation was only 17%, (from 1135 nmoles O_2/min/mg protein to 947 nmoles O_2/min/mg protein), whereas the drop in the rate was 67% in the case of *T.ferrooxidans*. It appears that this organism is an efficient iron oxidiser whether it is grown on iron or sulphur. A similar pattern emerges when the sulphur oxidation rates are examined, in that they differ very little whether the organism is grown on iron or sulphur.

When grown on ferrous iron, strain ALV had a similar oxygen uptake rate on ferrous iron to both *T.ferrooxidans* and strain BC1. However, when this organism was grown on elemental sulphur its rate of iron oxidation was reduced far more than the iron oxidation rate in strain BC1. This reduction in rate was not as great a reduction as occurs in *T.ferrooxidans*. Strain ALV also appeared to be in between the two organisms with respect to its sulphur oxidation rates. This rate was increased from 57.4 nmoles O_2/min/mg protein to 183.5 nmoles O_2/min/mg protein when the organism was grown on elemental sulphur. Although this increase was greater than for BC1 it is not as large an increase as observed in the sulphur oxidation rates of *T.ferrooxidans*.

The most immediately obvious point about the oxidation rates of strain LM2 was that its maximum iron oxidation rate was the best of all the organisms studied. As would be expected this rate occurred when the organism was grown on ferrous iron. The sulphur oxidation rate was much reduced in response to growth on ferrous iron, down to the level of

iron-grown strain BC1. It was in its response to growth on elemental sulphur that strain LM2 differed most from the other organisms tested. Although the sulphur rate was still fairly slow it did represent a seven-fold increase in uptake rate in response to growth on sulphur.

As might be expected the obligate sulphur-oxidising strain BC13 had the fastest sulphur oxidation rate of all the organisms tested, although the rate of sulphur-grown *T.ferrooxidans* was still fairly close. (336 nmoles O_2/min/mg protein compared to strain BC13's rate of 410 nmoles O_2/min/mg protein.)

DISCUSSION

In an initial paper on the oxidation abilities of *T.ferrooxidans*, Warburg experiments demonstrated similar results (Beck, 1960) to those presented here. However the concentration of the cells is given as mg cellular nitrogen/ml, which prevents accurate comparison. Beck also found that the consumption of oxygen ceased below the theoretical level, for example only 252 µl of oxygen was consumed in a flask containing 50 mM ferrous iron, whereas the theoretical value would be 608 µl. A similar effect also happened with 100 mM ferrous iron. At this concentration the amount of cells added was varied and it was found that this made no difference to the final oxygen uptake - even with three times the number of cells the final consumption only

increased from 514 to 523 µl, when the theoretical maximum is 1216 µl. The rate of uptake was decreased by decreasing the protein concentration but not the final oxygen consumption. The results in the present study came closer to the theoretical values. It would appear that the explanation for this lower than expected oxygen consumption is not due to substrate limitation at the low concentrations in the final stages of the experiment. Neither is it likely to be due to the inhibition effects of ferric iron since it would appear from Beck's work that tripling the number of cells has no effect on the final uptake values.

This set of results shows a large amount of variation between the organisms capable of iron and sulphur oxidation. Not only are their growth and solubilisation rates on various substrates different, but more significantly, their oxidation capacities are very different. This is particularly highlighted by the specific oxygen uptake rates. The intention of this particular investigation was to study the effect of growth history on the organisms' oxidation of ferrous iron or sulphur. Several different patterns of response were evidenced by the organisms tested. Throughout the discussion in the rest of this thesis the term constitutive is taken to mean a system whose activity is altered little or not at all by a given substrate.

The nature of the iron and sulphur oxidation systems of *T.ferrooxidans* was much as was expected from observations of its growth and solubilisation rates and earlier work on its oxidation capacities. It appears that the iron and sulphur

oxidation systems are enhanced by growth on the appropriate substrate and that growth on one substrate greatly alters the ability of the organism to oxidise the other. However the fact that low levels of oxidation of either substrate are always possible means that the organism must always be expressing the necessary systems at very low levels. The work of Sugio *et al.* (1985 - 1991) on the FIR system and its role in sulphur oxidation is not supported here. They have suggested that the iron oxidation system in *T.ferrooxidans* is vital in the oxidation of elemental sulphur and that during growth on sulphur the iron oxidation system is induced because of this. If this were the case it is not likely that the ability of the organism to oxidise ferrous iron when grown on sulphur would be reduced by over 65%.

Apart from being a slightly faster ferrous iron oxidiser and much poorer sulphur oxidiser, strain BC1's main difference to *T.ferrooxidans* is in the response of its iron oxidation system to growth on sulphur. The drop in rate of iron oxidation of only 16.5% is highly suggestive of a mainly constitutive system. Although the sulphur oxidation system is not much affected by growth on ferrous iron, and so would also appear to be constitutive, this system as a whole is very poor. It is hardly surprising that this strain grows so poorly on sulphur with such a comparatively poor oxidation rate. This poor sulphur oxidation is particularly interesting in the light of the suggestion that, in the case of *T.ferrooxidans*, the better sulphur-oxidising strains are better chalcopyrite solubilisers (Groudev, 1985). Strain BC1

is not only a faster pyrite oxidiser, as evidenced by the iron solubilisation rate, it is also a faster chalcopyrite oxidiser (data not shown). This may suggest a different method of oxidation or degree of sensitivity. In the light of the suggestion by Norris (1988) that higher temperatures can prevent the formation of a passivation layer on the mineral, it may be that the lack of an efficient sulphur oxidation system is not a handicap to strain BC1, since it would not require an efficient sulphur-oxidising system to remove the elemental sulphur coating the mineral.

The oxidation systems of strain ALV are not as affected by growth substrate history as *T.ferrooxidans*, and yet are more influenced than those of strain BC1. It would appear that whilst some increase in activity of the appropriate oxidation system occurs during growth on a particular substrate, the activity of the 'redundant' oxidation system is not reduced to the extent seen in *T.ferrooxidans*. These results may seem to indicate that this organism should grow well on pyrite, since it has reasonable rates of both iron and sulphur oxidation. However this serves to highlight the complex situation that exists during mineral oxidation, because this organism's low ferrous iron affinity and low tolerance for ferric iron means that it grows very poorly on pyrite.

Strain LM2 shows a similar pattern of response to *T.ferrooxidans* in that both its iron and sulphur oxidation systems appear to be enhanced by growth on the appropriate substrate. Growth on ferrous iron causes a nearly nine-fold

increase in the rate of iron oxidation in comparison to growth on sulphur, whereas there is a seven-fold increase in sulphur oxidation when the organism is grown on sulphur.

There does appear to be some correlation between the specific iron oxidation rates and ferrous iron doubling times, and the sulphur oxidation rates and the rates of sulphate production from elemental sulphur. Iron-grown *T.ferrooxidans*, strain BC1 and strain ALV all have doubling times on ferrous iron lying between about 6 and 8 hours. Their specific oxidation rates from the same growth history all lie in the region of 1000 - 1100 nmoles O_2/min/mg protein. The slowest rate of sulphate production during elemental sulphur growth was that of strain BC1, which also had the slowest sulphur oxidation rate, only 14 nmoles O_2/min/mg protein. However, the situation is not as simple for the other sulphur oxidation results. The sulphate production rate of sulphur-grown *T.ferrooxidans* and strain ALV on sulphur were fairly similar, being 121.2 mg/l/hour and 91.4 mg/l/hour respectively. Their sulphur oxidation rates are, however, quite different. *T.ferrooxidans*' rate was nearly double that of strain ALV - 336 nmoles O_2/min/mg protein compared to 183 nmoles O_2/min/mg protein.

Although there does appear to be a correlation between iron oxidation rate and doubling times, there does not seem to be such a correlation between affinity and oxidation rate. The iron oxidation rates for the organisms discussed above lie in the area 1000 - 1100 nmoles O_2/min/mg protein, yet whilst the apparent affinities for ferrous iron of

T.ferrooxidans and strain BC1 are fairly similar, 1.34 mM and 1.04 mM respectively, that of strain ALV is quite different, being 2.96 mM (Norris *et al.*, 1988).

The diversity of the organisms found in leaching environments is thus obvious from even such basic studies. In particular, the expression of the iron- and sulphur-oxidising systems shows several different patterns, from the systems of *T.ferrooxidans*, whose activities are very much affected by growth substrate, to the system of strain BC1, which is apparently a basically constitutive system. The rates of sulphur and iron oxidation also show a great variety, but the relationship between growth rates, solubilisation rates, substrate affinity and oxidation rates is not clear, particularly with respect to the solid substrates where other factors such as the role of attachment may have an important role.

CHAPTER FOUR.

MESOPHILE AND MODERATE THERMOPHILE MIXED CULTURE LEACHING.

1.0. SHAKE FLASK LEACHING.

Initial experiments on the leaching capabilities of the moderate thermophiles were carried out using shake flask cultures. In tandem with these, mesophiles were also investigated in order to provide a direct comparison of leaching activity with that of the better known organisms. In any commercial application for the mineral-oxidising acidophiles, the culture is likely to be a mixed culture, since the mineral, nutrients and reactors will not be sterilised prior to use. It is therefore important that the interactions that occur between organisms in this environment be studied. Accordingly much of this work concentrated on mixed culture leaching.

1.1. Pure Culture Leaching of Pyrite.

Both the moderate thermophiles and the mesophiles were first investigated as pure cultures to provide the comparison for the later mixed culture leaching. This work

involved autotrophically-grown cultures in shake flasks with the appropriate conditions for the organism involved, as described in materials and methods. All cultures were inoculated from pyrite medium.

1.1.1. Thiobacillus ferrooxidans.

This organism showed a typical leaching pattern (Figure 26). The lag phase lasted approximately twenty four hours, after which the rate of leaching, measured as a function of iron release, reached 12.3 mg/l/h. The rate began to level off after about 170 hours and reached a maximum yield of 2700 mg/l. The same lag phase occurred before sulphate release, but the levelling off of the sulphate production did not occur until after approximately 190 hours. The maximum rate of sulphate production was 41 mg/l/h and the final yield approximately 8000 mg/l. The final pH of the solution was 1.4, a drop of 0.6 pH from the original pH. Within a week the bacteria were numerous, motile and occurred as single cells only. Their appearance was similar after two weeks.

1.1.2. Leptospirillum ferrooxidans.

The lag phase before leaching with this organism was the longest of all the organisms, lasting approximately 80 hours (Figure 27). After this the leaching rate was not high, never going above 2.5 mg/l/h. There was no levelling off of the rate before the experiment was finished, after 220 hours. The final pH at this time was 1.5, a drop of 0.5.

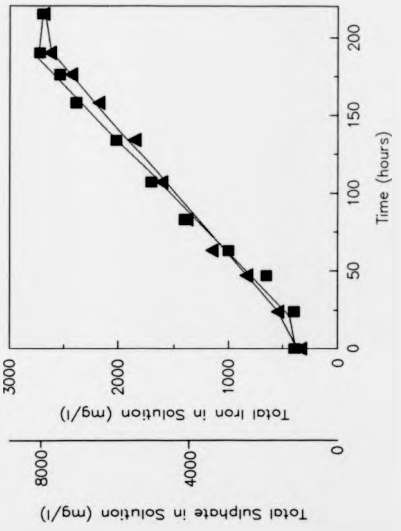


Figure 26. The leaching of pyrite by *T.ferrooxidans*. The production of soluble iron (■) and sulphate (▲) was assayed by atomic absorption spectrophotometry. The organism was grown on 1% pyrite at 30°C.

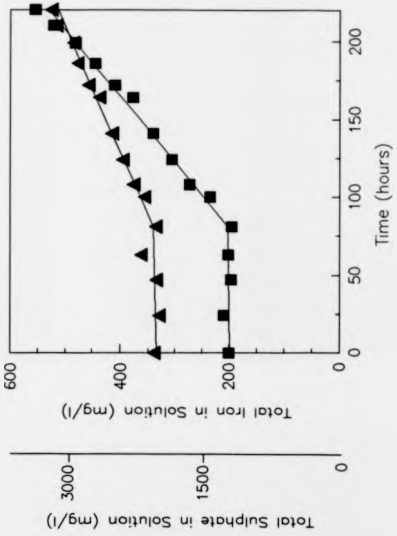


Figure 27. The leaching of pyrite by *L.ferrooxidans*. The production of soluble iron (■) and sulphate (▲) was assayed by atomic absorption spectrophotometry. The organism was grown on 1% pyrite at 30°C.

As with *T.ferrooxidans* the sulphate production showed the same lag phase as the iron solubilisation. The rate of production never exceeded 8.5 mg/l/h and again the production did not level off before the end of the experiment. Despite this low leaching rate, the cells looked healthy. A high proportion of *L.ferrooxidans* becomes attached to the mineral, and therefore the number of cells that could be seen by microscopic examination was low. After two weeks the cells were healthy, motile and occurred singly. There was a notable increase in the number of visible cells between the first and second weeks.

1.1.3. Strain BC1.

The leaching curve with BC1 was similar to that of *T.ferrooxidans* (Figure 28). The lag phase was a little shorter, at 16 hours, after which the rate of iron solubilisation reached a maximum of 11.5 mg/l/h, very close to the 12.3 mg/l/h of *T.ferrooxidans*. The final yield of iron in solution after completion of leaching was 2900 mg/l, slightly higher than that with *T.ferrooxidans*. The sulphate concentration was also similar to that of *T.ferrooxidans*. The lag phase appeared to be slightly longer than for iron production; approximately 20 hours. The rate of sulphate production was 38.7 mg/l/h and the final yield was approximately 8100 mg/l. The final pH of the solution was 1.2, a drop of 0.8. Within a week the cells were very numerous. However they were of an unusual morphology; they were elongated to 5 - 7 times longer than when grown on

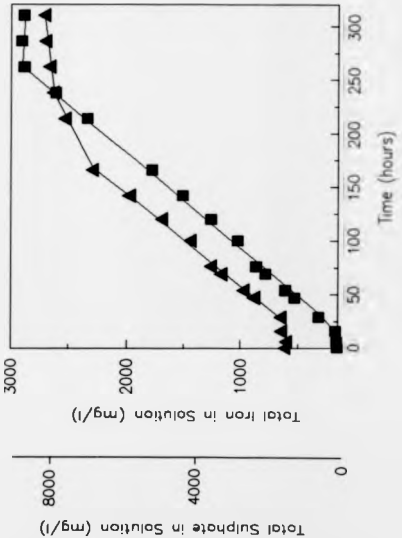


Figure 28. The leaching of pyrite by strain BC1. The production of soluble iron (■) and sulphate (▲) was assayed by atomic absorption spectrophotometry. The organism was grown autotrophically on 1% pyrite at 45°C.

ferrous iron and other samples of pyrite of different origin (data not shown). SDS PAGE analysis confirmed that the culture was still pure and comparison to previous results with the now depleted old batch of mineral showed no significant differences in leaching rates. The cells occurred mainly singly with some pairs and short chains. They were all non-motile. After the second week the cells were the same length and occurred singly and in pairs but with some much longer chains.

1.1.4. Strain BC13.

The leaching graph shows that as was expected there was no iron release above that of the control level (Figure 29). However, sulphate was produced, with a maximum rate of 5.4 mg/l/h. Although BC13 cannot oxidise iron and therefore cannot solubilise pyrite, these results show that it still grew with pyrite as substrate. This was presumably because the organism oxidised some 'free' elemental sulphur that existed in the impure mineral concentrate. Acid production was indicated by the drop in pH, from 2.0 to 1.7 at the close of the experiment. Within a week microscopic examination showed numerous, single, motile cells. After a further week, however, the bacteria looked much less healthy. The cells were less motile, were larger and rounder and also occurred in pairs. The number of cells visible was much reduced.

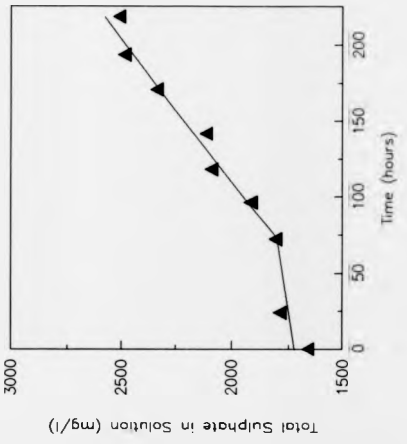


Figure 29. The growth of strain BC13 on pyrite. The production of sulphate (▲) was assayed by atomic absorption spectrophotometry. The organism was grown autotrophically on 1% pyrite at 45°C.

1.2. Mixed Culture Leaching of Pyrite.

The mixed culture leaching involved not just a mixture of iron oxidisers and sulphur oxidisers but also a mixture of mesophiles and moderate thermophiles. For this reason all the experiments were carried out at 35°C, a temperature at which it was expected the oxidation capabilities of the mesophiles would not be adversely affected.

1.2.1. Thiobacillus ferrooxidans and Strain BC13.

It would appear (Figure 30) that the coupling of the sulphur-oxidising strain BC13 to *T.ferrooxidans* actually decreased the ability of the culture to solubilise the mineral. The lag phase of the iron production was about thirty hours, after which the rate increased to a maximum of 6.6 mg/l/h, almost half that of the 12.3 mg/l/h for the pure *T.ferrooxidans* culture (Figure 26). The sulphate production showed a lag of about 24 hours and then the rate of production increased to 20.6 mg/l/h. Again this was much lower than that with *T.ferrooxidans* in pure culture which was 41 mg/l/h. The pH drop was the same as for the pure culture, dropping 0.6 units to pH 1.4. The cells were similar in appearance and therefore a simple visual examination to determine the ratio of the two organisms was not possible. The culture did display some pairs and chains of organisms, neither of which was typical for these species, but these were not visible after the first week.

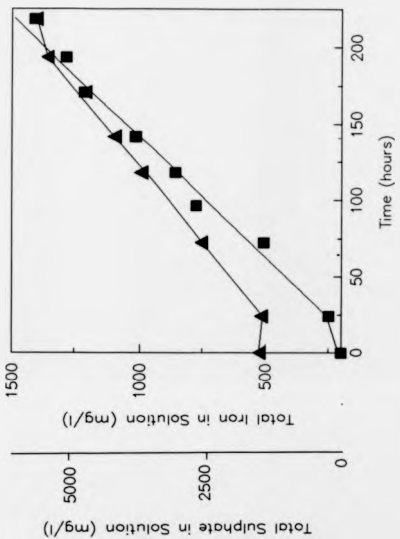


Figure 38. The leaching of pyrite by a mixed culture of *T.ferrooxidans* and strain BC13. The production of soluble iron (■) and sulphate (▲) was assayed by atomic absorption spectrophotometry. The organisms were grown autotrophically on 1% pyrite at 35°C.

1.2.2. Leptospirillum ferrooxidans and Strain BC13.

The lag phase for the production of iron was increased in comparison to that of *L.ferrooxidans* in pure culture (Figure 31). The mixed culture took 100 hours to begin leaching whereas the pure culture took 80 hours. However the final rate of iron solubilisation was increased to 2.7 mg/l/h, an improvement on the *L.ferrooxidans* leaching rate. The rate of sulphate production was also increased, from 8.5 to 10.12 mg/l/h, after a lag phase of approximately 70 hours. The culture pH dropped from 2.0 to 1.4, slightly lower than for *L.ferrooxidans* in pure culture. Since the cells of *L.ferrooxidans* are vibrioid it was possible to distinguish them from the short rods of BC13 but the tendency of the former to attach to the pyrite still precluded an estimate of the ratio of the two organisms in the culture. However, after the first week the *L.ferrooxidans* that were visible were not healthy. After the second week they looked far healthier, and occurred not just singly but also in pairs and chains. The BC13 remained healthy and motile throughout both weeks.

1.2.3. Strain BC1 and Strain BC13.

As with the mix of *T.ferrooxidans* and BC13, the solubilisation of the mineral was apparently slowed by the addition of the sulphur oxidiser (Figure 32). The lag phase was of the same duration but the fastest rate of iron solubilisation reached was only 7.1 mg/l/h, compared to 11.5 mg/l/h for the pure culture. The sulphate production in this

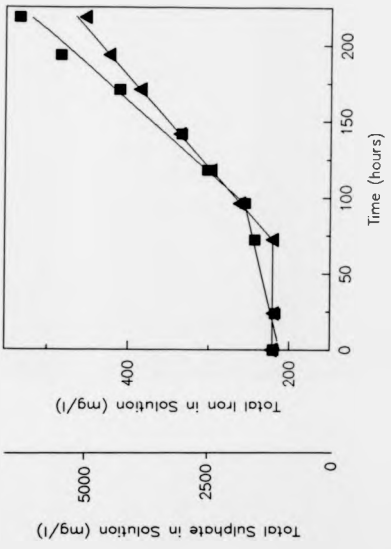


Figure 31. The leaching of pyrite by a mixed culture of *L.ferrooxidans* and strain BC13. The production of soluble iron (■) and sulphate (▲) was assayed by atomic absorption spectrophotometry. The organisms were grown autotrophically on 1% pyrite at 35°C.

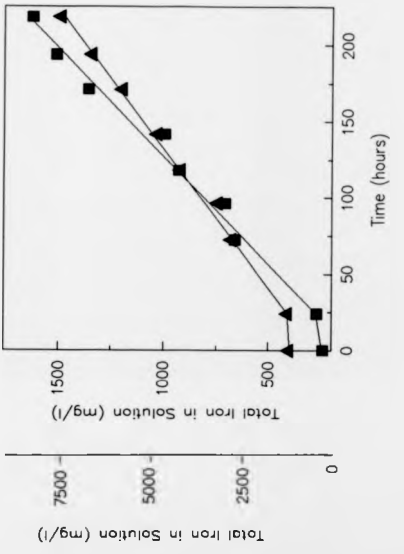


Figure 32. The leaching of pyrite by a mixed culture of strain BC1 and strain BC13. The production of soluble iron (■) and sulphate (▲) was assayed by atomic absorption spectrophotometry. The organisms were grown autotrophically on 1% pyrite at 35°C.

flask followed the same pattern. The lag was the same length as for the iron solubilisation, both in the mixed and pure culture. After the lag the rate of production never exceeded 25.3 mg/l/h, again lower than the pure culture. The pH of the flask at the end of the experiment was 1.4 a drop of 0.6. This final pH is higher than that of BC1 alone. Due to the unusual morphology of BC1 when grown on this pyrite it was possible to distinguish both species easily under the microscope. After a week both species were still present. BC1 accounted for approximately 60% of the culture. At the end of the second week, however, the BC13 easily outnumbered the BC1, and accounted for approximately 75 - 80% of the cells. The BC1 always occurred in the non-motile elongated form. The BC13 remained in its normal healthy form of single highly motile short rods.

2.0. MINERAL LEACHING IN AN AIRLIFT REACTOR

2.1. Mixed Culture Leaching of Chalcopyrite in Shake Flasks.

In order to develop further the investigation into the capabilities of the moderate thermophiles in mixed culture leaching it was decided to use reactors, in the form of air-lift vessels, to more closely simulate an industrial process. To this end it was decided to use the copper-bearing mineral chalcopyrite since pyrite is not a directly

commercially important mineral. In order to establish any differences between shake flask and fermentor it was necessary to first undertake shake flask experiments with chalcopyrite.

2.1.1. Strain BC1.

Strain BC1 showed little or no lag phase (Figure 33). The leaching rate, measured as a function of copper solubilisation, reached a maximum of 1.35 mg/l/h. The final pH of the flask was 1.6, a drop of 0.4 pH units. Throughout the experiment the cells looked healthy, although numbers only slowly increased. They were mainly single cells with some pairs and were all non-motile.

2.1.2. Strain BC13.

As was expected for the non-iron oxidiser, the release of copper from the mineral was never higher than the control (Figure 33). However, this organism was perfectly capable of growing on the mineral, as microscopic examination showed. The drop in pH from 2.0 to 1.7 suggested that the organism was utilising free elemental sulphur in the mineral concentrate. Within four days the culture was better established, at least numerically, than strain BC1. The organisms continued to thrive until the end of the experiment. They were always present as highly motile, short, singular rods.

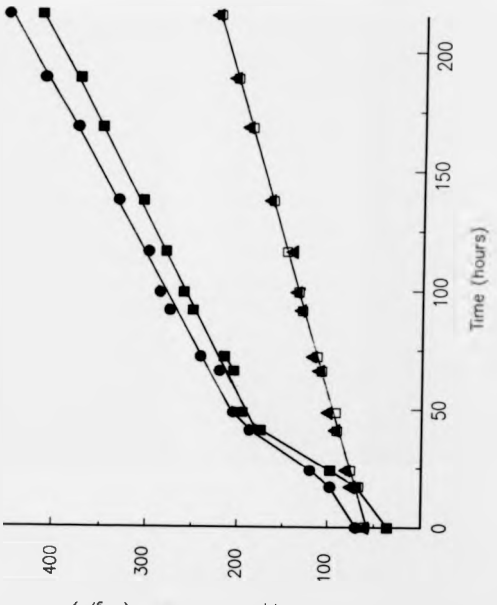


Figure 33. The shake flask leaching of chalcopyrite by strain BC1 (■), strain BC13 (□), and a mixed culture of strain BC1 and strain BC13 (●). The control, (no organism), is also shown (▲). The production of soluble copper was assayed by atomic absorption spectrophotometry. The organisms were grown autotrophically on 1% chalcopyrite at 45°C.

2.1.3. Strain BC1 and Strain BC13.

The mixed culture on chalcopyrite showed a different trend to the mixed cultures on pyrite (Figure 33). The addition of the sulphur oxidiser appeared to have little effect on the rate of leaching. Again there was little or no lag phase. The leaching rate reached 1.41 mg/l/h, only slightly higher than the 1.35 mg/l/h recorded for the pure culture of BC1. The final pH was also the same as the pure culture, pH 1.6. At all times the bacteria were healthy and numerous, but since the cells looked fairly similar, it was not possible to determine the relative numbers of each species.

2.2. Mixed Culture Leaching of Chalcopyrite in Air-Lift Reactors.

The leaching behaviour of the moderate thermophiles BC1 and BC13 were further studied in airlift reactors. This was intended to give a closer approximation to the conditions which might prevail in an industrial process. The chalcopyrite concentration was also increased to 5% (w/v) with this in mind. In order to understand more fully the reactions that occurred in the reactor and the contribution of the bacteria, readings were taken of more parameters than previously. Two reactors were used, one containing a pure culture of BC1 and the second a mixed culture of BC13 and BC1. These reactors were run with 1% mineral for 92 hours to enable the bacteria to become established. The remainder of

the mineral (4%) was then added to the active established cultures in order to avoid the lag phases that tended to result when higher concentrations of mineral were freshly inoculated.

2.2.1. Copper Solubilisation.

Although the BC1 showed a small lag phase after the addition of the mineral, the mixed culture showed none at all (Figure 34). The rate of leaching was similar however. The pure culture maximum rate was 13.04 mg/l/h and that of the mixed culture 14.24 mg/l/h. The yields of copper at the end of the experiment were 1.95 g/l for the strain BC1/BC13 mixture and 1.75 g/l for strain BC1. Since the rates of leaching were so similar, it appears that this difference in yield was due to the lag in production seen with the pure culture. However, the leaching in the BC1 culture probably did not quite reach completion so it is possible that the yield may have reached the same level as the mixed culture, in which case the only advantage of using the mixed culture would be the shorter duration of the process.

2.2.2. pH Changes.

After the addition of the mineral the pH of the pure culture solution increased rapidly (Figure 35). This reduction in acidity peaked after ten hours at pH 2.95, and was then followed by further acidification over the next 140 hours to reach pH 1.83. The mixed culture, however, showed no significant increase in pH throughout the duration of the

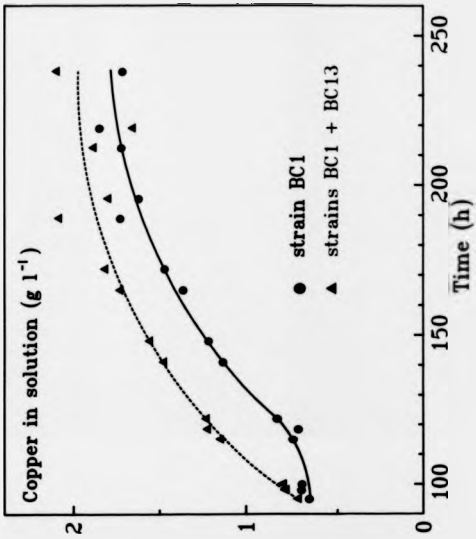


Figure 34. The production of soluble copper during chalcopyrite leaching by strain BC1 (●) and a mixed culture of strain BC1 and strain BC13 (▲) in an airlift reactor containing 5% mineral. Temperature was 45°C and the organisms grown autotrophically. Copper content was assayed by atomic absorption spectrophotometry.

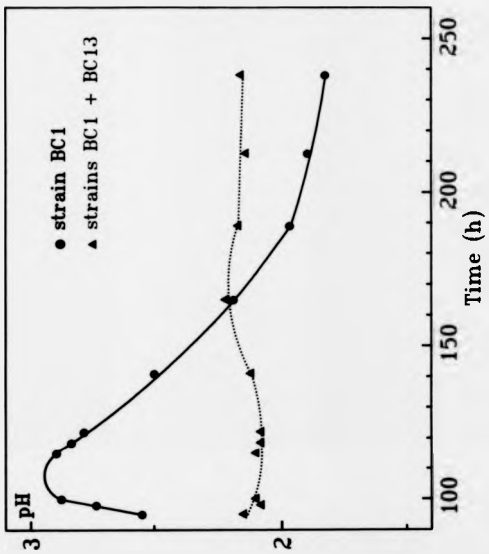


Figure 35. The change in pH during chalcopyrite leaching by strain BC1 (●) and a mixed culture of strain BC1 and strain BC13 (▲) in an airlift reactor containing 5% mineral. Temperature was 45°C and the organisms grown autotrophically.

experiment. The level stayed remarkably constant at around pH 2.1, even after the addition of the mineral. It would appear that the inclusion of BC13 helps maintain a constant pH, although it may be that this buffering effect relates to the pH difference, and therefore buffering capacity , of the two cultures.

2.2.3. Total and Ferrous Iron in Solution.

Unlike the pattern of copper solubilisation the concentration of iron in solution showed marked differences between the two reactors (Figure 36). In the mixed culture there was no lag phase and the soluble iron level quickly increased. The maximum rate of leaching measured in this way was 42.67 mg/l/hour. The yield of total iron in solution peaked at 4.8 g/l after 190 hours. This yield then dropped to 4.25 g/l at the end of the experiment. In the pure culture almost 3 days passed before the appearance of increased iron levels in the solution. Initially the concentration of iron in solution dropped to reach a minimum of 0.3 g/l before increasing to reach a maximum yield of 1.4 g/l.

The concentration of ferrous iron in solution was also very different in the pure and mixed cultures. The BC1 culture demonstrated a slow oxidation of the ferrous iron, such that 45 hours after the addition of the mineral no ferrous iron was detectable. In contrast in the mixed culture the levels of ferrous iron increased after the mineral was added, thus comprising almost all of the iron in

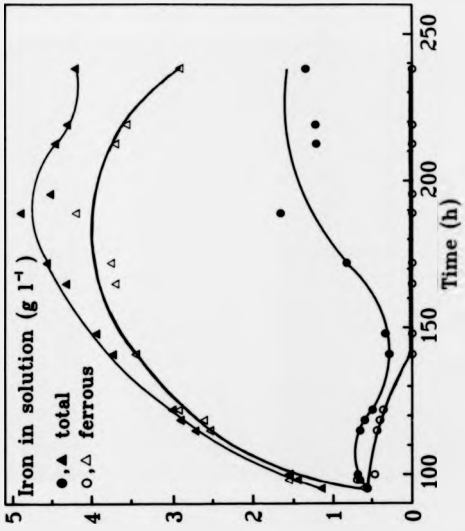


Figure 36. The production of total soluble iron and ferrous iron during chalcopyrite leaching by strain BC1 (● , ○ respectively) and a mixed culture of strain BC1 and strain BC13 (▲ , △ respectively) in an airlift reactor containing 5% mineral. Temperature was 45°C and the organisms grown autotrophically. Soluble iron content was assayed by atomic absorption spectrophotometry, ferrous iron by ceric sulphate assay.

solution. This rate slowed faster than the total iron production rate however, and after 170 hours the levels began to drop again, having peaked at nearly 4 g/l.

2.3. Strain BC1 Iron Oxidation Capacity Towards the Completion of the Chalcopyrite Leaching.

One possible explanation for the high levels of ferrous iron in the mixed culture reactor may have been that strain BC1 somehow lost the capability to oxidise the iron. In order to test this hypothesis cells were removed from both the pure and mixed cultures, washed, and their ability to oxidise iron tested by adding ferrous iron and measuring its rate of oxidation. The results indicated that the iron-oxidation capacity of the mixed culture was indeed significantly reduced (Figure 37). Within seventeen hours the BC1 from the pure culture reactor had removed all the ferrous iron from the flask, at a rate of 2.9 mM/hour. The mixed culture reactor bacteria however had oxidised comparatively little at an oxidation rate of 0.4 mM/hour. An equal volume mix of the two cultures gave a rate of 1.4 mM/hour, almost exactly half way between the two, as would be expected if the cultures had these inherently different iron oxidising capacities rather than that the presence of BC13 was sufficient to inhibit the activity of strain BC1.

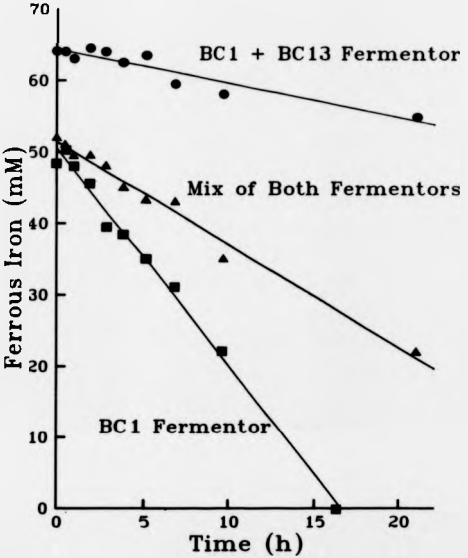


Figure 37. Ferrous iron oxidation by washed cell suspensions of cells from the pure culture strain BC1 reactor (■), the mixed culture strain BC1 and strain BC13 reactor (●), and an equal mix of cells from the two reactors (▲). Ferrous iron was assayed by the ceric sulphate assay. Cultures were incubated at 45°C.

3.0. MIXED CULTURE RESPIROMETRY.

As well as looking at the ability of mixed cultures to solubilise metals, the oxygen uptake of the two moderate thermophile strains BC1 and BC13 were examined whilst they were oxidising minerals, in order to determine whether the mixed culture demonstrated any evidence of interactions. This was done by Warburg Respirometry on washed cell suspensions of approximately equal protein concentration.

3.1. *Pure Culture Oxygen Uptake.*

Both strains were examined in pure culture along with *T.ferrooxidans* to provide a comparison with a well studied mineral-oxidising acidophile. Of the two moderate thermophiles, the rate of oxygen uptake was greater for strain BC13 (Figure 38). After 200 minutes the culture had consumed 345 μl O_2, whereas strain BC1 had only consumed 294 μl O_2. This slower rate of oxidation by strain BC1 is due to the nature of the substrate, i.e. a solid mineral instead of a solution of ferrous iron. The rate of consumption by *T.ferrooxidans* was slower than either of the two moderate thermophiles, and after 200 minutes only 223 μl of oxygen had been consumed.

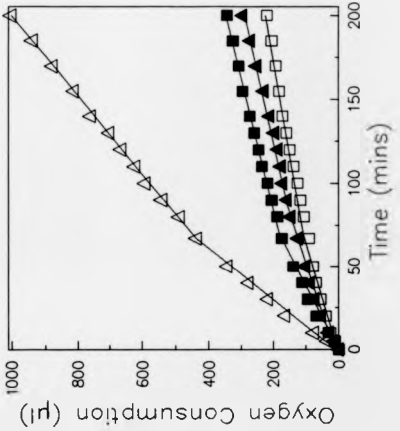


Figure 38. The oxygen uptake of washed cell suspensions of *T.ferrooxidans* (□), strain BC1 (▲), strain BC13 (■) and a mixed culture of strains BC1 and BC13 (△) on 5% pyrite (w/v), as determined by Warburg respirometry.

3.2. Mixed Culture Oxygen Uptake.

The mixed culture flask, containing equal amounts of strains BC1 and BC13 showed an extremely fast oxygen uptake rate (Figure 38). After 200 minutes the volume of oxygen consumed was 1006 µl. This is greater than the total oxygen consumption of the two individual cultures and may indicate some form of chemical or biological interaction between the two strains.

DISCUSSION.

Perhaps the most significant point to make from the results of the pure culture pyrite leaching is that the rates of leaching of *T.ferrooxidans* and strain BC1 are virtually identical. This is in direct contradiction to experiments described earlier, (chap. 3, 1.1.), which showed strain BC1 solubilising the mineral at a much faster rate than *T.ferrooxidans*. This is despite the fact that both experiments were conducted in the same manner. Strain BC1 did give a greater yield and a reduced lag phase but these appear from this experiment to be the only advantages to the use of this strain. Marsh and Norris (1983b) reported that strain BC1 at 37°C had the same leaching rate as *T.ferrooxidans* at 30°C, but an increase of temperature to 45°C gave strain BC1 a much faster rate of pyrite solubilisation. Strain BC1's unusual morphology does

indicate that it was reacting unfavourably to the presence of something in the mineral, but comparisons of its leaching rates on earlier batches of mineral and on the batch used for the majority of this work showed no significant difference in rates or yields. The organism has already been shown to undergo a morphological change in response to growth on yeast extract (Norris *et al.*, 1980) but this has never previously been observed during growth on pyrite.

The fact that the sulphur-oxidising strain BC13 was capable of growth on the minerals used is not really a surprise once the nature of the mineral concentrate is considered. The sulphate levels and reduced pH clearly demonstrated that this organism was oxidising the reduced sulphur which was contained in the concentrate but was not breaking down metal-sulphide bonds of the pyrite or chalcopyrite fractions.

Of the three organisms grown in mixed culture with strain BC13, strain BC1 showed the fastest rate of pyrite solubilisation, followed by *T.ferrooxidans*. However the difference in rates between these two organisms was not great, an increase from 6.6 mg/l/hr to 7.1 mg/l/hr. The addition of the sulphur oxidiser did not appear to have had a large effect on the final pH of the leach solutions when compared to the pure cultures. In the case of the *T.ferrooxidans*/strain BC13 mixture the final pH was the same and with the *L.ferrooxidans*/strain BC13 culture the drop in pH was only 0.1 unit lower than the pure culture. The strain BC1/strain BC13 mix actually shows less of a drop, by 0.2

units, than with the pure strain BC1 culture. However, care must be taken when comparing the pure and mixed cultures, since not only was strain BC13 added for the mixed culture work, but the temperature was also changed. The likely effects of this are discussed below.

The most significant point of these results is that there appeared to be little or no advantage to the addition of a sulphur oxidiser to the iron-oxidising bacteria. Indeed, in the case of the pyrite leaching there was a small reduction in the rate of leaching, except for the *L.ferrooxidans*/BC13 mixture, which showed some improvement. However, in this case the *L.ferrooxidans* was growing particularly poorly and so it is not possible to draw any firm conclusions from this. Unfortunately, because the investigation into pure culture pyrite leaching was undertaken at different temperatures from the investigation into mixed culture pyrite leaching, neither is it possible to draw any firm conclusions on the effect of adding strain BC13 to pure cultures. Norris *et al.* (1986) have published data, in graph form, that show that the doubling time of strain BC13 on sulphur increases from approximately 8 hours at 45°C to 11 hours at 35°C. However, no data is available for its growth rate on pyrite over a range of temperatures. Such data, again in graph form, is available for *T.ferrooxidans* and strain BC1. The iron solubilising doubling time of *T.ferrooxidans* increases from approximately 34 hours at 30°C to approximately 36 hours at 35°C, whilst that of strain BC1 increases from approximately 25 hours at

45°C to approximately 34 hours at 37°C (Marsh and Norris, 1983b). These figures clearly demonstrate that, especially in the case of strain BC1, the change in temperature will have had an effect on the leaching rates. Strain BC1 will have been particularly affected, since this shows the greatest drop in doubling time. The drop in iron solubilisation rate seen in strain BC1 leaching when strain BC13 was added, from 11.5 mg/l/hour to 7.1 mg/l/hour, was therefore more likely to be due to the temperature change.

Other workers have reported benefits from the use of mixed cultures and in particular from the use of a sulphur oxidiser in conjunction with the necessary iron oxidiser, for example Balashova *et al.* (1974) and Norris (1988). As already discussed, the usual explanation for the influence of sulphur oxidisers is that they remove the passivation layer from the surface of the mineral. It has also been hypothesised that elevated temperatures increase the rate of solubilisation partially by the removal of the passivation layer (Norris, 1988).

In those cases where it was possible to tell the two organisms apart care must be taken in interpreting the results. Sulphur is a far better substrate than iron for growth in terms of available energy in the substrate, and it may be that this is all that was reflected in the fact that the BC13 in the cultures always ended up outnumbering the iron oxidiser.

The strain BC1 shaken flask culture with chalcopyrite as the substrate showed several differences in comparison to

the organism grown on pyrite, and these are probably due to the nature of the mineral. The pH drop was not as severe as in the pyrite flask, only being 0.4 units rather than 0.8. Norris *et al.* (1980) reported that during chemoheterotrophic growth of strain TH1 on 1% chalcopyrite (w/v) the pH actually increased slightly (0.01 unit) over the period of the experiment. The pH of the control flask actually increased from pH 2.0 to pH 2.36, demonstrating that as well as bacterial iron oxidation causing an increase in pH, purely chemical reactions can be responsible for this lack of pH drop in the flasks.

The pure culture of BC13 in the shake flasks once again demonstrated growth on the 'free' sulphur available in the mineral, as it did during the pyrite solubilisation experiments. When used in mixed culture with strain BC1, there was no beneficial effect on the rate of leaching. The final pH also remained unaffected by the addition of strain BC13.

The reactor work showed much better rates of copper leaching as might be expected. The rate in the mixed culture reactor was increased ten-fold over the rate in the shake flask. The reactors were subject to less disturbance than the flasks during readings, they contained a higher concentration of mineral, were much better mixed and contained a higher concentration of bacteria that had become well established before the major addition of substrate. Although the rates were improved in comparison to the flask experiments, there did not appear to be any real improvement

due to the addition of the sulphur-oxidising strain BC13 to a pure culture of strain BC1. The copper yield was slightly higher at the end of the experiment for the mixed culture but this was more likely to be due to the smaller lag phase seen with this culture, since the rates of copper leaching were similar in the two reactors.

The addition of the sulphur oxidiser presumably helped maintain a constant pH by the production of sulphuric acid, although this is likely to be a simplistic argument given the complexity of the situation. It is an important consideration though, given the fact that the pH in the strain BC1 reactor reached a level that could have affected the growth and activity of these organisms. Considering potential applications it could well be worth adding strain BC13 to a reactor simply to keep the pH from affecting strain BC1, whether or not the organism has a direct effect on leaching rate.

It is the iron levels and oxidation rates that were the least explicable. The total soluble iron in the mixed culture reactor was initially nearly all in the ferrous form, as demonstrated by the proximity of the total and ferrous iron concentrations. Even when the concentrations of the total and ferrous iron differed, the concentration of soluble non-ferrous iron was never greater than 1 g/l. The pure culture, on the other hand, showed an initial reduction in soluble ferrous and total iron. Again the proximity of the concentrations of total soluble iron and ferrous iron showed that most of the iron was present in the ferrous

form. Within 45 hours of the addition of the mineral the concentration of ferrous iron could not be detected by the ceric sulphate assay. It thereafter remained undetectable by this method, presumably because the ferrous iron produced as a consequence of mineral dissolution was instantly re-oxidised. In this way the level of ferrous iron remained beyond the limits of detection whilst the level of total iron rose to 1.4 g/l. Although there is more iron in solution in the mixed culture reactor, most of this is in the ferrous form. If the ferrous iron levels are discounted then the pure strain BC1 reactor can be seen to contain more ferric iron than the mixed culture reactor. The much reduced capability of the mixed culture to oxidise iron has resulted in this major difference between the mixed and pure culture. It could be that strain BC13 is producing a metabolic product that is inhibiting the iron oxidation system of strain BC1. The inhibition of *T.ferrooxidans* iron oxidation and growth by various organic acids has already been discussed; it would appear that a wide range of these are capable of affecting *T.ferrooxidans*.

If strain BC13 is producing such a product it would appear that it takes time to build up to toxic levels. The strain BC1 cells taken from the pure culture reactor and mixed with the strain BC1/strain BC13 cells taken from the mixed culture reactor did not demonstrate any inhibition of iron oxidation ability. If there had been any inhibition then the rate observed would have been less than half that observed with the unmixed cells from the strain BC1 reactor.

The much reduced ability of strain BC1 to oxidise the ferrous iron may mean that it had to rely on elemental sulphur oxidation in conjunction with reduced iron oxidation in order to survive, since this strain was still visible in the reactor at the end of the run. This would be a good example of why nutritional versatility is important, both commercially and in evolutionary terms.

The effect of pH on the solubility of ferric iron compounds has already been discussed in the Introduction, but it is worth noting that the initial pH of the two reactors was different, as was the subsequent rate of acidification. The virtually constant pH level in the mixed culture reactor meant that the pH effect on the concentrations of total soluble and ferrous iron remained unaltered throughout. However the pH in the pure culture reactor was continually changing and this must have had some effect on the concentrations of total soluble iron and ferrous iron. In general the lower the pH the less likely the ferric iron is to precipitate and therefore the lower pH in the mixed culture should increase the concentration of Fe^{3+}.

The actual rates of copper release were not significantly different in the reactors, despite the fact that there was presumably only low levels of the mineral oxidising agent ferric iron being produced by the bacteria. However, the increasing amounts of ferrous iron indicates that the mineral was being oxidised chemically, and it may be that this was due to ferric iron present in the medium

from the period before the addition of the mineral, assuming that strain BC1 was not immediately inhibited by strain BC13.

The most likely explanation for the increased oxygen uptake demonstrated by a mixed culture of strains BC1 and BC13 on pyrite is that the mineral solubilisation action of strain BC1 is making available more sulphur for strain BC13. In pure culture strain BC13 can only oxidise the 'free' sulphur in the mineral. In the mixed culture, strain BC1 breaks down the mineral crystals, thereby releasing more substrate for strain BC13 and so causing greater oxygen uptake.

CHAPTER FIVE.

INVESTIGATION OF THE COMPONENTS OF THE IRON- AND SULPHUR-OXIDISING SYSTEMS.

1.0. IDENTIFICATION OF SUBSTRATE OXIDATION PROTEINS BY COMPARATIVE SDS POLYACRYLAMIDE GEL ELECTROPHORESIS.

In this study this technique was used to investigate proteins produced specifically during growth on ferrous iron and elemental sulphur. The mesophile *T.ferrooxidans* and several strains of moderate thermophiles were studied. In this way it was hoped to identify key proteins involved in the oxidation of ferrous iron and compare the oxidation systems in the moderate thermophiles with that in *T.ferrooxidans*. This approach to the investigation of the proteins was expected to reveal whether particular proteins would show enhanced expression or were constitutive.

1.1. Thiobacillus ferrooxidans.

The protein banding pattern of *T.ferrooxidans* when grown on ferrous iron or sulphur or pyrite is shown (Figure 39). The pattern showed several interesting features which are dissimilar from results already reported for this

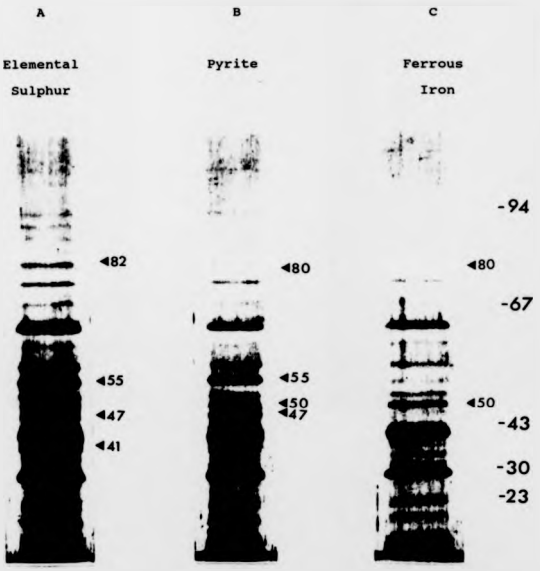


Figure 39. SDS-Polyacrylamide gel of *T.ferrooxidans* grown on elemental sulphur (A), pyrite (B) and ferrous iron (C). Molecular weights of markers and significant bands are given in kD. Cells were grown and harvested as detailed in materials and methods.

organism under these conditions. The major protein differences are tabulated (Table 4).

The more immediately obvious proteins are those apparently involved in sulphur oxidation. There appear to be four bands whose expression was enhanced by the presence of elemental sulphur, with the molecular weights of 82, 55, 47 and 41 kD. The majority of the bands are large and heavily stained, although the 55 kD protein bands of sulphur- and pyrite- grown cells are somewhat fainter. All these bands are equally visible in the sulphur and pyrite tracks.

The protein bands showing as a response to growth on ferrous iron show as much fainter bands, although one of them, the 50 kD, is still easily discernible. This band can also be seen in the sulphur-grown cells but more protein appears present in the tracks of iron and pyrite-grown cells. The 80 kD protein is very faint in both the iron- and pyrite-grown cells, particularly the pyrite-grown cells, and is not at all visible in the sulphur-grown cells. Two previous publications on iron-induced proteins reported proteins of different molecular weights: these were 63 kD (Fukumori *et al.*, 1988) and 92 kD (Mjoli and Kulpa, 1988).

A feature of these results is that the profile for pyrite-oxidising cells is not the sum of profiles of the ferrous iron- and elemental sulphur-grown profiles. This is despite the fact that pyrite-grown cells will be oxidising both iron and sulphur. The pyrite-grown cells contain both the bands whose expression was enhanced by the presence of ferrous iron (80 and 50 kD), but only two of the bands to

ELEMENTAL SULPHUR	PYRITE	FERROUS IRON
82		
	80	80
55	55	
	50	50
47	47	
41		

Table 4. The molecular weights (in kD) of SDS-PAGE protein bands induced in *T.ferrooxidans* by growth in the presence of the indicated substrate.

found in sulphur-grown cells (55 and 47 kD). The 82 and 41 kD proteins do not appear to be necessary for sulphur oxidation when the cells are grown on pyrite.

1.2. Strain BC1.

The potential induction of specific proteins in relation to growth on iron or sulphur was investigated in both autotrophically- and chemoheterotrophically-grown cells. Additionally cells were grown on yeast extract as sole substrate for comparison with the protein profiles of cells grown chemoheterotrophically with yeast extract and either iron or sulphur.

1.2.1. Heterotrophically-Grown Strain BC1.

The polyacrylamide concentration for this gel was 12% (Figure 40). It was run at 40mA for approximately five and a half hours, and then silver stained.

A feature of the gels of heterotrophically-grown organisms was the proliferation of bands in comparison to the same organism grown autotrophically. However in this case this particular feature is not well demonstrated here, where, even allowing for the extra sensitivity of the silver staining, there are approximately the same number of bands in the tracks of both autotrophically- and heterotrophically- grown cells (See also Figure 41).

Once again the proteins showing an increase in concentration in response to growth on elemental sulphur are

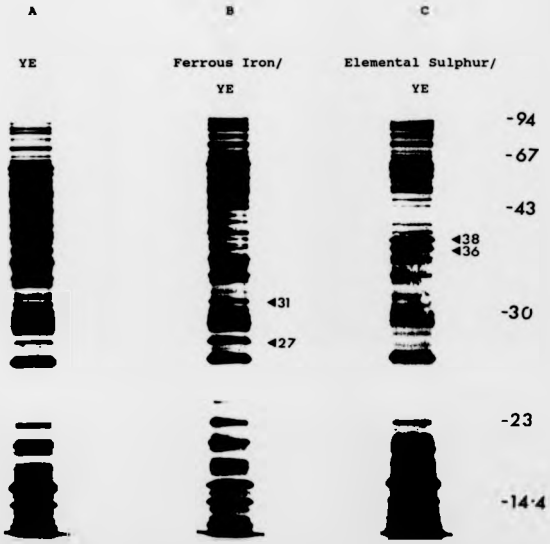


Figure 40. SDS-Polyacrylamide gel of strain BC1 grown on yeast extract (A), ferrous iron/yeast extract (B) and elemental sulphur (C). Molecular weights of markers and significant bands are given in kD. Cells were grown and harvested as detailed in materials and methods.

1.2.2. Autotrophically-Grown Strain BC1.

The polyacrylamide concentration for this gel was 12% (Figure 41). It was run at 40mA for approximately five hours and then Coomassie stained.

There are two protein bands that appear to be related to growth on sulphur and which are easily visible, one with an apparent molecular weight of 40 kD, the other with an apparent molecular weight of 33kD. The 40 kD protein band follows the general pattern so far observed with this organism in that it is also present in the ferrous iron-grown cells but in a lower concentration. The 33 kD polypeptide is also present in both tracks but as before it is more heavily stained in the sulphur-grown cells.

A fainter band does appear in the ferrous iron-grown cells. This 30 kD polypeptide is visible in both tracks but is produced in greater quantities in ferrous iron-grown cells.

There seems to be no major differences between heterotrophically- and autotrophically-grown cells in their response to substrate iron. The autotrophically-grown cells produced a band at 30 kD, whereas the heterotrophically-grown cells showed a band at 31 kD. However , the 27 kD protein is not visible in the autotrophically-grown cells but this may possibly be due to the difference in staining sensitivity. Pairs of bands demonstrating enhanced expression by sulphur in the heterotrophically-grown cells and autotrophically-grown cells had molecular weights of 38 and 36 kD and of 40 and 33

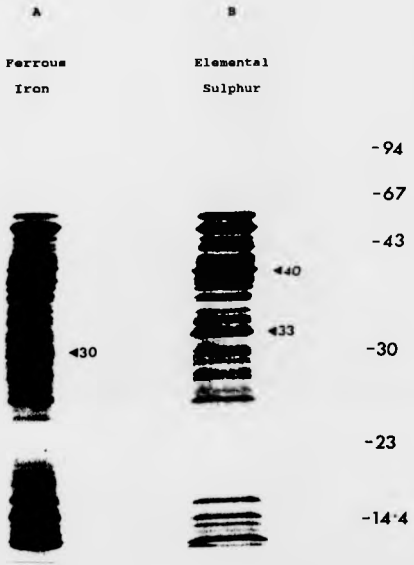


Figure 41. SDS-Polyacrylamide gel of strain BC1 grown autotrophically on ferrous iron (A) and elemental sulphur (B). Molecular weights of markers and significant bands are given in kD. Cells were grown and harvested as detailed in materials and methods.

kD respectively. Considering the accuracy and reproducibility of this technique these could be the same protein bands.

1.3. Strain LM2.

The protein profiles of autotrophically-grown strain LM2 with either iron or sulphur as the substrate are shown (Figure 42). Polyacrylamide concentration was 12%. The gel was run at 40mA for four hours and then stained with Coomassie stain.

The polypeptides that demonstrate an increase in concentration due to the presence of elemental sulphur are of very similar molecular weights to those showing the same response to sulphur in strain BC1. In the same manner as strain BC1 the 31 kD band is present in both tracks but is more heavily stained in the track of the sulphur-grown bacteria. The other band has an apparent molecular weight of 33 kD and is only visible in the track of sulphur-grown cells, although there may be a very faint corresponding band in the track of the iron-grown bacteria.

In contrast to *T.ferrooxidans* and strain BC1 any polypeptides enhanced by growth on ferrous iron were not visible. None of the bands seen in the track of the iron-grown cells appear to be present in a greater concentration than in the track of the sulphur-grown bacteria.

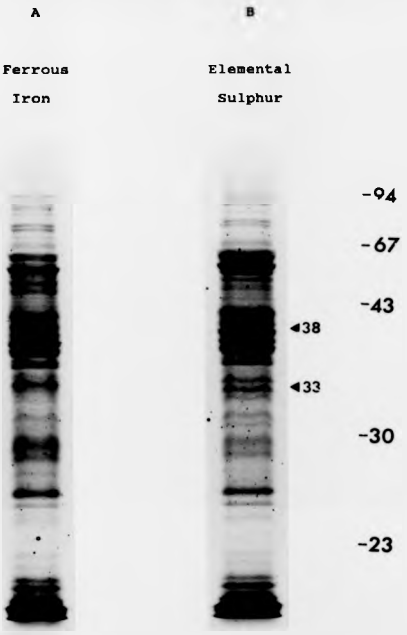


Figure 42. SDS-Polyacrylamide gel of strain LM2 grown autotrophically on ferrous iron (A) and elemental sulphur (B). Molecular weights of markers and significant bands are given in kD. Cells were grown and harvested as detailed in materials and methods.

1.4. Strain TH3.

Polyacrylamide concentration was 12%. The gel was run at 35mA for five and a half hours and then Coomassie stained (Figure 43). Strain TH3 did not clearly show specific protein bands as a result of growth on elemental sulphur. There is however one faint band visible with a molecular weight of 34 kD. This is slightly more heavily stained than the corresponding bands that are visible in the iron/yeast extract and yeast extract tracks.

There are three polypeptides produced in greater concentration during growth on ferrous iron, all of which are also to be seen in the sulphur/yeast extract and yeast extract tracks. The 36 kD band is probably the most obvious, as the 27 kD and 25 kD bands are very faint.

1.5. Comparison of Molecular Weights of Polypeptides Whose Expression is Enhanced by Growth on Iron or Sulphur.

The polypeptides found in different organisms in response to growth on iron or sulphur are listed in Table 5.

The polypeptides 'induced' by ferrous iron ranged in apparent molecular weight between 30 and 80 kD. However in the moderate thermophiles this range was reduced to 25 to 36 kD. This pattern also holds true for the bands found in sulphur-grown cells. There is a wide range of molecular weights from 33 to 83 kD until one looks only at the moderate thermophile results, in which case the range drops

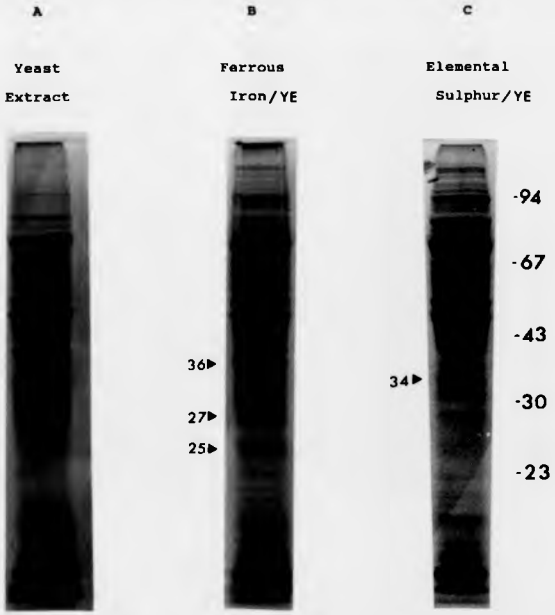


Figure 43. SDS-Polyacrylamide gel of strain TH3 grown on yeast extract (A), ferrous iron/yeast extract (B) and elemental sulphur/yeast extract (C). Molecular weights of markers and significant bands are given in kD. Cells were grown and harvested as detailed in materials and methods.

SUBSTRATE	ORGANISM								
	T.ferrooxidans			B C 1			L M 2	T H 3	
IRON	8 0								
	5 0								
								3 6	
				3 1					
				2 7				2 7	
								2 5	
SULPHUR	8 2								
	5 5								
	4 7								
	4 1								
				3 8					
				3 6					
								3 4	
							3 3		
							3 1		

Table 5. A comparison of the molecular weights (in kD) of SDS-PAGE protein bands induced in iron- and sulphur-oxidising bacteria by growth on the indicated substrate. All organisms were grown autotrophically, except the obligate heterotroph, strain THJ.

to 33 to 40 kD.

DISCUSSION.

The protein banding patterns observed in *T.ferrooxidans* indicate the inducible nature of at least some components of the iron- and sulphur-oxidising systems in this organism. In this way these results show good correlation with the oxidation data, which also indicated induced systems.

Mjoli and Kulpa (1988) have shown a 92 kD protein induced by the presence of ferrous iron that is located in the outer membrane. The band is not clearly visible in whole cell extracts and it could be that the band is also present in the gel shown here but since this region of the gel is faint anyway the band is not visible at all. If the band contains a protein or proteins involved in iron oxidation then, in view of the importance of this system to the organism, its presence or absence is not likely to be due to a strain difference. An examination of the gel patterns of whole cell extracts published by Mjoli and Kulpa shows no major differences to the gel shown here, although detailed examination is not possible.

Fukumori *et al.* (1988) have apparently purified an iron-oxidising enzyme from *T.ferrooxidans*, which has a molecular weight of 63 kD. The authors do not, however, indicate whether this enzyme is induced by the presence of iron. There is no indication of an induced protein band of

to 33 to 40 kD.

DISCUSSION.

The protein banding patterns observed in *T.ferrooxidans* indicate the inducible nature of at least some components of the iron- and sulphur-oxidising systems in this organism. In this way these results show good correlation with the oxidation data, which also indicated induced systems.

Mjoli and Kulpa (1988) have shown a 92 kD protein induced by the presence of ferrous iron that is located in the outer membrane. The band is not clearly visible in whole cell extracts and it could be that the band is also present in the gel shown here but since this region of the gel is faint anyway the band is not visible at all. If the band contains a protein or proteins involved in iron oxidation then, in view of the importance of this system to the organism, its presence or absence is not likely to be due to a strain difference. An examination of the gel patterns of whole cell extracts published by Mjoli and Kulpa shows no major differences to the gel shown here, although detailed examination is not possible.

Fukumori *et al.* (1988) have apparently purified an iron-oxidising enzyme from *T.ferrooxidans*, which has a molecular weight of 63 kD. The authors do not, however, indicate whether this enzyme is induced by the presence of iron. There is no indication of an induced protein band of

this weight in the gel discussed here, and neither do Mjoli and Kulpa indicate the presence of such a band in their work.

Sulphur:ferric iron oxidoreductase is comprised of two 23 kD protein subunits (Sugio *et al.*, 1987), and is apparently induced during growth on sulphur (Sugio *et al.*, 1986). There is no evidence of induced proteins at this molecular weight. Furthermore Sugio *et al.* (1988a) have also stated that their strain of *T.ferrooxidans*, AP19-3, has a constitutive iron oxidation system. This strain was demonstrated to have lost only 37.5% of its iron oxidation capacity after 22 consecutive subculturings on a sulphur-salts medium.

Although sulphur oxidation is involved in the solubilisation of pyrite, only two of the four bands enhanced in expression in *T.ferrooxidans* by the oxidation of elemental sulphur are enhanced in the pyrite-grown cells. A possible explanation for this is that the sulphur present in pyrite is in the form of sulphide, S^{2-}, and this can be chemically oxidised to sulphite, SO_3^{2-}, whereas the chemically stable elemental sulphur cannot be under the prevalent conditions. If this were the case then less elements of the sulphur oxidation system would be required by pyrite-grown cells. This explanation would mean that the pyrite-grown cells would have a much reduced ability to oxidise elemental sulphur since they would not have enough of the necessary components of the sulphur oxidation system. However, in Warburg oxygen uptake experiments, pyrite-grown

T.ferrooxidans showed no reduced sulphur oxidation capacity in comparison with sulphur-grown cells (data not shown).

An alternative explanation could lie in the role of the enhanced proteins. They need not play a direct role in the oxidation systems, but have an associated role. For example, the necessity for attachment in pyrite oxidation has already been discussed and it may be that some or all of the proteins 'induced' may be attachment proteins. It is possible that extra proteins seen in sulphur-grown cells are required for attachment to, or as 'wetting agents' for, the highly hydrophobic surface of elemental sulphur. Phosphotidylinositol has been identified as a 'wetting agent' produced by *T.thiooxidans* in order to reduce this hydrophobicity (Schaeffer and Umbreit, 1962) and it is not inconceivable that *T.ferrooxidans* also produces a 'wetting agent'. This is not, however, likely to be a protein.

The protein banding pattern for strain BC1 is also supportive of the hypothesis put forward in chapter three that the oxidation systems in this organism are constitutive. Those proteins that are apparently involved in iron oxidation are always present when the organism is grown on either substrate, although the increase in staining density when the organism is grown on iron would indicate some enhancibility. The situation for strain LM2 is, however, very different. The oxygen uptake experiments seemed to suggest enhancible systems for both iron and sulphur oxidation. However, although the organism shows protein bands that increase in concentration during growth

on elemental sulphur, it shows no such bands during growth on ferrous iron. It could be that although the system is constitutive, its activity is much reduced during growth on elemental sulphur, and requires time to regain its activity. Alternatively, given that the recently isolated cytochrome involved in iron oxidation in *L.ferrooxidans* (Hart *et al.*, 1991) is not visible in SDS-PAGE gels of whole cell extracts (A. Hart, personal communication) it could be that the induced proteins are masked by others in the same band. This would imply that they are not produced in large quantities. With the exception of one faint band that may not be a real result, strain TH3 did not produce any bands in response to growth on elemental sulphur, and yet produced three bands in response to growth on ferrous iron, which would seem to indicate that the situation is reversed with respect to strain LM2.

As with all the facultatively autotrophic organisms, strain BC1 grows faster and with a greater yield if grown chemoheterotrophically. The gel results indicated that there was little difference in the protein make-up whether the organism was grown autotrophically or heterotrophically. It was therefore considered reasonable to use heterotrophically-grown cells in more detailed studies of the iron oxidation system in this organism. The extra protein bands often associated with heterotrophic growth where considered to be much less important than the advantage of a much improved growth yield.

Table 5 provides a clear overview of the proteins

produced by different organisms in response to growth on iron or sulphur. The main point of this table is to illustrate the diversity of this response.

As before the diversity of the organisms is well demonstrated. The moderate thermophiles show several different responses to growth history in their iron and sulphur oxidation systems. These responses range from those similar to *T.ferrooxidans* to that of strain BC1, with its basically constitutive oxidation systems. The situation is not as clear cut as it might be, however, particularly with respect to the example of strain LM2, where the situation requires more study. Even the well-studied *T.ferrooxidans* demonstrated a hitherto unreported response in the contrasting sulphur oxidation banding patterns. It seems that whilst there may a general correlation between the effect of growth history on the oxidation rates and its effect on the protein make-up of these organisms, there are no definitives.

2.0. IDENTIFICATION OF MAJOR RESPIRATORY CHAIN COMPONENTS BY OPTICAL SPECTROSCOPY.

Optical spectroscopy was used to reveal the electron transport components of the moderate thermophiles, again with *T.ferrooxidans* as a reference organism. It was hoped to show that the moderate thermophiles did indeed use a

different ferrous iron oxidation system to *T.ferrooxidans*, as had seemed likely from the comparison of the protein banding patterns after SDS PAGE experiments. As well as studying whole cells, cell fractions were also examined to enable any cytochromes found to be assigned to a general cellular location.

2.1. Thiobacillus ferrooxidans.

Whole cell difference spectra of *T.ferrooxidans* grown on ferrous iron and on pyrite are illustrated (Figure 44). Both samples were oxidised with ammonium persulphate and reduced with dithionite, to give reduced minus oxidised spectra.

The presence of a Soret peak at 419 nm and an α and ß-peak at 551 nm and 520 nm respectively is indicative of a c-type cytochrome. The 441 nm Soret peak and 596 nm α-peak is typical of a cytochrome a_1.

When the soluble fraction of *T.ferrooxidans* was examined, a spectrum typical of the blue copper protein rusticyanin (Cobley and Haddock, 1975) was found in the oxidised sample (Figure 45). This demonstrated the value of investigating the spectrum of fractions, since in whole cell preparations the cytochrome a_1 α-peak masks the rusticyanin absorption peak.

The cell fractions that were investigated were the soluble fraction and the membrane fraction of ferrous iron-grown cells (Figure 46). The spectra are both reduced

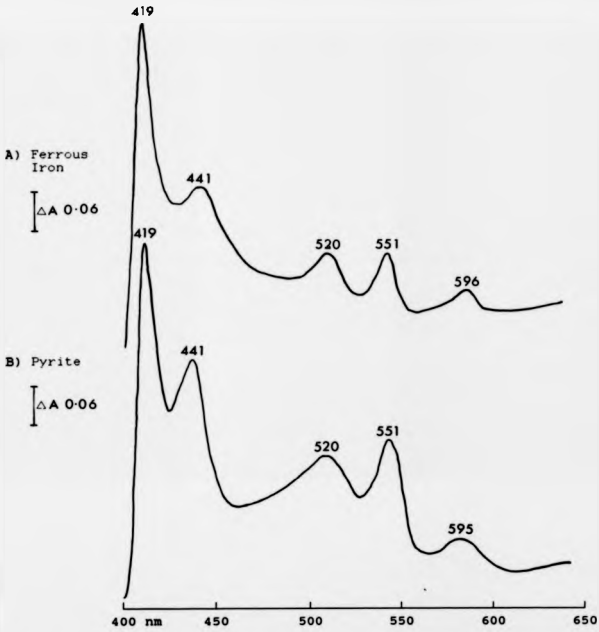


Figure 44. Reduced minus oxidised whole cell spectra of *T.ferrooxidans* grown on 50mM ferrous iron (a) and 5% pyrite (b). Absorbance maxima are marked in nm. Protein concentrations were as follows, (mg/ml) - (A) 2.14, (B) 1.93.

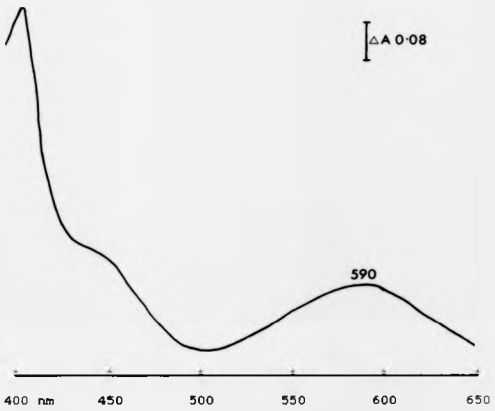


Figure 45. Oxidised spectra of the soluble fraction of *T.ferrooxidans* grown on ferrous iron, showing the typical 590nm peak of rusticyanin. Protein concentration was 4.61 mg/ml.

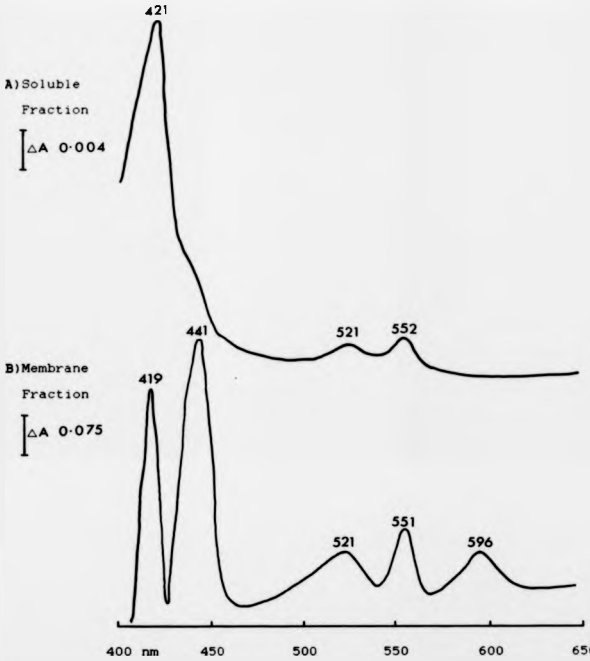


Figure 46. Reduced minus oxidised spectra of the soluble (a) and membrane (b) fractions of ferrous iron-grown *T.ferrooxidans*. The absorbance maxima are given in nm. Protein concentrations were as follows, (mg/ml) - (A) 0.95, (B) 2.49.

minus oxidised, using the same reagents as above.

The soluble fraction showed the peaks associated with a c-type cytochrome. There were no clear absorption peaks for the a_1 cytochrome, although a shoulder can be seen on the cytochrome c Soret peak that corresponds to the wavelength of the cytochrome a_1 Soret peak. This is probably due to minor contamination of the sample.

The membrane fraction shows evidence for both of the cytochromes, although there appears to be comparatively less cytochrome c than in the whole cells. The a_1 signal is as clear as it was in the whole cell spectra.

These results confirmed that the c-type cytochromes in *T.ferrooxidans* exist in both the soluble and membrane fractions but the terminal oxidase is entirely associated with the membrane. Rusticyanin was also entirely associated with the soluble fraction, as reported previously (Cobley and Haddock, 1975).

2.2. Strain BC1.

Whole cell spectra of strain BC1 were obtained at two different temperatures: room temperature and with liquid nitrogen, (77 K), in an attempt to improve the resolution.

2.2.1. Room temperature spectra.

The whole cell spectra indicate the major cytochromes present during growth on ferrous iron and pyrite (Figure 47). The spectra were oxidised with sodium hexachloroiridate

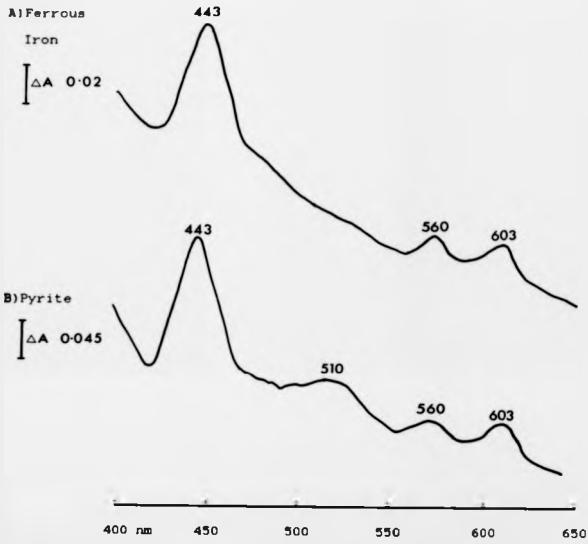


Figure 47. Reduced minus oxidised whole cell spectra of BC1 grown autotrophically on 50mM ferrous iron (A) and 5% pyrite (B). Absorbance maxima are indicated in nm. Protein concentrations were as follows, (mg/ml) - (A) 1.89, (B) 3.25.

and reduced with sodium dithionite.

The spectrum of the ferrous iron-grown cells shows major peaks at 443 nm, 560 nm and 603 nm. The 443 nm and 603 nm correspond to the Soret and α-peaks respectively of a cytochrome aa_3. The 560 nm peak may be a β-peak or be the signal for a completely different cytochrome. Unlike *T.ferrooxidans* there is no evidence for any c-type cytochromes. The pyrite-grown cells exhibit the same peaks plus a further peak at 510 nm, which is the only qualitative difference exhibited by either organism between ferrous iron- and pyrite-grown cells.

The only fraction spectrum from ferrous iron-grown cells that showed any evidence of cytochrome content was the membrane fraction (Figure 48). The spectrum shows exactly the same pattern as the whole cell spectrum, indicating that the major cytochromes to be found in this organism are to be found in the membrane.

There appears to be major differences in the electron transport systems of *T.ferrooxidans* and strain BC1. Not only is the terminal oxidase different but the latter does not appear to have any small soluble components whereas *T.ferrooxidans* is reliant on a soluble cytochrome c and rusticyanin.

2.2.2. Liquid Nitrogen Spectra.

This investigation was undertaken with ferrous iron-grown whole cells. The oxidant for this spectrum was ammonium persulphate and the reductant was dithionite.

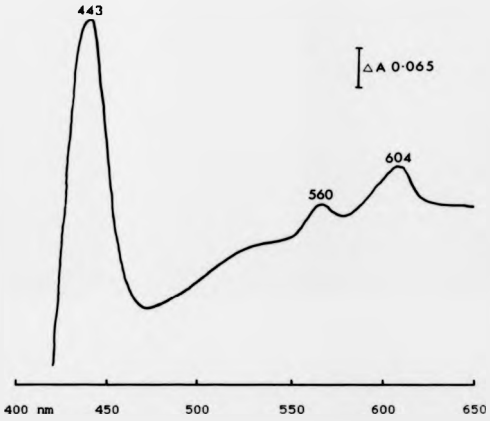


Figure 48. Reduced minus oxidised spectrum of the membrane fraction of ferrous iron - grown BC1. The cells were grown chemoheterotrophically in 50 mM Fe^{2+} and 0.01% YE. Absorbance maxima are marked in nm. Protein concentration was 2.68 mg/ml.

The spectrum is the same as the room temperature spectrum, with one small difference (Figure 49). There is a small shoulder on the Soret peak at approximately 450 nm. By narrowing the wavelength scan it is possible to get a closer look at this shoulder (Figure 49, inset). It is not visible on any other spectrum obtained with this organism at room temperature and there remains the possibility that it corresponds to the 560 nm peak so far observed in all the whole cell and membrane preparations of this organism.

DISCUSSION.

The results obtained with *T.ferrooxidans* are in good agreement with earlier published work. Cytochrome c was partially purified by Vernon *et al.* (1960) and shown to have absorption peaks at 417 nm, 523 nm and 552 nm. It has since been suggested that *T.ferrooxidans* contains two c-type and one c_1-type cytochrome (Tikhonova *et al.*, 1967). Cytochrome c_{552} has been purified and characterized (Sato *et al.*, 1989) and the reduced minus oxidised absorption peaks reported as 417 nm, 523 nm and 552 nm. The same cytochrome purified from *T.thiooxidans* (Takakuwa, 1975) demonstrated peaks at 418 nm, 523 nm and 552nm.

The absorption maxima for the terminal oxidase, cytochrome a_1, also show good agreement with other published values, for example both Ingledew and Cobley (1980) and Blaylock and Nason (1963) have reported a value of 597 nm

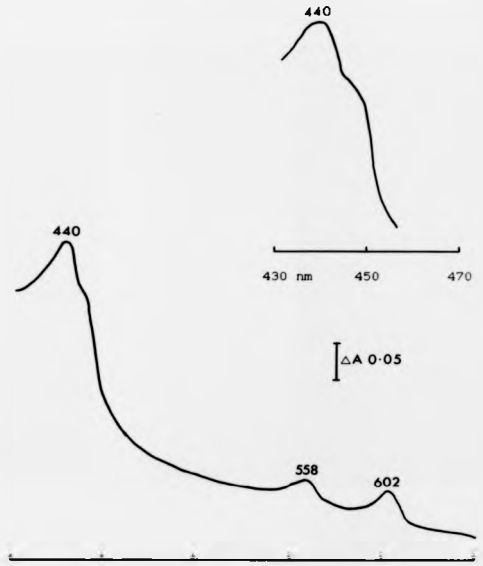


Figure 49. Liquid nitrogen reduced minus oxidised spectrum of whole cells of ferrous iron-grown BC1. Cells were grown chemoheterotrophically in 50 mM Fe^{2+} and 0.01% YE. Absorbance maxima are marked in nm. Protein concentration was 4.29 mg/ml. Inset shows a magnification of the 430 nm to 470 nm region, demonstrating the shoulder on the cytochrome aa_3 sorat peak.

for the α-peak.

The absence of cytochrome $\underline{a}_1$ in the soluble fraction is entirely consistent with the hypothesis of Ingledew *et al.* (1977) that the site of oxygen reduction is the cell membrane. The presence of cytochrome $\underline{c}$ in both the soluble and membrane fractions suggests that it exists in two forms, a soluble and membrane bound form. The degree of absorbance seen in the membrane fraction presented here is not likely to be due to contamination. This again is consistent with the proposal of Ingledew *et al.* (1977) that electrons are passed from a soluble cytochrome $\underline{c}$ to a membrane bound form and thence to cytochrome $\underline{a}_1$. The existence of two different locations for $\underline{c}$-type cytochromes is not accounted for in some later electron transport hypotheses, for example Blake *et al.* (1988) or Sato *et al.* (1989), both of which only made use of soluble cytochrome $\underline{c}$ in their work.

There appears to be little variation in the major components of the electron transport chain during growth on pyrite as compared to growth on ferrous iron. The technique is largely qualitative and therefore minor differences in peak height are difficult to interpret. It may appear that the cytochrome $\underline{a}_1$ Soret peak in particular is absorbing more strongly in the pyrite-grown cells but this difference is not great enough to imply any real change in the cytochrome $\underline{a}_1$ content.

The spectra of strain BC1 show much difference from that of *T.ferrooxidans*. The absorbance peaks at 443 nm and 603 nm are typical of cytochrome $\underline{aa}_3$. *Thiobacillus novellus*

is known to have a cytochrome $\underline{aa}_3$, with reduced minus oxidised peaks at 442 nm and 602 nm (Yamanaka and Fujii, 1980) and the Gram positive *Bacillus subtilis* has also been shown to contain a cytochrome $\underline{aa}_3$, which, when purified, showed absorbance peaks at 443 nm and 601 nm (DeVrij *et al.*, 1983). The cyanobacterium *Anabaena variabilis* has an $\underline{aa}_3$-type cytochrome that not only demonstrates reduced minus oxidised peaks at 440 nm and 604 nm, but also has a third peak at 517 nm (Häfele *et al.*, 1988). Wakagi *et al.* (1989) also showed a third peak in the spectrum for the terminal oxidase of *Sulfolobus acidocaldarius*. The three peaks occurred at 441 nm, 583 nm and 603 nm and it was concluded that this was a cytochrome $\underline{aa}_3$. Unfortunately, the authors had not analysed the 'cytochrome $\underline{a}_{583}$' to see whether it actually contained a heme a group. These results are interesting since the strain BC1 spectra also show a third peak, although this occurs at 560 nm. It may be that this peak is due to the cytochrome aa_3 itself, to the presence of a second chromophore or it may be that it is due to an associated cytochrome. Absorbance at this wavelength is typical of both b- and c-type cytochromes.

Buse *et al.* (1989) have shown a $\underline{c}_1\underline{aa}_3$ cytochrome in *Thermus thermophilus* that exhibited α-peaks at 549 nm and 603 nm. A cytochrome c has been shown to be associated with the terminal oxidase in *Bacillus firmus* (Kitada and Krulwich, 1984) although in this case the reported absorption maxima for the reduced complex were 445 nm, 518 nm, 548nm and 598 nm. Sone *et al.* (1979) purified a

cytochrome $\underline{caa_3}$ from the thermophilic organism PS3 which showed reduced minus oxidised absorbance peaks at 418 nm, 445 nm, 521 nm, 550 nm and 604 nm. It was later claimed that the c-type cytochrome was actually a cytochrome $\underline{c_1}$ and that the whole complex had α-peaks at 547 nm and 604 nm (Baines and Poole, 1985). A recently isolated terminal oxidase from *T.thermophilus* was shown to be a cytochrome ba_3 (Zimmerman *et al.*, 1988). This displayed reduced minus oxidised peaks at 427 nm, 442 nm, 530 nm, 560 nm and 613nm. It appeared to be a single 35 kD protein and was tightly bound to the membrane. However, in these cases of cytochrome complexes, the cytochrome associated with the terminal oxidase will obviously still display a Soret peak, for example the 427 nm peak of the cytochrome $\underline{ba_3}$ of PS3 is typical of a cytochrome b. The absence in the room temperature strain BC1 whole cell and membrane spectra of a second Soret peak strongly suggests that the 560 nm peak is not due to a second cytochrome present with cytochrome $\underline{aa_3}$.

The spectrum obtained at 77°K shows a shoulder on the Soret peak for the cytochrome $\underline{aa_3}$. It could be that this is the Soret peak associated with the 560 nm peak, which was previously masked by the cytochrome $\underline{aa_3}$ peak. This masking of peaks is not uncommon and the use of low temperature spectra is often used to uncover peaks hidden at room temperature. For example, Barr *et al.* (1990) published reduced minus oxidised spectra for both *T.ferrooxidans* and *L.ferrooxidans* at room temperature and 77°K. In the case of *T.ferrooxidans*, a single peak at 551 nm was resolved at 77°K

into two peaks at 548 nm and 552 nm. The 579 nm peak of *L.ferrooxidans* was shown to consist of 573 nm and 585 nm peaks. If the 560 nm peak was due to a separate cytochrome b then the associated Soret peak would not be in the region of 450 nm. Kurokawa *et al.* (1989) isolated cytochrome b_{559} from *Nitrobacter winogradskyi* and showed that the reduced cytochrome had α-peaks at 528 nm and 559 nm and a Soret peak at 426 nm. If the 560 nm peak was due to a cytochrome c then this too would have a Soret peak in a different region from that demonstrated here: it would typically be in the 415-425 nm region.

In pyrite-grown strain BC1, a fourth peak appears at 510 nm. Unlike the responses seen in the electrophoregrams, where the protein bands showing enhanced concentration need not correspond to proteins involved directly in oxidation, changes seen in spectra are far more likely to be connected to changes in the actual oxidation systems. The major difference between ferrous iron oxidation and pyrite oxidation is obviously that in the latter sulphur oxidation is also implied. It could be that the organism is producing a chromophore involved in the oxidation of sulphur. It is not inconceivable that this 'induced' chromophore is represented by the 38 kD protein band seen to be enhanced in the sulphur-grown cells in the electrophoregram.

The absence of any detectable soluble electron carriers in strain BC1 is probably due to the nature of the cell surface. In the Gram negative *T.ferrooxidans* there is a requirement for the transport of the electrons liberated

from ferrous iron at the cell surface to be transported to the inner membrane, the location of the terminal oxidase. Hence the presence of cytochromes c and rusticyanin in the periplasmic space. However, in Gram positive strain BC1 there is no requirement for electron transport across the periplasmic space and so it is not surprising that there are no visible soluble electron carriers. The absence of a periplasmic space obviously does not mean the absence of several membrane bound electron carriers. From the Gram positive PS3 Sone *et al.* (1989) purified cytochrome c_{551} from the membrane and suggested that it was responsible for transporting electrons from cytochrome bc_1 (b_6f) complex to cytochrome o, the terminal oxidase.

CHAPTER SIX.

PARTIAL PURIFICATION OF COMPONENTS OF THE FERROUS IRON-OXIDISING SYSTEMS.

1.0. THE PARTIAL PURIFICATION OF RUSTICYANIN.

In order to continue the comparison of *T.ferrooxidans* and strain BC1 the purification of rusticyanin from *T.ferrooxidans* was undertaken. It was hoped that this would give a control for, and confidence in, the techniques to be used in the investigation into the iron oxidation system of strain BC1. The already documented techniques would also serve as an introduction to the protein purification processes.

1.1. Spectra of broken cells.

In order to determine that the rusticyanin in the cells could be detected, spectra where obtained for the soluble fraction where the rusticyanin was expected to be located.

A sample oxidised with ammonium persulphate showed that rusticyanin was indeed present in the soluble fraction (Figure 50a). This scan also shows a Soret peak that

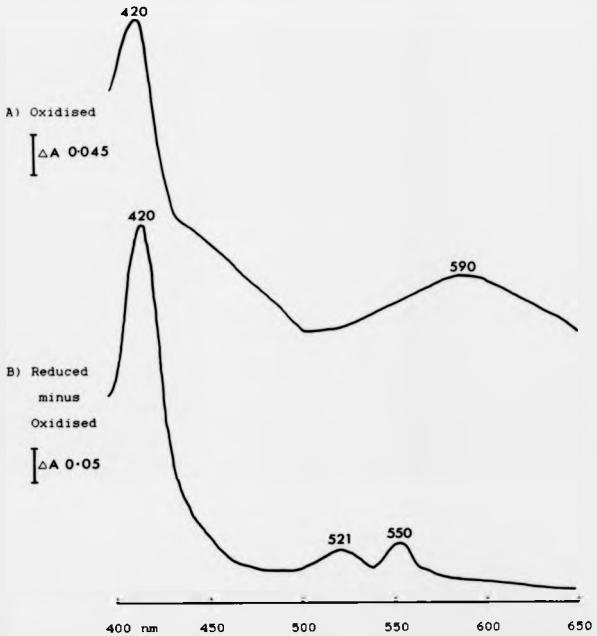


Figure 50. Comparison of an oxidised (A) and reduced minus oxidised spectra (B) of the soluble fraction of iron-oxidising *T.ferrooxidans*. The rusticyanin is only visible in the oxidised sample. Absorbance maxima are marked in nm. Protein concentration was 3.35 mg/ml.

corresponds to the Soret peak of oxidised cytochrome c. To look more closely at the cytochrome c content of the sample a scan was run under different conditions - before the spectra was run the sample was reduced with dithionite to give a reduced minus oxidised spectra. This spectra clearly shows the presence of cytochrome c in the sample (Figure 50b). In its reduced state the rusticyanin does not show in the spectra.

1.2. Elution Profile of Rusticyanin from a CM Sephadex Column.

After the sample had been passed down a CM Sephadex column an elution profile was plotted, (Figure 51), using the absorbance parameters of protein content (280 nm) and rusticyanin content (590 nm). The samples were first oxidised with ammonium persulphate so that the presence of rusticyanin could be detected by absorption at 590 nm.

The profile shows that the rusticyanin was released from the column after the NaCl concentration exceeded 300 mM. The protein peak that coincided with the rusticyanin peak accounted for only a small proportion of the total protein on the column.

After the rusticyanin was removed from the column further NaCl was added until the concentration reached 1M. This treatment did not elute any fractions showing an absorption peak at 590 nm.

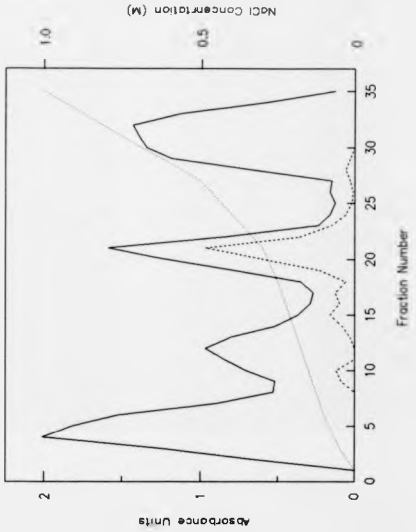


Figure 51. Elution profile following ion exchange chromatography on a CM Sephadex column of the soluble fraction of *T.ferrooxidans*. Fractions were collected and assayed spectrophotometrically for protein (A_{280}, ——) and rusticyanin (A_{590}, ---). NaCl concentration is also shown (·····).

1.3. SDS PAGE Analysis of Column Fraction.

The fractions that contained significant 590 nm absorption (fractions 20, 21, and 22) were pooled and then run on an SDS polyacrylamide gel (Figure 52). The concentration of this gel was 12%, and was run at 30mA for four hours. The gel was then silver stained.

There are a total of nine bands shown on the profile, of molecular weights ranging from 16 to 61 kD. Purified rusticyanin has a molecular weight of 16 kD (Cox and Boxer, 1978) and the darkest, largest band on the gel is of the same weight and is probably therefore the partially purified rusticyanin.

1.4. Spectrophotometric Analysis of Column Fraction.

Spectra were also run of the pooled fractions, in order to attempt to find the 590 nm peak of oxidised rusticyanin. The sample was oxidised with ammonium persulphate. The spectra produced by the fraction shows only one peak (Figure 53). This peak was the 590 nm peak of rusticyanin and therefore indicates that rusticyanin is present in the sample. The peak has, however, a much lower overall absorption value than the original sample, indicating that much of the rusticyanin was either lost or denatured during the preparation.

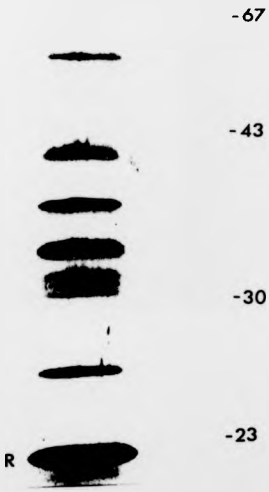


Figure 52. SDS-Polyacrylamide gel of partially purified rusticyanin, (R). Molecular weights of markers are given in kD. The sample was prepared as detailed in materials and methods.

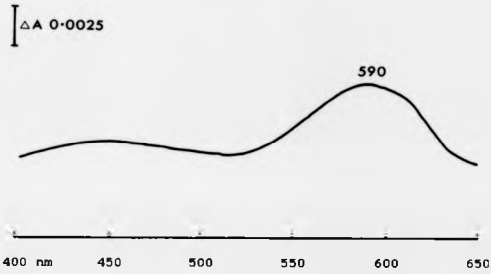


Figure 53. Oxidised spectra of the pooled fractions from a CM Sephadex column loaded with the soluble fraction of *T.ferrooxidans*. These fractions showed absorbance at 590nm, which demonstrated the presence of rusticyanin. Absorbance maximum is given in nm. Protein concentration was 0.52 mg/ml.

DISCUSSION.

It would appear that rusticyanin was indeed partially purified. The oxidised spectra of the pooled fractions definitely showed the presence of the peak associated with rusticyanin, whilst on the SDS polyacrylamide gel the largest, clearest band occurred at the same molecular weight as that of rusticyanin. Unfortunately it appears that although the protein was present, there was either very little of it or it was denatured. This is supported by the fact that the sample itself had apparently been decolourised, whereas in the original report such a sample had a deep blue colour (Cox and Boxer, 1978), although it could be bleached on denaturation.

2.0. EXAMINATION OF THE MEMBRANE OF STRAIN BC1.

The moderate thermophile chosen for further investigation of its ferrous iron oxidation system was strain BC1 because this had given the clearest and most interesting results in initial studies. This organism had been shown to be amenable to both SDS PAGE and spectrophotometric analysis. The major proteins involved in the oxidation system were apparently associated solely with the membrane; there were, perhaps unfortunately, no soluble components to be concerned with. The method of regulation of

its ferrous iron- and elemental sulphur-oxidising systems was significantly different from that of *T.ferrooxidans*. Unlike *T.ferrooxidans* it could be grown heterotrophically and such growth would not affect its production of the ferrous iron-oxidising proteins.

2.1. Preparation of Membranes of Strain BC1.

For this investigation membranes were prepared from both ferrous iron-oxidising cells and, for use as a control, from yeast extract-grown cells. Yields were particularly low, especially from the yeast extract-grown cells. An average of approximately 100mg of membrane could be extracted from forty litres of ferrous iron/yeast extract-grown cells that had oxidised 70 - 80% of the available iron.

The membranes from the iron-oxidising cells were a deep red/brown colour, whereas the membranes from yeast extract-grown cells were a pale green colour. This difference in colour may have simply reflected the presence of iron in the membrane of the iron-grown cells or may have been due to a protein being produced by the iron-grown cells that gave such a colouration. After the membrane had been spun down, the supernatant from the iron/yeast extract-grown cells had a slight brown coloration but the yeast extract-grown cells gave no colour at all in their supernatant.

The membranes were routinely frozen to -20^{o}C. Reduced

minus oxidised spectra of thawed samples showed little or no difference to those of unfrozen samples (Figure 54). The SDS PAGE work also showed no appreciable difference between frozen and unfrozen cells. It was therefore concluded that freezing of the samples would not affect the results of this particular investigation.

2.2. Iron Content of Membrane and Soluble Fractions.

In order to be certain that the difference in the colours of the fractions of the iron/yeast extract- and yeast extract-grown cells was due to the iron content, as seemed highly likely, the various fractions were assayed for iron, using atomic absorption spectrophotometry (Table 6). The technique of boiling in concentrated nitric acid was used to ensure the release of the iron from the proteins to allow its quantification.

The iron/yeast extract-grown cells showed a six times greater concentration of iron in the membrane than in the supernatant. There was actually little difference between the nitric acid treated and untreated samples. The yeast extract-grown cells showed an almost total absence of iron, within the limits of detection of this technique. There does appear to be a greater concentration in the membrane but at this concentration the quantitative accuracy of this technique is impaired.

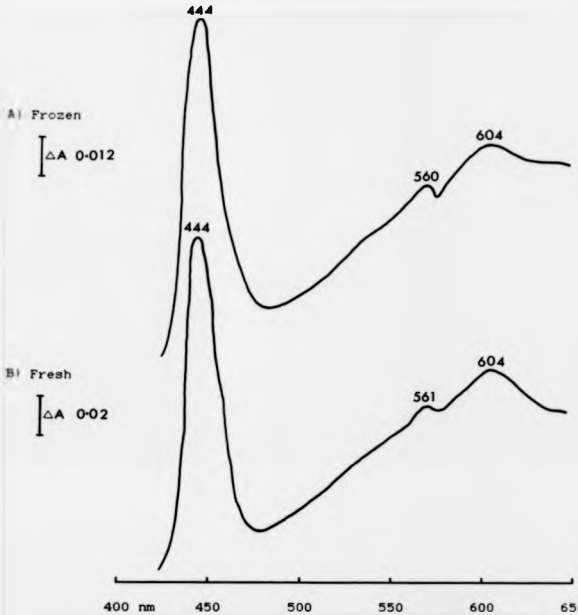


Figure 54. Reduced minus oxidised spectra of freeze/thawed (A) and freshly prepared (B) samples of ferrous iron-grown BC1. The cells were grown autotrophically in 50 mM Fe^{2+}, and the frozen sample stored at $-20^{0}C$ for several days before thawing. Protein concentrations were as follows, (mg/ml) - (A) 1.42, (B) 1.61.

TREATMENT	SUBSTRATE	FRACTION	SPECIFIC IRON CONC. (μmoles/mg protein)
NITRIC ACID	Fe^{2+}/YE	Supernatant	0.14
		Membrane	1.01
	YE	Supernatant	0.01
		Membrane	0.02
UNTREATED	Fe^{2+}/YE	Supernatant	0.14
		Membrane	0.82
	YE	Supernatant	0.00
		Membrane	0.00

Table 6. The specific iron content of the membrane and supernatant fractions of strain BC1 grown chemoheterotrophically on Fe^{2+}/ YE or heterotrophically on YE. Cells were either treated by boiling in concentrated nitric acid or untreated.

2.3. Iron Distribution Within Gel Tracks.

It had already been determined that the organism produced certain polypeptides in response to the presence of ferrous iron. To determine whether these polypeptides were linked in some manner to the iron, the iron content of various regions of an SDS polyacrylamide gel was determined after whole cell extracts had been run. Both iron/yeast extract- and yeast extract-grown cells were used.

A major difference in the distribution of iron in the track containing the iron/yeast extract-grown cells occured in the region of the 30 kD marker (Figure 55). This is the region in which the two major polypeptides lie whose expression is enhanced as a consequence of iron oxidation. In contrast, the yeast extract-grown cells showed not only an overall lower iron content, the eletrophoresed extract did not yield any peaks in iron distribution throughout the gel track. This indicated that at least one of the polypeptides involved in iron oxidation in this organism was associated with iron.

DISCUSSION.

The yield of strain BC1 membrane was low, for example Kitada and Krulwich (1984) obtained 1.5 to 2 grams of membrane protein from 32 litres of heterotrophically-grown *Bacillus firmus* cells. However, although the yield of

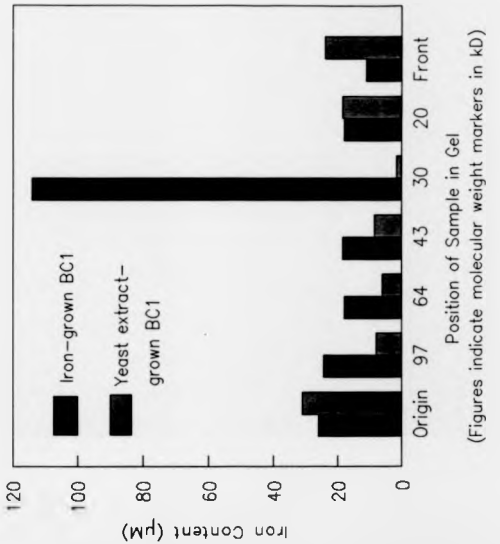


Figure 55. The distribution of iron in the SDS polyacrylamide gel tracks of whole cell extracts of ferrous iron/yeast extract- and yeast extract-grown strain BC1. The gel tracks were divided into regions, each corresponding to an area above and below a molecular weight marker, as detailed in materials and methods. Each region was assayed for iron.

membrane is low, it is worth noting that had strain BC1 been grown autotrophically the yield would have been far smaller and this therefore demonstrates the advantage of being able to grow the organism heterotrophically.

The difference in colour between the initial cell suspensions from the two different growth media was eventually reflected in the two different colours of the membranes obtained from these cells. The assay of iron content of the fractions from the two batches of cells showed that the difference in colour was indeed due to the presence of iron in the membrane. It would appear that treating the cells with nitric acid gave better results for this assay, although the improvement was not major.

The experiment to detect the presence of iron in the SDS-PAGE gel has no precedent. It does seem that it is a valid technique however, and has provided an interesting result. It is perhaps debatable whether the boiling of the gel pieces in nitric acid released all of the iron present in the gel but in this particular case this was not important. It was not intended to determine the total amount of iron in the gel track, only to demonstrate that the iron-grown cells contained proteins that were associated with iron, and that this was not so for the yeast extract-grown cells. The vast difference between the result obtained from the iron-grown cells in the 30 kD region of the gel and any of the other areas also strongly suggests the validity of this technique, since such a large difference is less likely to be due to experimental error.

The results presented here indicate that at least one of the proteins enhanced in strain BC1 by the presence of ferrous iron is in some way chemically associated with iron. It would appear that the higher levels of iron found in the membranes of iron-grown cells is entirely due to this association with the protein/proteins, since in other regions of the gel there is comparatively little difference between iron/yeast extract- and yeast extract-grown cells.

3.0. SOLUBILISATION OF THE MEMBRANE OF STRAIN BC1.

In order to further investigate the proteins that could be involved in the ferrous iron oxidation system, such proteins needed to be released from the membrane, so a series of experiments was undertaken to solubilise the membrane.

3.1. Initial Use of Detergents.

Initially a selection of detergents was used, coupled to an increase in the pH of the membrane-containing solution. (The original pH was that at which the membranes had been prepared, pH7). Despite using a wide range of detergents little success was achieved (Table 7). After treatment, spectra were run of the resulting supernatants and resuspended membrane pellets. These were particularly

Table 7. Percentage composition of the pellet and supernatant after the membrane fraction of iron- grown strain BC1 was treated with various detergents. The membrane's absorbance at 280 nm (protein), 604.5 nm (cyt. aa_3) and the iron content were measured before treatment. After treatment the same parameters were taken of the resulting membrane pellet and supernatant.

DETERGENT	FRACTION	pH	% A_{280}	% $A_{604.5}$	% IRON
DDGP	Supernatant	8	34.9	0.9	8.6
	Membrane	8	65.1	99.1	91.4
CHOLATE	Supernatant	8	48.3	3.0	23.5
	Membrane	8	51.7	97.0	76.5
PROPAN-2-OL	Supernatant	8	29.6	0.5	1.0
	Membrane	8	70.4	99.5	99.0
OTG	Supernatant	8	46.7	0.8	12.4
	Membrane	8	53.3	92.2	87.6
TRITON X - 100	Supernatant	9	48.0	2.0	29.0
	Membrane	9	52.0	98.0	71.0
OEG	Supernatant	9	28.5	1.1	25.1
SMC	Supernatant	9	18.1	2.1	15.4
	Membrane	9	91.9	97.9	94.6

important in the case of those treatments that appeared to be releasing large amounts of protein, since they were a method of detecting the presence of the cytochromes. As this was a preliminary investigation it was considered sufficient to estimate protein and cytochrome $\underline{aa}_3$ concentrations by absorption measurements at 280 nm and 604.5 nm respectively. Iron levels were determined by atomic absorption spectrophotometry.

The dodecyl-ß-D-glucopyranoside (DDGP) appears to have released about 35% of the protein into the supernatant, but only 9% of the total iron, and only 1% of the absorption at 604.5 nm, the α-peak absorption maximum for the cytochrome $\underline{aa}_3$. It therefore did not release the polypeptides of interest.

The cholate results show nearly half of the protein in the supernatant, and 25% of the iron. However an examination of the spectra of both the membrane and soluble fractions shows that the detergent has destroyed the proteins (Figure 56).

Propan-2-ol liberated 30% of the protein but showed a very poor release of the proteins of interest. Only 1% of the iron was released in this way and 0.5% of the 604.5 nm absorption. This was the poorest release of iron or the 604.5 nm absorption demonstrated by any of the initial detergents tested.

Octyl-ß-D-thioglucopyranoside (OTG) showed a more promising result. After treatment approximately 50% of the protein was found in the supernatant, along with over 12% of

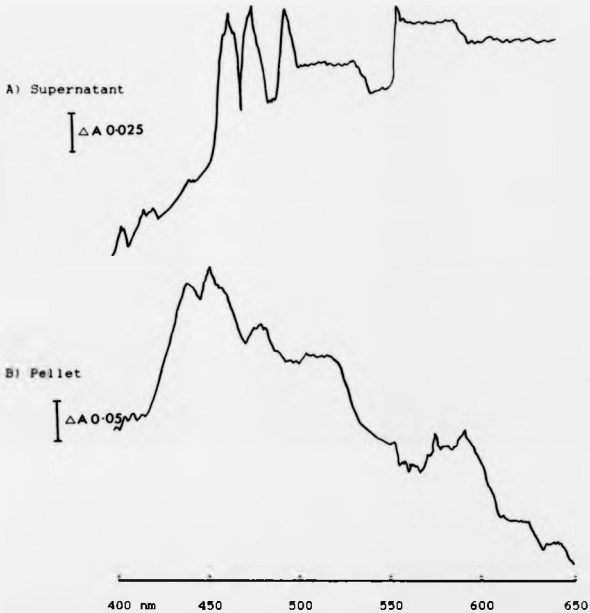


Figure 56. Reduced minus oxidised spectra of the resulting supernatant (A) and pellet (B) after a membrane pellet of strain BC1 was resuspended in a cholate solution, and then recentrifuged.

the iron. However the 604.5 absorption was still less than 1% in the supernatant. Unlike the cholate, where 50% protein recovery in the supernatant coincided with destruction of the membrane proteins, the spectra for the membrane after treatment with OTG shows the normal pattern. The supernatant spectrum however showed no sign of any cytochrome activity (Figure 57).

Sarkosyl caused the greatest release of protein of all the treatments. Over 70% of the protein was found in the supernatant after treatment. 65% of the iron was released by this detergent, along with 20% of the 604.5 nm absorption. However the spectra for the treated membrane and the resulting supernatant show that, as with cholate, the proteins were ruined by this treatment (Figure 58).

Triton X-100 also liberated nearly 50% of the protein. The release of iron and the 604.5 nm absorption was also good; 29 and 2% respectively. Unfortunately the spectra once again indicated that the resulting supernatant and membrane fraction had no active chromophores (Figure 59).

The sodium monocaprate (SMC) and octyl-ethyl-gluconoride (OEG) were used in conjunction. The membrane suspension was treated first with the OEG and the resultant membrane pellet then treated with SMC. After both treatments approximately 50% of the proteins had been removed, of which nearly 30% was removed in the first treatment with OEG. The two treatments together achieved the best removal of iron, just over 40% being released. Again the first treatment with OEG removed the majority. This dual treatment also produced

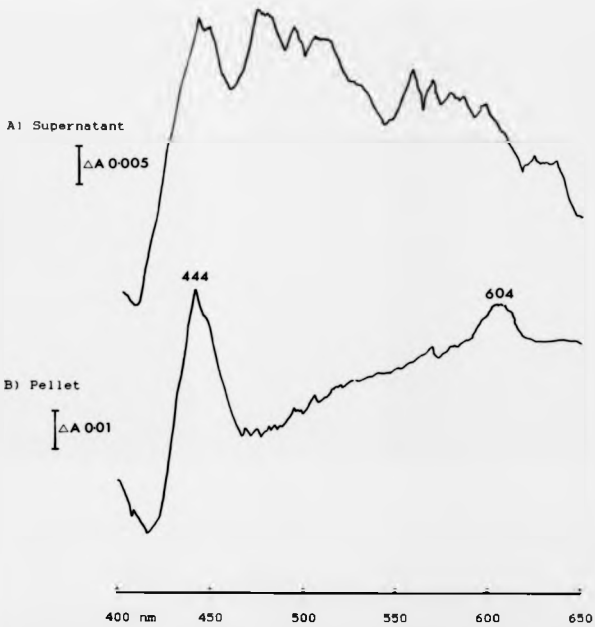


Figure 57. Reduced minus oxidised spectra of the resulting supernatant (A) and pellet (B) after a membrane pellet of strain BC1 was resuspended in an OTG solution, and then recentrifuged. The absorption maxima are given in nm.

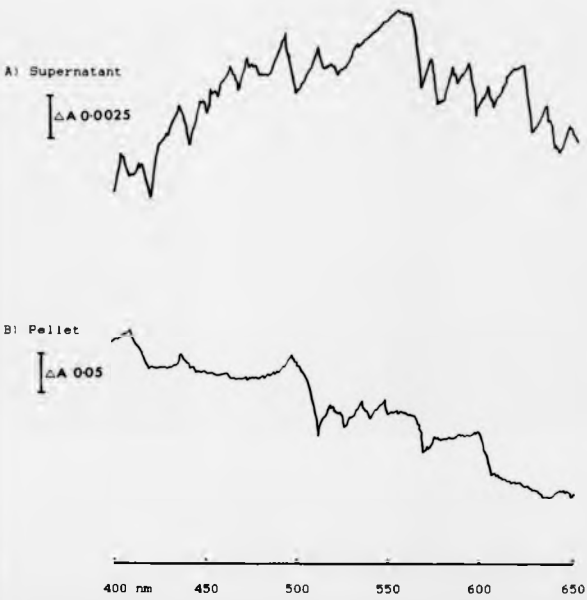


Figure 58. Reduced minus oxidised spectra of the resulting supernatant (A) and pellet (B) after a membrane pellet of strain BC1 was resuspended in a sarkosyl solution, and then recentrifuged.

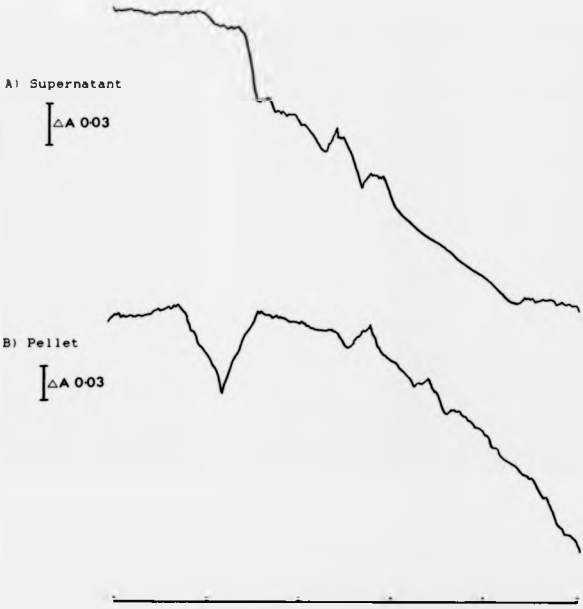


Figure 59. Reduced minus oxidised spectra of the resulting supernatant (A) and pellet (B) after a membrane pellet of strain BC1 was resuspended in a solution of Triton X-100 and then recentrifuged.

the highest removal of the 604.5 nm absorption, the total removal amounting to 3.2%. This time it was the second treatment that released the greatest amount. An examination of the spectra reveals that the only sign of any cytochrome occurred in the remaining membrane pellet, where the Soret peak for the aa_3 cytochrome can still be seen (Figure 61). Neither of the supernatants give any indication of the presence of cytochromes (Figures 60 and 61).

3.2. Use of Sonication, Heat and pH in Membrane Solubilisation.

As the results of the detergent treatments did not seem particularly promising, various other methods of membrane disruption were investigated. It was necessary to use a chemical method in conjunction with these methods so one of the detergents used previously was selected. Although the cholate had probably denatured the cytochromes during treatment, it had still shown a high degree of protein and iron release, and so was selected for this investigation (Table 8). As before spectra were run of the resulting supernatants and membrane pellets.

Sonication proved to be no more effective at releasing protein from the membrane suspension than cholate on its own. However both the iron release and the increase in 604.5 nm absorption in the supernatant indicate that this method does significantly increase the effect of the detergent. The iron release, at 34%, was the best of any of the treatments

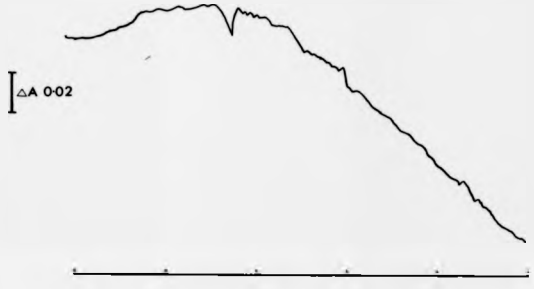


Figure 60. Reduced minus oxidised spectra of the resulting supernatant after a membrane pellet of strain BC1 was resuspended in an OEG solution, and then recentrifuged.

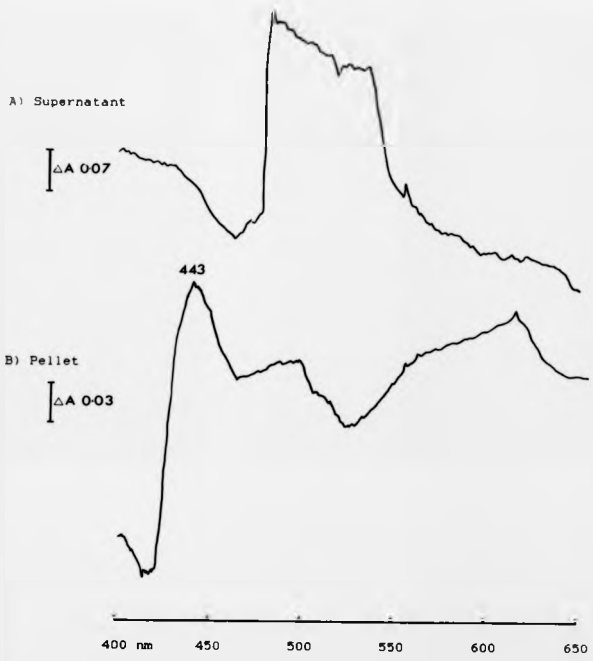


Figure 61. Reduced minus oxidised spectra of the resulting supernatant (A) and pellet (B) after a membrane pellet of strain BC1 was resuspended in a solution of SMC and then recentrifuged. The absorption maximum is given in nm.

TREATMENT	FRACTION	pH	% A_{280}	% $A_{604.5}$	% IRON
SONICATION	Supernatant	8	48.5	5.0	34.1
	Membrane	8	51.5	95.0	65.9
HEAT ($60^{o}C$)	Supernatant	8	48.2	2.5	28.2
	Membrane	8	51.8	97.5	71.8
ALKALINITY	Supernatant	9	48.6	6.7	30.0
	Membrane	9	51.4	93.3	70.0

Table 8. Percentage composition of the pellet and supernatant after the membrane fraction of iron- grown strain BC1 was treated with a cholate solution and various membrane disruption methods. The membrane's absorbance at 280 nm (protein), 604.5 nm (cyt. aa_3) and the iron content were measured before treatment. After treatment the same parameters were taken of the resulting membrane pellet and supernatant.

used. A major drawback to this method was the tendency of the membrane suspension to froth. Initially it caused nearly all of the solution to foam, but even with adjustments to the treatment it proved impossible to get this down to an acceptable level. The spectra show some evidence of cytochrome $\underline{aa}_3$ in the supernatant in the shape of a small Soret peak at 443 nm, although the membrane does show a better such peak (Figure 62). Although in both cases the spectra are clearer than when cholate was used on its own, there is still much evidence of the proteins being denatured by the detergent.

The effect of heat was also to enhance the action of the cholate. As with the sonication, the amount of protein released was not increased, but there was an increase in iron release by about 5%. The absorption at 604.5 nm was reduced slightly however, from 3% for cholate on its own to 2.5% for this treatment. The spectra for both the supernatant and the membrane show little evidence for cytochromes in either, although there is some evidence for a Soret peak at 444 nm in the supernatant (Figure 63).

As with both the treatments outlined above, increasing the pH to 9 did not increase the release of protein from the membrane solution; this remained at about 50%. This method did enhance the removal of iron from the membrane and gave the best increase in the 604.5 nm absorption readings in the supernatant. This increase in 604.5 nm absorption was the best of all of the techniques at this stage in the work. The spectra for this method show that the supernatant contains

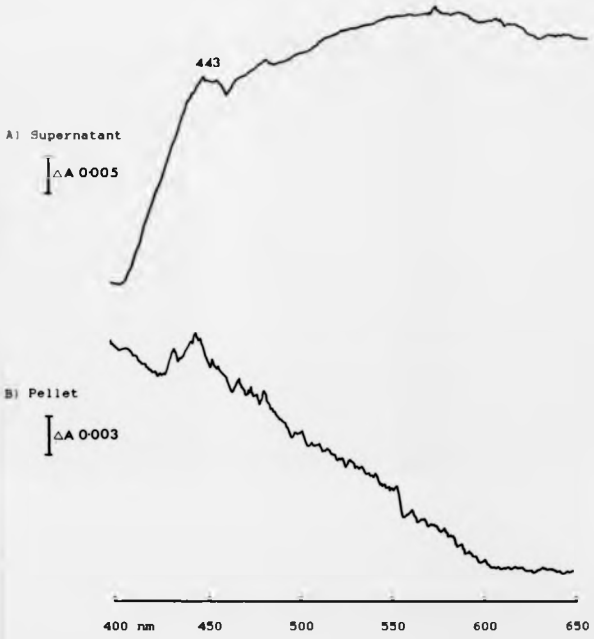


Figure 62. Reduced minus oxidised spectra of the resulting supernatant (A) and pellet (B) after a membrane pellet of strain BC1 was resuspended in a cholate solution, sonicated and then recentrifuged. The absorption maximum is given in nm.

Figure 63. Reduced minus oxidised spectra of the resulting supernatant (A) and pellet (B) after a membrane pellet of strain BC1 was resuspended in a cholate solution, heated to 60^{0}C and then recentrifuged. The absorption maximum is given in nm.

no visible sign of a cytochrome, whereas the membrane showed one of the clearest cytochrome $\underline{aa}_3$ results from any of these experiments (Figure 64).

3.3. Combination of Detergents With pH and Temperature.

It appeared that the application of sonication, heat or pH in conjunction with the detergents increased the solubilisation of the membrane. Since the use of cholate nearly always resulted in the loss of any recognisable cytochrome spectra, it was decided to try other detergents in conjunction with temperature and pH alterations. Sonication was discounted due to its tendency to produce so much foam from the sample. From the initial use of detergents it appeared that OTG would be a good alternative to cholate, especially since it did not appear to interfere with the cytochrome $\underline{aa}_3$ in the membrane. Some marked increases were observed using this combined approach (Table 9). As well as the OTG other detergents were also tried. As before spectra were run of the resulting supernatants.

Nonidet proved to be difficult to work with. The pellets produced after treatment were always very loose and required careful handling to ensure that the supernatant could be separated from the membrane. The supernatant itself was frequently layered and this could give rise to even more problems in separating the supernatant from the pellet. The nonidet used at pH 9 demonstrated little difference in protein release from that of the best of the other

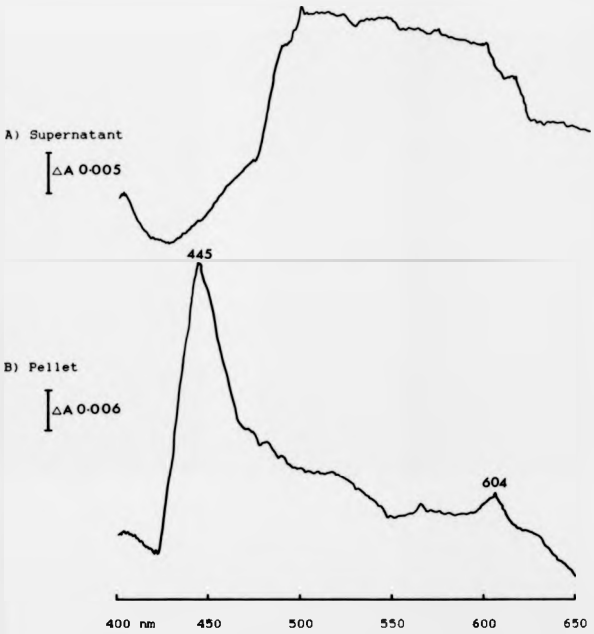


Figure 64. Reduced minus oxidised spectra of the resulting supernatant (A) and pellet (B) after a membrane pellet of strain BC1 was resuspended in a cholate solution, the pH adjusted to 9 and then recentrifuged. The absorption maximum is given in nm.

Table 9. Percentage composistion of the pellet and supernatant after the membrane fraction of iron-grown strain BC1 was treated with a given detergent and either temperature or pH disruption methods. The membranes absorbance at 280 nm (protein), 604.5 nm (cyt. aa_3) and the iron content were measured before treatment After treatment the same parameters were taken of the resulting membrane pellet and supernatant. (RT = room temperature).

DETERGENT	FRACTION	pH	TEMP.	% A_{280}	% $A_{604.5}$	% IRON
NONIDET AND HEAT	Supernatant	8	60°C	61.4	6.0	21.7
	Membrane	8	60°C	38.6	94.0	78.3
NONIDET AND ALKALI	Supernatant	9	RT	49.0	59.4	24.3
	Membrane	9	RT	51.0	40.6	75.7
TAURINE/ BETAINE AND HEAT	Supernatant	8	60°C	48.8	4.0	34.1
	Membrane	8	60°C	51.2	96.0	65.9
TAURINE/ BETAINE AND ALKALI	Supernatant	9	RT	51.7	2.2	40.1
	Membrane	9	RT	48.3	97.8	59.9
OTG AND HEAT	Supernatant	8	60°C	49.1	1.5	25.1
	Membrane	8	60°C	50.9	98.5	74.9
OTG AND ALKALI	Supernatant	9	RT	51.2	5.0	35.2
	Membrane	9	RT	48.8	95.0	64.8

detergents already tried but when used at high temperature there was a definite increase in the amount of protein to be found in the supernatant. Over 60% of the protein was released from the membrane solution in this manner, which was the best result for any of the techniques tried. The pattern is reversed for the 604.5 nm absorption. In this case the best result was obtained with the high pH, where the increase in absorption in the supernatant was again the best result of any of those tried, giving a large increase to 59%. The iron release in both was very similar and not particularly good in comparison to some of the initial detergents, neither being above 25%. The spectra were much improved. The temperature experiment showed a clear spectrum for cytochrome aa_3, although the peaks were not high (Figure 65a). The corresponding membrane spectrum is a very clear cytochrome aa_3 pattern (Figure 65b), and it would therefore appear that the nonidet did not interfere with the cytochromes to the extent that cholate did.

The taurine/betaine mix demonstrated no real advantages over any of the other methods. Both at a high temperature and a high pH the incidence of protein in the supernatant was still around 50%. Treatment with this mix and a temperature increase gave more 604.5 nm absorption in the supernatant than with a pH increase, but this 4% was still not as high as the nonidet or the OTG. Iron release was better in the pH-treated sample where it reached 40% compared to the 34% of the temperature treated sample. This level of iron release was the only advantage of this method

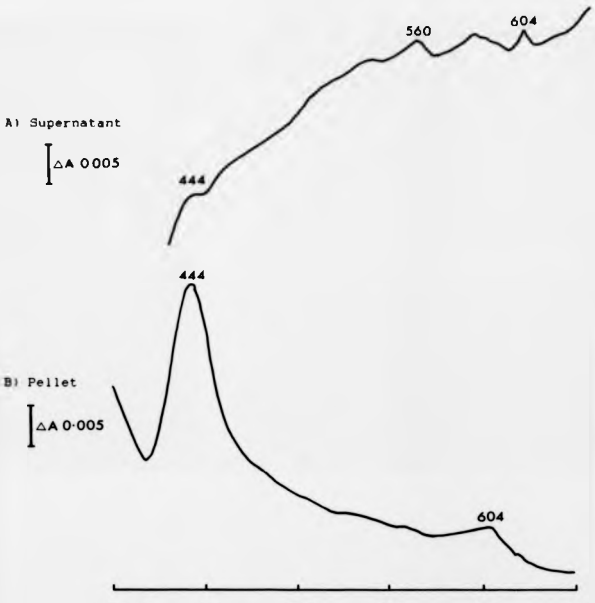


Figure 45. Reduced minus oxidised spectra of the resulting supernatant (A) and pellet (B) after a membrane pellet of strain BC1 was resuspended in a nonidet solution, heated to 60°C and then recentrifuged. The absorption maxima are given in nm.

over that of nonidet. The spectra were again very clear. There is a small peak in the supernatant of the temperature treated sample at 446 nm, indicative of cytochrome $\underline{aa}_3$, but comparison with the corresponding trace for the remaining membrane showed that there is far more in the membrane (Figure 66). Despite the fact that more 604.5 nm absorption was recorded in the supernatant of the temperature treated sample, it is the pH treated sample that showed the best cytochrome $\underline{aa}_3$ spectrum in the supernatant (Figure 67). The membrane treated in this way also gave a very clear spectrum for cytochrome $\underline{aa}_3$.

The OTG in conjunction with pH showed the best overall solubilisation of any of the techniques and methods used, although both the pH and the temperature alterations gave no significant increase over protein liberation, both being in the region of 50%. The effect of the temperature change was not as marked as that of the pH, as can be seen from the level of 604.5 nm absorption and the iron release. In the case of the temperature experiment only 1.5% of the total 604.5 nm absorption was in the supernatant, compared to 5% in the case of the pH experiment. Similarly, the increase in iron concentration in the supernatant of the pH treated membrane was not as high as for the temperature method. Only 25% of the iron was recovered in the supernatant compared to 35% in the case of the higher pH treatment. It was in the results of the spectrum that the OTG/pH treatment really showed a difference. The supernatant spectrum clearly showed Soret and α-peaks for cytochrome $\underline{aa}_3$ (Figure 68). These were

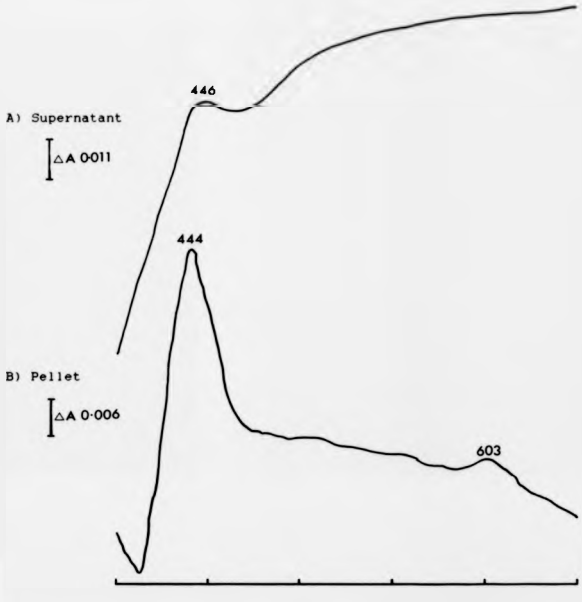


Figure 66. Reduced minus oxidised spectra of the resulting supernatant (A) and pellet (B) after a membrane pellet of strain BC1 was resuspended in a taurine/betaine solution, heated to 60°C and then recentrifuged. The absorption maxima are given in nm.

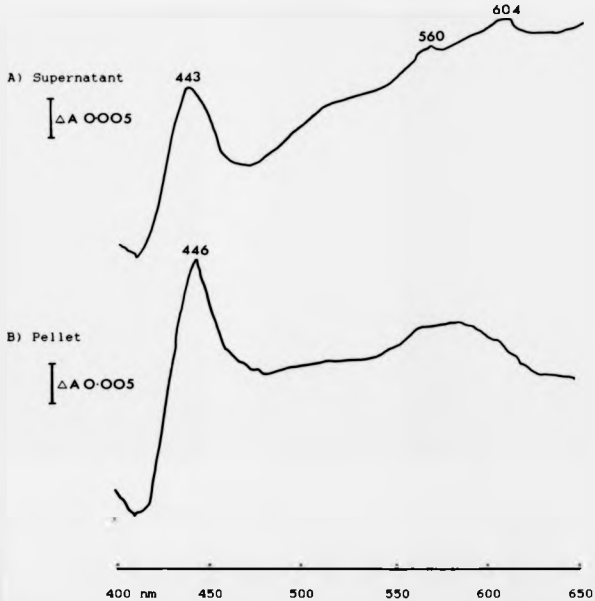


Figure 67. Reduced minus oxidised spectra of the resulting supernatant (A) and pellet (B) after a membrane pellet of strain BC1 was resuspended in a taurine/betaine solution, the pH adjusted to 9 and then recentrifuged. The absorption maxima are given in nm.

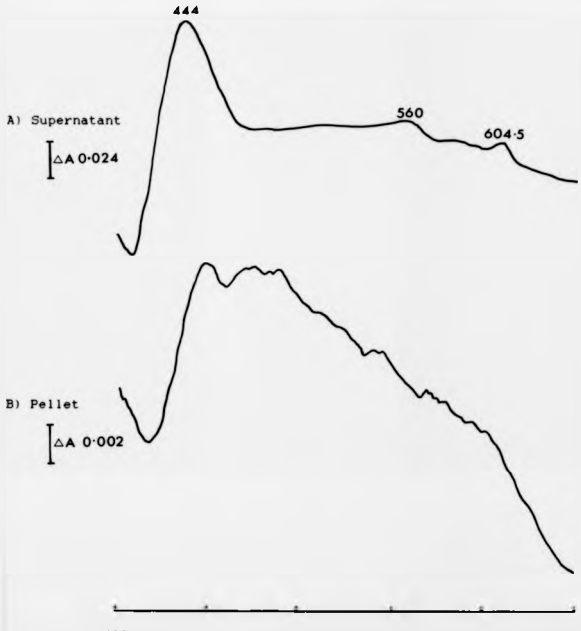


Figure 68. Reduced minus oxidised spectra of the resulting supernatant (A) and pellet (B) after a membrane pellet of strain BC1 was resuspended in an OTG solution, the pH adjusted to 9 and then recentrifuged. The absorption maxima are given in nm.

the best results obtained for any of the supernatants in the solubilisation investigation. This occurred despite the fact that the recovery of 604.5 nm absorption in the supernatant was lower than for some other treatments. This illustrates the fact that such a measurement could only be used as a general guide to cytochrome content, and is why spectra were run of all samples. The corresponding membrane spectrum also showed some cytochrome aa_3 but this signal was not as clear as that of the supernatant.

This method was improved by small adjustments in the incubation times, level of pH and concentration of the OTG. Using the optimised method the supernatant of approximately 100 mg of membrane became very brown. If the membrane pellet was then treated a second time, the supernatant was clear and the pellet had lost its colour.

DISCUSSION.

There are basically two types of detergent, non-ionic and ionic. Non-ionic detergents are widely used in membrane solubilisation because of their effectiveness and the fact that membranous proteins usually retain their activity. The non-ionic detergents used in this study included DDGP, Triton X-100 and OTG.

Triton X-100 is a commonly used detergent in the solubilisation of membrane-bound proteins. 1.5% Triton X-100 was used by Buse *et al.* (1989) in their purification of

cytochrome $\underline{c_1aa_3}$ from *T.thermophilus* and also, at a concentration of 4%, by Kitada and Krulwich (1984) in the solubilisation of the terminal oxidase from *B.firmus*. Sone *et al.* (1979) also used Triton X-100 in their preparation of cytochrome $\underline{caa_3}$ from PS3 but in conjunction with treatment by a cholate/deoxycholate mix. Yoshida *et al.* (1984) used 2% Triton X-100 in their extraction of cytochrome $\underline{caa_3}$ from *T.thermophilus*. The same extraction process could also be used to extract the membrane bound cytochromes c_{555} and c_{549}. Although Triton normally gives good retention of activity, in the case of the strain BC1 cytochrome $\underline{aa_3}$ it appeared to destroy the protein. Another problem with Triton is that it interferes with A_{280} readings, so that none of the readings at this wavelength can be relied upon.

ß-D-alkyl glucosides such as DDGP and OTG are also fairly regularly used in the solubilisation of membranes. The terminal oxidase from *B.subtilis* was solubilised by 1% dodecyl-ß-D-maltoside (DeVrij *et al.*, 1983), whilst Kai *et al.* (1989) were able to remove the terminal oxidase from *T.ferrooxidans* membrane with 1.5% octylthioglucoside. In another example of mixing detergents, Häfele *et al.* (1988) used 1% octyl-ß-D-thioglucoside in conjunction with 0.2% deoxycholate in solubilising cytochrome $\underline{aa_3}$ from *A.variabilis*. As well as having the advantages of effectiveness and the retention of protein activity that are seen in Triton, these compounds are also UV transparent and so do not interfere with A_{280} readings. Further advantages include a generally higher critical micelle concentration

(CMC) and a lower average molecular weight for the micelle than that of Triton.

Deoxycholate and cholate are ionic detergents which have a low CMC and micelle molecular weight, although this does depend somewhat on the pH and ionic strength of the solution. They generally do not have much effect in solubilising tightly bound membrane proteins, but are often used in the preliminary extraction of unwanted proteins.

Hunter *et al.* (1989) used some unusual membrane solubilisers in their study of the membrane bound cytochrome c_4 in *Pseudomonas stutzeri* and *Azotobacter vinelandii*. Both 30% propan-2-ol and 1 - 2 molar iodide removed the cytochrome from *P.stutzeri*, although the propan-2-ol was not effective with either *A.vinelandii* or, in this case, strain BC1 membranes.

It was not likely that sonication, heat or pH changes on their own would be capable of solubilising the cytochrome aa_3, but the study did highlight this and helped to indicate what treatment was likely to work in conjunction with a detergent. In most of the examples of treatments used by other authors given above it was found that there was no need for any extra treatment apart from the incubation in the detergent of choice. Exceptions include Häfele *et al.* (1988) who also agitated membranes in the presence of biobeads and Buse *et al.* (1989) who homogenised membranes in the presence of the Triton. There can be no doubt that in the case of strain BC1 however, the elevation of the pH increased the effectiveness of the OTG.

Baines and Poole (1985) undertook a similar study into the relative capabilities of detergents to solubilise the membranes of the PSJ. This as yet unamed organism is Gram-positive, spore forming and thermophilic. They found that at Triton X-100 concentrations below 0.8%, although around 50% of the protein was released no cytochrome $\underline{aa}_3$ was solubilised. Even at 1.0% only approximately 5% of the cytochrome had been released. Strain BC1 membranes behaved rather differently in that the release of cytochrome $\underline{aa}_3$ was far better than this when Triton X-100 was used, as measured by A_{604}. With ß-D-dodecylglucoside far better solubilisation of the PSJ membranes occurred. Concentrations as low as 0.4% caused more solubilisation than 1% Triton X-100, whilst at 1% over 40% of the cytochrome $\underline{aa}_3$ had been released. This all highlights the fact that, as was also seen with the strain BC1 membranes, the extent of release of proteins from membranes by detergents depends greatly on their mode of action and is therefore selective. Even a high percentage of protein release is not a guarantee that there is any significant release of a specific protein, particularly if the protein is tightly bound, as many of the cytochromes $\underline{aa}_3$ seem to be.

A second treatment of the membrane with OTG gave no improvement in its solubilisation. Both the supernatant and the pellet were colourless. This combined with the fact that there was very little scanning spectroscopy evidence for cytochrome $\underline{aa}_3$ in the supernatant after solubilisation is in conflict with the $A_{604.5}$ readings of the pellet and

supernatant after solubilisation. The latter may seem to suggest that only 5% of the terminal oxidase is in the supernatant after solubilisation. However, the spectra are far more likely to show an accurate picture of the distribution of the cytochrome since it is a more specific detection method.

4.0. COLUMN CHROMATOGRAPHY OF MEMBRANE EXTRACT.

4.1. Gel Filtration.

Having refined the OTG/pH treatment (see section 3.3) until a high level of membrane solubilisation was occuring, the resulting membrane extract was passed down several types of gel filtration columns. This was done in order to try to isolate the cytochrome $\underline{aa}_3$ and the putative `iron protein'.

Before the samples were added to the columns spectra were run in order to ascertain that cytochrome could be readily detected. All of the spectra for this work were obtained following initial oxidation with sodium hexachloroiridate rather than ammonium persulphate, since this gave better results. Reduction of samples was still achieved with dithionite. Protein assays were also carried out. Initially in the chromatography column elution profiles, A_{420}, A_{440} and $A_{604.5}$ were used in order to detect the presence of cytochrome $\underline{aa}_3$. It was found, however, that

the readings for A_{420} were appreciably higher than for A_{440} (data not shown) and so the use of A_{440} discontinued. The spectrum of the membrane before treatment was well defined (Figure 69a). After solubilisation, the resulting supernatant also gave a very clear cytochrome $\underline{aa}_3$ spectrum (Figure 69b). The protein assays showed that the concentration of protein in the membrane suspension after treatment but before centrifugation was 7000 µg/ml, whereas after centrifugation the supernatant contained 6000 µg/ml. This meant that 85.7% of the protein in the membrane originally was released into the supernatant upon treatment. It appears that the OTG in the sample after treatment did not interfere with the absorption of the sample, neither did the buffer or the raised pH. Dialysis of the solubilised membrane had no effect on the difference spectra.

4.1.1. Sephadex G-75 Column.

The sample was then added to a Sephadex G-75 column. The fractions collected from this column were analysed for protein by absorption at 280 nm and cytochrome $\underline{aa}_3$ by absorption at 420 nm and 604 nm. These results were then plotted to give an elution profile (Figure 70). No separation of the proteins was indicated by this elution profile. There was a sharp peak for the 420 nm and 604 nm absorptions in fraction 6. Although the protein showed only one peak, this was a very broad peak and it was clear that at least some of the protein could be removed from the fractions containing the cytochrome. However the more

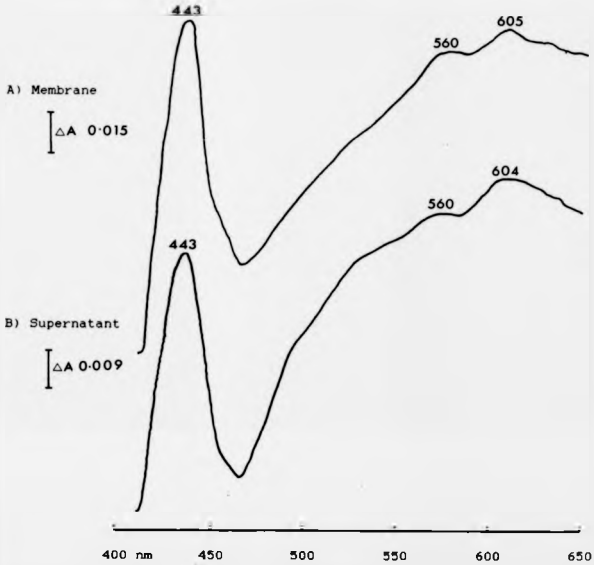


Figure 69. Reduced minus oxidised spectra of strain BC1 membrane before solubilisation (A) and the supernatant after solubilisation (B). The solubilised protein was passed through a Sephadex G-75 column. Membrane was solubilised by the OTG / pH method (see section 3.3). Absorbance maxima are given in nm. Protein concentrations were as follows, (mg/ml) (A) 1.75, (B) 1.5.

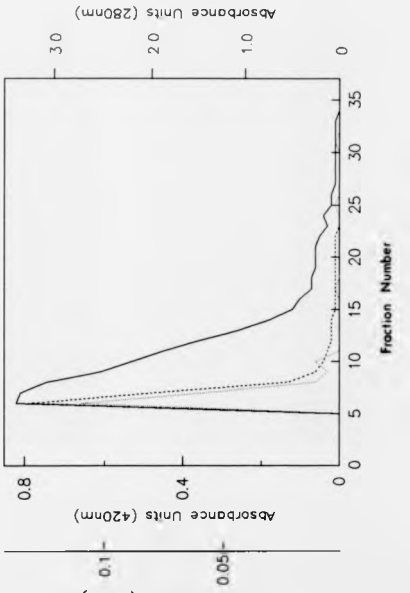


Figure 76. The elution profile following gel filtration by Sephadex G-75 of a solubilised membrane extract of iron-grown strain BC1. Fractions were assayed spectrophotometrically for protein (A_{280}, ——), and cytochrome $\underline{aa}_3$, ($A_{604.5}$, ·····, A_{420}, ---). Membrane extract prepared as detailed in materials and methods.

important observation was that the cytochrome appeared to have come straight through the column and been released in the void volume. This implied that the cytochrome was of a greater molecular weight than 75 kD, the cut-off point for this particular column matrix. A reduced minus oxidised spectrum of a sample of fraction six confirmed that the cytochrome $\underline{aa}_3$ was within this peak (Figure 71a).

4.1.2. Sephacryl S300 Column.

Because the cut-off point of the Sephadex G-75 was too low a column with a cut-off of 300 kD was used. This was a Sephacryl S300 column. As with the previous column, an elution profile was plotted using the same absorption parameters (Figure 72). In addition, the concentration of iron in each sample was assayed by atomic absorption spectrophotometry. An examination of this profile reveals that once again the cytochrome $\underline{aa}_3$ came down in the void volume, the peak being at fraction twenty. This peak also coincided not only with one of the two protein peaks but also with the major iron peak. A second broader protein peak occurred around fraction 28. There was no corresponding 420/604 nm peak but the iron concentration of the fractions did stop decreasing and only continued to decrease when the protein content once again dropped. It appeared that the cytochrome was even larger than 300 kD, although better separation was possible with this column than with the Sephadex G-75. The reduced minus oxidised spectrum for fraction 20 clearly showed that this fraction did indeed

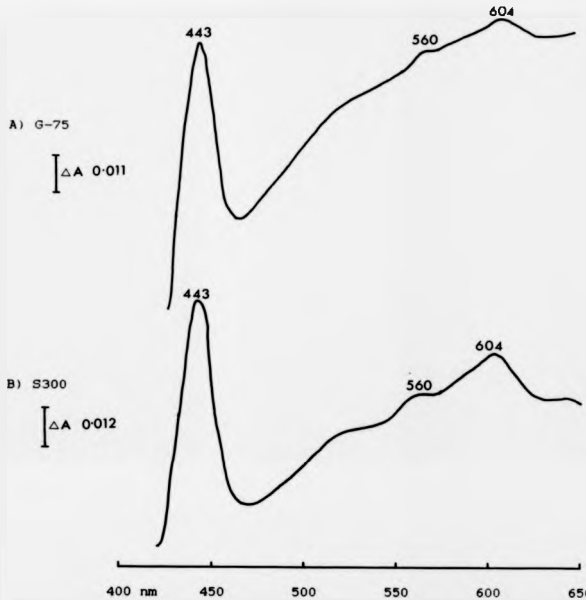


Figure 71. Reduced minus oxidised spectra of void volume fraction containing the cyt $\underline{aa}_3$ after solubilised strain BC1 membrane was passed down a Sephadex G-75 column (fraction 6) (A) and a Sephacryl S300 column (fraction 20) (B). Absorbance maxima are given in nm. Protein concentrations were as follows, (mg/ml) - (A) 2.48, (B) 1.4.

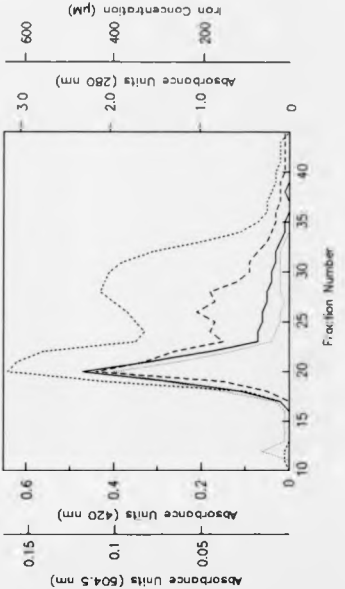


Figure 71. The elution profile following gel filtration by Saphacryl S300 of a solubilised membrane extract of iron-grown strain BC1. Fractions were assayed spectrophotometrically for protein (A_{280}, – – –), and cytochrome aa_3, ($A_{604.5}$, · · · · ·, A_{420}, ——). Iron concentration was measured by atomic absorption spectrophotometry (— —). Membrane extract prepared as detailed in materials and methods.

contain the cytochrome $\underline{aa}_3$ (Figure 71b). A spectrum for fraction 28 did not show any evidence of any cytochrome.

4.1.3. Sepharose CL-6B.

Subsequent gel filtration steps were carried out using a Sepharose CL-6B column matrix. This has an exclusion limit of four million kD. Once again an elution profile was built up using the same parameters as with the S300 column (Figure 73). This profile showed three peaks, although none of these peaks were completely separate. The first two protein peaks had corresponding peaks for both cytochrome $\underline{aa}_3$ and for iron. The third protein peak had no such corresponding levels of either cytochrome or iron. Reduced minus oxidised spectra of all three peaks were obtained. The first peak showed very little sign of the cytochrome, although there did appear to be a small Soret peak at 444 nm (Figure 74a). The second peak gave a much clearer, more positive cytochrome $\underline{aa}_3$ spectrum, with both the Soret and α-peaks visible along with the 560 nm peak (Figure 74b). The third peak did not show any sign of a cytochrome. It appeared from this that the cytochrome was still eluting in the void volume, despite the extremely large exclusion limit of this column matrix. However it was true to say that reasonable separation was now occurring.

4.2. Ion Exchange.

As well as using gel filtration in an attempt to

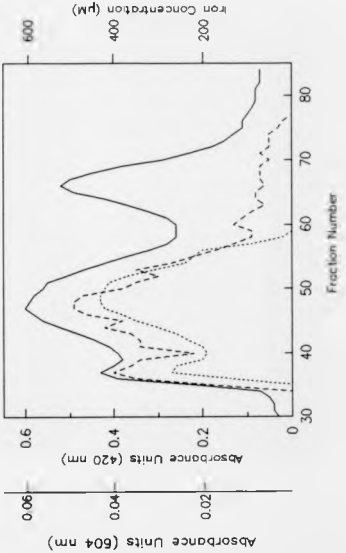


Figure 73. The elution profile following gel filtration by Sepharose CL-6B of a solubilised membrane extract of iron-grown strain BC1. Fractions were assayed spectrophotometrically for protein (A_{280}, ——), and cytochrome aa_3, ($A_{604.5}$, – –). Iron concentration was measured by atomic absorption spectrophotometry (— —). Membrane extract prepared as detailed in materials and methods.

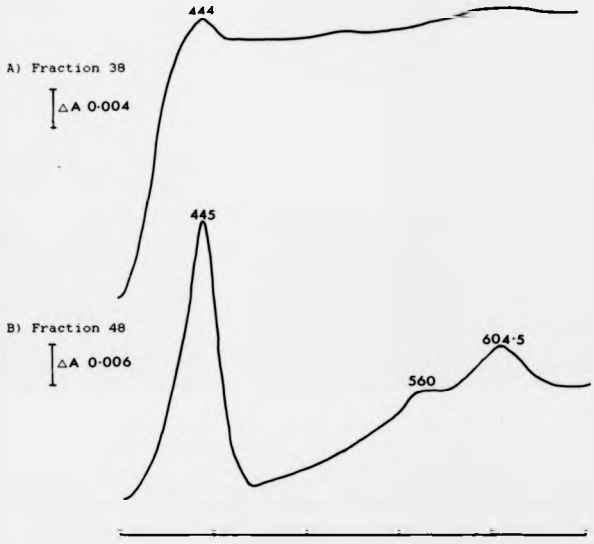


Figure 74. Reduced minus oxidised spectra of the two fractions containing 604.5 nm absorbance and iron after solubilised membrane from strain BC1 was passed down a CL-6B column. Absorbance maxima are given in nm. Protein concentrations were as follows, (mg/ml) - (A) 0.67, (B) 0.51.

achieve protein separation, ion exchange columns were also set up. As with the gel filtration experiments elution profiles were plotted for each matrix, and reduced minus oxidised spectra obtained for any peaks with correct absorption profiles. The oxidant for all fractions was sodium hexachloroiridate and the reductant was dithionite. Spectra of the sample before and after solubilisation indicated that cytochrome $\underline{aa}_3$ was present in large amounts in both. Protein assays showed a slightly lower level of solubilisation, approximately 70% of the protein in the membrane was to be found in the supernatant after the solubilisation process. (Protein concentration of the membrane suspension before spinning was 8400 µg/ml, whereas after spinning the concentration in the supernatant was 5900 µg/ml)

4.2.1. DEAE Sepharose Column.

The first column tried was an anion exchanger, DEAE Sepharose. The elution profile from this column showed two large protein peaks and two smaller ones (Figure 75). Both the larger peaks, around fraction 4 and fraction 24, had corresponding peaks for the cytochrome absorption wavelengths, but the fraction 4 peak contained much higher levels. The two smaller peaks, at fraction 36 and fraction 41 showed no real levels of absorption at either cytochrome absorption wavelength, A_{420} or $A_{604.5}$. The first peak contained the proteins that were washed straight through and were therefore positively charged or uncharged. The second

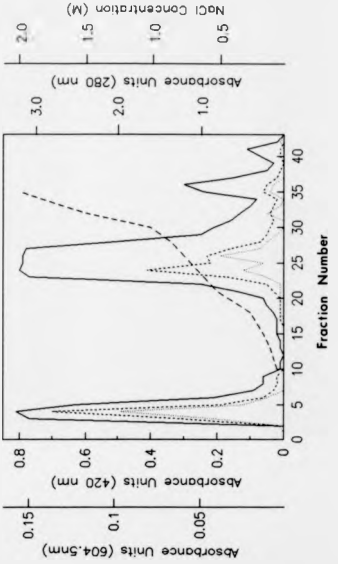


Figure 75. The elution profile following ion exchange chromatography by DEAE Sepharose of a solubilised membrane extract of iron-grown strain BC1. Fractions were assayed spectrophotometrically for protein (A_{280}, ——), and cytochrome aa_3, (A_{420}, ---, $A_{604.5}$, · · · · ·). NaCl concentration gradient is also given (— —). Membrane extract prepared as detailed in materials and methods.

peak required 500 - 600mM salt to remove it from the column matrix. Spectra for fractions 4 and 24 were then obtained. The spectrum for fraction 24 showed little sign of any cytochrome $\underline{aa}_3$ (Figure 76b). There was a small ridge that occurred at approximately 450 nm that may have been due to the cytochrome but at a very low concentration. Fraction 4 gave a very clean spectrum. The peaks were smooth and there was very little background noise. It would appear that the cytochrome passed straight through the column, but that much of the other proteins were removed during this process, which led to a much clearer spectrum being obtained (Figure 76a).

4.2.2. CM Sephadex Column.

Fractions 3,4,5 and 6 were pooled and applied to the cation exchange column matrix CM Sephadex. In this case the elution profile showed only one protein peak, at fractions 2 and 3 (Figure 77). This peak coincided with the only peaks for 420 nm and 604 nm, indicating that the cytochrome within the sample was all contained within this protein peak. The spectrum of fraction 2 showed that the cytochrome was still present in the sample and confirmed that it had not bound to the column (Figure 78).

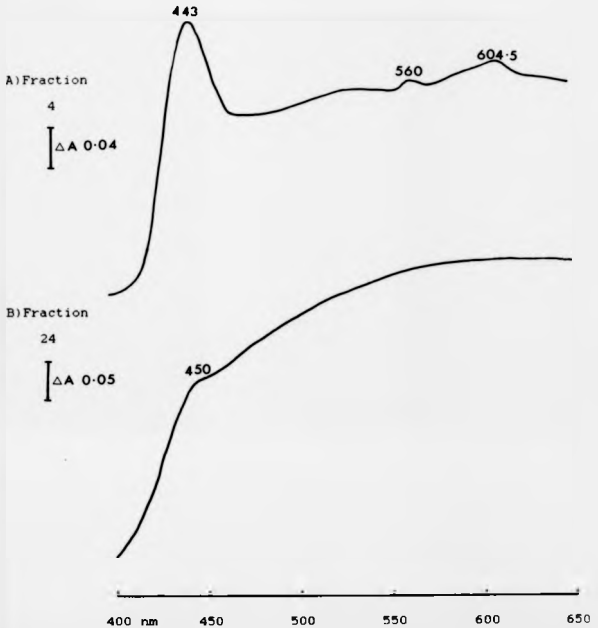


Figure 7d. Reduced minus oxidised spectra of the two fractions containing 604.5 nm absorbance after solubilised membrane from strain BC1 was passed down a DEAE Sepharose column. Absorbance maxima are given in nm. Protein concentrations were as follows, (mg/ml) - (A) 2.68, (B) 1.0.

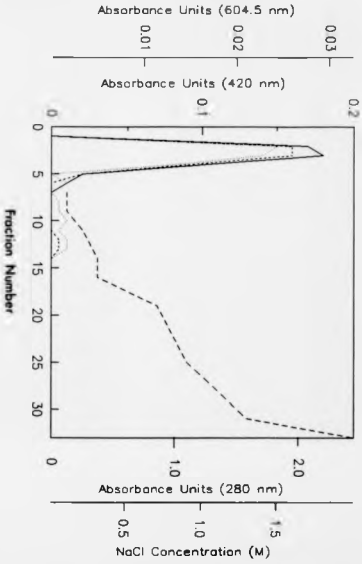


Figure 77. The elution profile following ion exchange chromatography by CM Sephadex of fractions 3, 4, 5, and 6 from the DEAE Sepharose treatment of membrane extract of iron-grown strain BC1 (Fig. 62). Fractions were assayed spectrophotometrically for protein (A_{280}, ——), and cytochrome aa_3, (A_{420}, ---, $A_{604.5}$, · · · ·). NaCl concentration gradient is also given (— —). Membrane extract prepared as detailed in materials and methods.

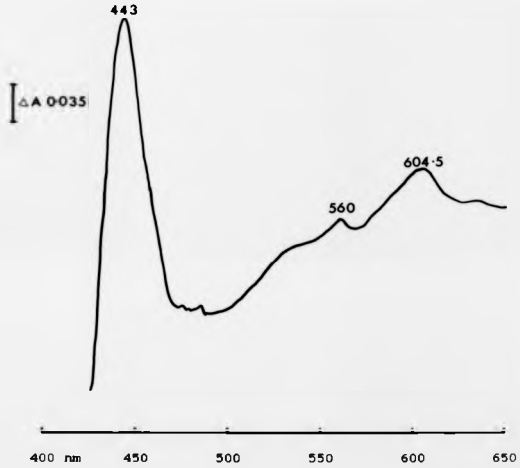


Figure 78. Reduced minus oxidised spectrum of fraction 2 after ion exchange chromatography by CM Sephadex (Fig. 64.) Absorption maxima are given in nm. Protein concentration was 0.85 mg/ml.

DISCUSSION.

The effectiveness of the solubilisation technique was shown by the high percentage of protein that was released from the membrane. DeVrij *et al.* (1983) reported a 70% solubilisation of the membrane proteins from *B.subtilis* which compares well with the 70 - 80% solubilisation shown by the technique employed on strain BC1.

During the preparation of the elution profiles it was noted that the absorption at 420 nm was higher than at 440 nm. From this it follows that the cytochrome $\underline{aa_3}$ was probably mainly in the oxidised form since this was the wavelength region at which many oxidised cytochrome $\underline{aa_3}$s absorb. It appears that the use of A_{420} and $A_{604.5}$ did not give an accurate picture of the distribution of the terminal oxidase, since some of the smaller peaks seen in the elution profiles at these wavelengths did not give valid cytochrome $\underline{aa_3}$ difference spectra. Yoshida *et al.* (1984) found the same principle applied to their purification of a cytochrome $\underline{c}$ from *T.thermophilus* - difference spectra of fractions giving elution profile peaks at the appropriate wavelength did not necessarily indicate the presence of cytochrome c.

The above attempts at purification of components of the iron-oxidising system of strain BC1 encountered several problems. It proved impossible to separate completely any of these components by gel filtration, although the Sepharose CL-6B column did demonstrate some separation. Given the void volume 'cut-off' for this column, it is highly unlikely that

the cytochrome $\underline{aa}_3$ was too large for separation. The cytochrome $\underline{aa}_3$ isolated from *B.subtilis* had an apparent molecular weight of 115 kD (DeVrij *et al.*, 1983), that of *B.firmus* 110 kD (Kitada and Krulwich, 1984) and that of *Rhodobacter sphaeroides* 117 kD (Azzi and Gennis, 1986). Smaller terminal oxidases include that of *Pseudomonas AM1* which has a molecular weight of 80 kD (Ludwig, 1987) and the $\underline{ba}_3$ complex of *T.thermophilus* which it would appear consists of a single 35 kD polypeptide (Zimmerman *et al.*, 1988). A likely explanation for the apparent size of the cytochrome is that the proteins are aggregating. It is known that non-ionic detergents such as OTG do not readily break protein-protein interactions. Consequently it seems likely that the proteins in the solubilised membrane are aggregating and thus causing the cytochrome $\underline{aa}_3$ and the 'iron protein' to pass straight through the column. A similar explanation would be that although most of the proteins were released into solution they were still bound to large amounts of membrane thus effectively increasing their size.

The results of the ion-exchange chromatography were also unexpected. The natural assumption when the elution profile and difference spectrum from the DEAE-Sepharose column were examined was that a cation-exchanger was needed and yet this too failed to bind the cytochrome $\underline{aa}_3$. There can be no doubt that the initial DEAE-Sepharose column was functioning well since it was capable of good protein separation. It would seem, however, that the aggregation of

proteins that probably produced the anomalous results in the gel filtration experiments was having less effect and allowing separation to occur. The presence of a relatively high concentration of ferric iron may have been at least partially responsible for the behaviour of the sample in the ion-exchange columns. It may have been possible to remove the iron with a chelating agent but the iron was being used a marker for the presence of the putative 'iron protein' and there was always the possibility that the iron was an integral part of the protein and therefore any attempt to remove it would damage the protein. In those elution profiles where the iron concentration was plotted it coincided with the A_{604} and A_{420} absorptions. Although there is no complete iron profile for the Sepharose CL-6B elution profile, the concentration of iron was determined for the individual peaks and was found to follow the same pattern, i.e. where there was a high A_{604}/A_{420} peak there was a correspondingly high iron concentration. This could suggest that it is the cytochrome $\underline{aa}_3$ that is the polypeptide that was demonstrated to be associated with iron but there is nothing else as yet to support this hypothesis.

The 560 nm peak seen in the solubilised membrane difference spectra was present in all the cytochrome $\underline{aa}_3$ difference spectra after the various column experiments. During the preparation of cytochrome $\underline{aa}_3$ from *B.subtilis*, DeVrij *et al.* (1983) produced a difference spectrum of the solubilised membrane that had peaks at 427 nm, 443 nm, 559 nm and 601 nm. After further purification steps the 427 nm

and 559 nm peaks disappeared, indicating that a membrane bound cytochrome c had been removed by the purification process. This removal of a peak in a difference spectrum could not be demonstrated in strain BC1 and it therefore seems likely that the 560 nm peak was indeed due to the cytochrome aa_3 itself.

5.0. AMMONIUM SULPHATE FRACTIONATION OF MEMBRANE EXTRACT.

Ammonium sulphate fractionation was attempted with a small sample of solubilised membrane. The drop in the absorption at 420 nm and 604 nm showed that after 50% ammonium sulphate saturation had been reached nearly all of the cytochrome had been removed (Figure 79).

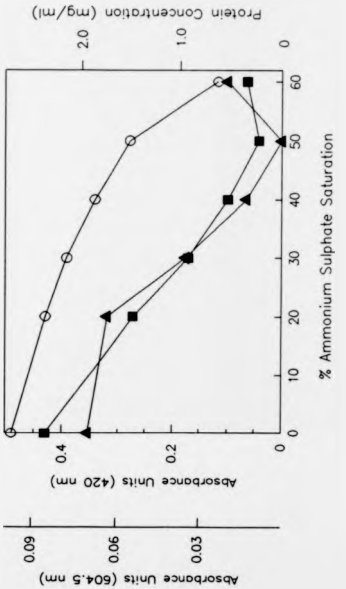


Figure 79. Ammonium sulphate fractionation of solubilised membrane extract of iron-grown strain BC1. As the ammonium sulphate concentration is increased, the protein concentration (○) decreases, as does the cytochrome aa_3 content, as measured spectrophotometrically - $A_{604.5}$ (▲) and A_{420} (■).

CHAPTER SEVEN.

R E D O X T I T R A T I O N S .

Redox titrations were carried out as a preliminary investigation into the characteristics of the isolated cytochrome aa_3 from BC1. As a control for the efficacy of the procedure the same was done for the c and a_1 cytochromes in *T.ferrooxidans* for which previous data are available.

1.0. Thiobacillus ferrooxidans.

Both the cytochrome c_{551} and cytochrome a_1 in this organism were examined. In order to ascertain that the cytochromes were present in sufficient quantities, spectra were run of the sample to be used for the redox titrations.

1.1. Cytochrome c α-peak.

The α-peak at 551 nm was the first to be examined. The spectrum for the sample used indicated that the cytochrome was present in sufficient levels and that it was active. As the potential of the solution dropped the peak height increased. This increase was measured in terms of absorbance units. The relative peak heights were plotted against the potential, (Figure 80), and the resulting graph was used to determine the mid-point redox potential for this cytochrome. The resulting potential of +320 mV was similar to other published values (See discussion).

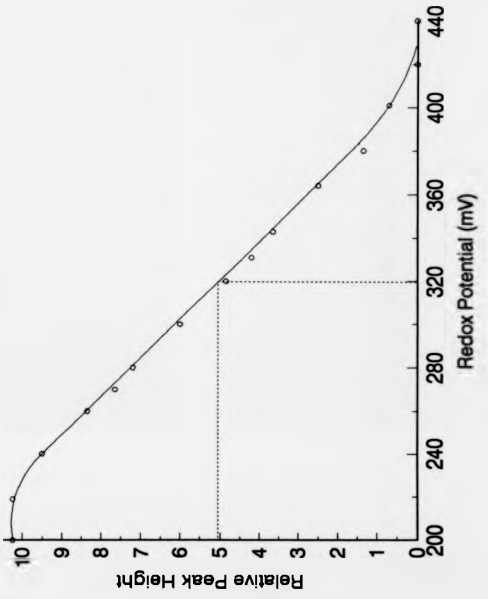


Figure 80. Relative peak height reduction of *T.ferrooxidans* cytochrome *c* α-peak in a soluble fraction preparation against redox potential obtained by reductive titration. Protein concentration was 6.13 mg/ml.

1.2. Cytochrome a_1 α-peak.

The α-peak of the terminal oxidase was examined in the same manner as cytochrome c. The initial reduced minus oxidised spectra for the sample used indicated that the sample contained sufficient active cytochrome. As might be expected the peak height began to increase at higher potentials than was the case with cytochrome c, indicating a higher mid-point redox potential. The plot of potential against increase in absorbance at 597 nm, (Figure 81), gives a mid-point redox potential of +497 mV, which again is comparable with other published data (See discussion).

2.0. Strain BC1.

This organism contains three readily observable cytochrome peaks in reduced minus oxidised spectra. Of these, two appear to be due to the presence of cytochrome aa_3. The α-peak of this cytochrome was investigated for the first time.

2.1. Cytochrome aa_3 α-peak.

The α-peak for this cytochrome was obtained at 604 nm in a reduced minus oxidised spectrum. The initial spectrum indicated that the sample to be used was active and present in sufficient concentration. The peak began to appear soon after the redox potential was dropped. The plot of redox potential against absorbance change at 603.5 nm allowed a mid-point redox potential of +528 mV to be calculated. A

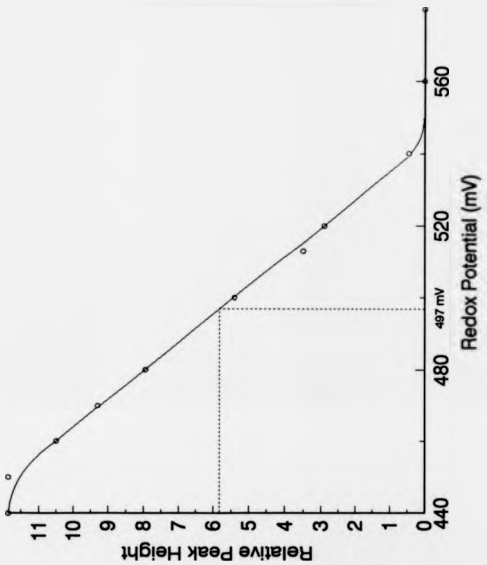


Figure 81. Relative peak height reduction of *T.ferrooxidans* cytochrome a_1 α-peak in a soluble fraction preparation against redox potential obtained by reductive titration. Protein concentration was 5.74 mg/ml.

subsequent repeat of this experiment gave a mid-point of +520 mV (Figure 82). The average mid-point redox potential of this cytochrome is therefore calculated as +524 mV, slightly higher than the value calculated for the terminal oxidase of *T.ferrooxidans*.

DISCUSSION.

The redox potentials for several of the constituents of the electron transport chain of *T.ferrooxidans* were calculated by Ingledew and Cobley (1980). These were calculated at two different pH values, pH 7.0 to determine the standard redox potential and pH 3.2, considered to be near to the physiological pH of the periplasm. In Ingledew and Cobley's work at pH 7.0, the cytochrome $\underline{c}_{551}$ peak indicated a redox potential of +300 mV, which is close to the value obtained in this study of +320 mV. However, Sato *et al.* (1989), working with the purified cytochrome reported a redox potential of +360 mV, which is higher and may illustrate the value of working with purified cytochrome but is more likely to be due to the rather different method used. These values are slightly higher than those reported for other c-type cytochromes, for example, the cytochrome $\underline{c}_{551}$ of PS3 has a redox potential of +225 mV (Sone *et al.*, 1989) and the cytochrome $\underline{c}_{552}$ of *T.thiooxidans* has a value of +247 mV (Takakuwa, 1975). There was also good agreement as regards the cytochrome $\underline{a}_1$. Ingledew and Cobley (1980)

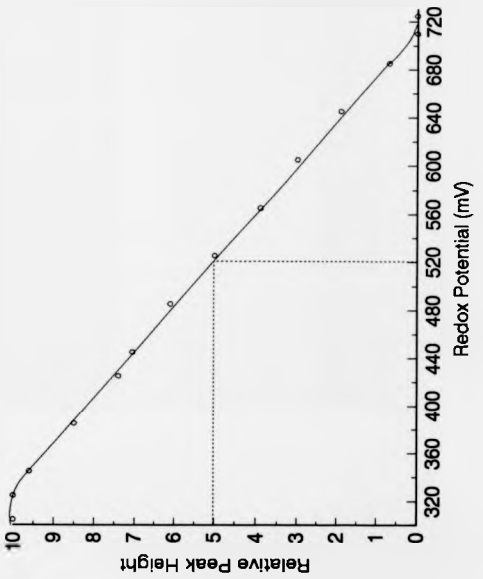


Figure #1. Relative peak height reduction of strain BC1 cytochrome aa_3 α-peak in a soluble fraction preparation against redox potential obtained by reductive titration. Protein concentration was 8.53 mg/ml.

found evidence for two cytochrome a_1s, which at pH 7.0 had redox potentials of +420 mV and +500 mV. The latter value is very close to the +497 mV recorded here.

The agreement with established *T.ferrooxidans* values not only served to confirm those results but more importantly demonstrated that the apparatus and technique used were suitable and accurate. Ingledew and Cobley (1980) found that the use of dithionite as a reductant gave rise to a black precipitate and so had to use other reductants, but the oxidant in both studies was the same, sodium hexachloroiridate.

CONCLUSIONS

C O N C L U S I O N S .

The known variety of sulphur- and iron-oxidising acidophilic organisms has greatly increased in recent years. It is therefore no surprise that the investigation into such organisms is still in its infancy. The basic investigations described at the beginning of the results section reflects this. Most of the isolates examined are little known and much is still to be learned about them. Indeed there is only one well studied mineral solubilising organism, *T.ferrooxidans*, and this is why this organism was used for comparative purposes at most stages of this work. The moderate thermophiles showed a wide range of nutritional versatility and demonstrated different doubling times or solubilisation rates on iron, pyrite and sulphur. In some cases the influence of past growth history on the capacity to oxidise either of these two substrates was very significant. The iron and sulphur oxidation capacity of strain BC1 appeared to be little influenced by its substrate history whereas *T.ferrooxidans* and strain LM2 were clearly influenced by growth history. It would appear that different strains tested could regulate their oxidation systems in a different manner. The variety in oxidation systems was evidenced in the SDS PAGE analyses, where the various strains investigated showed different proteins involved in the oxidation of iron or sulphur.

Optical spectroscopy indicated that strain BC1 did not contain any major soluble electron transport components.

This probably reflects the Gram-positive nature of the organism. Iron oxidation must occur within the cell membrane, and since a Gram-positive organism lacks a true periplasm within its membrane, it is not likely to have soluble electrons carriers in its iron oxidation system. The cell fraction spectra indicated that the only major chromophore was a membrane-bound cytochrome $\underline{aa}_3$. This cytochrome was slightly unusual in that, as well as the expected α-peak at 604 nm, it also appeared to have a second peak at 560 nm. The column chromatography experiments suggested that this was not due to an associated cytochrome, as did the evidence of the spectra themselves.

SDS PAGE analysis had shown two protein bands that increased in concentration after growth on iron. The investigation into the iron content of these proteins in the gel suggested that at least one of the proteins was bound to iron or had iron as part of its integral struture. The column chromatography experiments may suggest that it was the terminal oxidase that was so associated, since the cytochrome-containing fractions were always the fractions with the highest iron content. However, considering the amount of separation that was possible in these experiments higher resolution of the proteins is required to confirm this hypothesis. The extensive investigation into the effects that detergents had on the terminal oxidase during solubilisation experiments on the membrane of strain BC1 illustrated the difficulties involved in working with a membrane-bound protein. However it was possible to

eventually find a technique which gave a good percentage of protein release, the cytochrome aa_3 included, and which left the cytochrome intact. In this way it was possible to determine the mid-point redox potential of the cytochrome, which was slightly higher than the terminal oxidase of *T.ferrooxidans*. A high redox potential is characteristic of a terminal oxidase, and also, because the redox potential of the Fe^{2+}/Fe^{3+} couple is so high, (771 mV), it is also probably necessary in an initial electron acceptor from this couple.

Effects of using mixed cultures in mineral oxidation were demonstrated. The addition of the sulphur-oxidising strain BC13, whilst it did not appear to have a major effect, certainly had some influence on mineral solubilisation by iron-oxidising bacteria. In the pyrite shake flasks this influence proved impossible to judge due to the conditions used in the experiments. However, the consequences of the addition of this strain could be determined on strain BC1 chalcopyrite leaching, both in shake flask and airlift reactor. In the chalcopyrite shake flasks there was a slight increase in leaching rate by the mixed culture. However, the airlift fermentor work provided the more intriguing results. The addition of strain BC13 helped to prevent the rise in pH seen in the pure strain BC1 culture, presumably due in some part to the organisms' high rate of sulphur oxidation, as demonstrated in the oxygen uptake experiments. However it did appear to have ultimately had a detrimental effect on the iron oxidation of strain

BC1. At the end of the experiment, strain BC1 had lost much of its iron oxidation capacity. It appeared that this loss of iron oxidation capacity was not brought about by the presence of strain BC13 rapidly, but over a period of time. This was demonstrated by the fact that the iron oxidation of washed cells from the pure strain BC1 reactor was not adversely affected by strain BC13 from the mixed culture during the 20 hours of the iron oxidation rate experiment undertaken after the reactors had finished. It can also be inferred from the fact that there was sufficient iron oxidation in the mixed culture reactor initially to give mineral solubilisation. In a final piece of evidence for a mixed culture phenomenon involving strains BC1 and BC13, it was discovered that a mixed culture of the two on pyrite had a higher than expected oxygen uptake rate, if the oxygen uptake rates of the two in pure culture are taken into account. A likely explanation for this is that the mineral solubilisation action of strain BC1 was releasing sulphur,and this meant that more substrate was available to strain BC13 in the mixed culture. The interactions are obviously complex and it will require much more work in order to understand exactly what is occurring.

The mineral-oxidising organisms studied in this work demonstrated much variety in both their iron- and sulphur-oxidising systems, and in their ability to solubilise minerals. In the case of strain BC1 methodologies were developed to initiate detailed study of components of the iron oxidation system.

REFERENCES

Ahonen L., Tuovinen O.H. (1989a) Microbiological oxidation of ferrous iron at low temperatures. *Appl. Environ. Microbiol.* **55**: 312 - 316.

Ahonen L., Tuovinen O.H. (1989b) Effect of temperature on the microbiological leaching of sulfide ore material in percolators containing chalcopyrite, pentlandite, sphalerite and pyrrhotite as main materials. *Biotechnol. Lett.* **11**: 331 - 336

Ahonen L., Tuovinen O.H. (1990) Kinetics of sulfur oxidation at suboptimal temperatures. *Appl. Environ. Microbiol.* **56**: 560 - 562.

Ahonen L., Tuovinen O.H. (1991) Temperature effects on bacterial leaching of sulfide minerals in shake flask experiments. *Appl. Environ. Microbiol.* **57**: 138 -145.

Aleem, M.I.H., Lees, H., Nicholas, D.J.D. (1963) Adenosine triphosphate-dependent reduction of nicotinamide adenine dinucleotide by ferro-cytochrome c in chemoautotrophic bacteria. *Nature.* **200**: 759 - 761.

Andrews G.F. (1988) The selective adsorption of *Thiobacilli* to dislocation sites on pyrite surfaces. *Biotechnol. Bioeng.* **31**: 378 - 381.

Andrews, G., Darroch, M., Hansson, T. (1988) Bacterial removal of pyrite from concentrated coal slurries. *Biotechnol. Bioeng.* **32**: 813 - 820.

Azzi, A., Gennis, R.B. (1986) Purification of the aa_3-type cytochrome oxidase from *Rhodopseudomonas sphaeroides*. *Methods Enzymol.* **126**: 138 - 145.

Bagdigian, R.M., Myerson A.S. (1986) The adsorption of *Thiobacillus ferrooxidans* on coal surfaces. *Biotechnol. Bioeng.* **28**: 467 - 479.

Baines, B.S., Poole, R.K. (1985) Cytochrome oxidases from the thermophilic bacterium PS3: Solubilization with various detergents, purification and preliminary characterisation. *In* Microbial Gas Metabolism: Mechanistic, Metabolic and Biotechnological Aspects. (Eds. R.K. Poole and C.S Dow) p124 - 137. Academic Press, London.

Balashova, V.V., Vedinina, I.Ya., Markosyan, G.E., Zavarzin, G.A. (1974) The auxotrophic growth of *Leptospirillum ferrooxidans*. *Mikrobiologiya*. **43**: 491 - 494.

Barr, D.W., Ingledew, W.J., Norris, P.R. (1990) Respiratory chain components of iron-oxidizing acidophilic bacteria.
FEMS Microbiol. Lett. **70**: 85 - 90.

Bärtels, C.-C., Chatzitheodorou, G., Rodrigeuz-Leiva, M., Tributsch H. (1989) Novel technique for investigation and quantification of bacterial leaching by *Thiobacillus ferrooxidans*.
Biotechnol. Bioeng. **33**: 1196 - 1204.

Beck, J.V. (1960) A ferrous-ion-oxidising bacterium. I. Isolation and some general physiological characteristics.
J. Bacteriol. **79**: 502 - 509.

Beck, J.V., Brown, D.G. (1968) Direct sulfide oxidation in the solubilization of sulfide ores by *Thiobacillus ferrooxidans*.
J. Bacteriol. **96**: 1433 - 1434.

Blake II, R.C., Shute E.A. (1987) Respiratory enzymes of *Thiobacillus ferrooxidans*. A kinetic study of electron transfer between iron and rusticyanin in sulfate media.
J. Biol. Chem. **262**: 14983 - 14984.

Blake II, R.C., White, K.J., Shute E.A. (1988) Electron transfer from Fe(II) to rusticyanin is catalysed by an acid stable cytochrome.
In Biohydrometallurgy, Proc. Int. Symp. (Norris P.R. and Kelly D.P., Eds.), pp. 103 - 111, Science and Technology Letters, Kew, U.K.

Blaylock, B.A., Nason, A. (1963) Electron transport systems of the chemoautotroph *Ferrobacillus ferrooxidans*. I. Cytochrome c-containing iron oxidase.
J. Biol. Chem. **238**: 3453 - 3462.

Brierley, J.A., Norris, P.R., Kelly, D.P., Le Roux, N.W. (1978) Characteristics of a moderately thermophilic and acidophilic iron-oxidising bacillus.
Eur. J. Appl. Microbiol. Biotechnol. **5**: 291 - 299.

Brock, T.D., Gustafson, J. (1976) Ferric iron reduction by sulfur- and iron-oxidizing bacteria.
Appl. Environ. Microbiol. **32**: 567 - 571.

Buse, G., Hensel, S., Fee J.A. (1989) Evidence for cytochrome oxidase subunit I and a cytochrome c-subunit II fused protein in the cytochrome 'c_1aa_3' of *Thermus thermophilus*. How old is cytochrome oxidase?
Eur. J. Biochem. **181**: 261 - 268.

Chang, R. Physical Chemistry with Applications to Biological Systems, 2nd ed. (Collier Macmillan, New York, 1981)

Cobley, J.G., Haddock, B.A. (1975) The respiratory chain of *Thiobacillus ferrooxidans*: the reduction of cytochromes by Fe^{2+} and the preliminary characterization of rusticyanin, a novel `blue' copper protein. *FEBS Lett.* **60**: 29 - 33.

Colmer A.R., Hinkle, M.E. (1947) The role of microorganisms in acid mine drainage: a preliminary report. *Science*. **106**: 253 - 256.

Colmer A.R., Temple, K.L., Hinkle, M.E. (1950) An iron-oxidizing bacterium from the acid drainage of some bituminous coal mines. *J. Bacteriol*. **59**: 317 - 328.

Cox, J.C., Boxer, D.H. (1978) The purification and some properties of rusticyanin, a blue copper protein involved in iron (II) oxidation from *Thiobacillus ferrooxidans*. *Biochem. J.* **174**: 497 - 502.

Cox, J.C., Nicholls, D.G., Ingledew, W.J. (1979) Transmembrane electrical potential and transmembrane pH gradient in the acidophile *Thiobacillus ferrooxidans*. *Biochem. J.* **178**: 195 - 200.

Cwalina, B., Wilczok, T., Weglarz, L., Dzierzewicz, Z. (1990) Activity of sulphite oxidase, thiosulphate oxidase and rhodanese in *Thiobacillus ferrooxidans* during covellite and chalcopyrite leaching. *Appl. Microbiol. Biotechnol.* **34**: 279 - 281.

Davidson, V.L., Kumar, M.A. (1989) Cytochrome c_{550} mediates electron transfer from inducible periplasmic c-type cytochromes to the cytoplasmic membrane of *Paracoccus denitrificans*. *FEBS Lett.* **245**: 271 - 273.

DeVrij, W., Azzi A., Konings, W.N. (1983) Structural and functional properties of cytochrome c oxidase from *Bacillus subtilis* W23. *Eur. J. Biochem.* **131**: 97 - 103.

Din, G.A., Suzuki, I. (1967) Mechanism of Fe^{++}-cytochrome c reductase of *Ferrobacillus ferrooxidans*. *Can. J. Microbiol.* **45**: 1547 - 1556.

Din, G.A., Suzuki, I., Lees, H. (1967) Ferrous iron oxidation by *Ferrobacillus ferrooxidans*. Purification and properties of Fe^{++} - cytochrome c reductase. *Can. J. Biochem.* **45**: 1523 - 1546.

DiSpirito, A.A., Dugan, P.R., Tuovinen, O.H. (1983) Sorption of *Thiobacillus ferrooxidans* to particulate material. *Biotechnol. Bioeng.* **25**: 1163 - 1168.

Dugan, P.R., Lundgren, D.G. (1965) Energy supply for the chemoautotroph *Ferrobacillus ferrooxidans*. *J. Bacteriol.* **89**: 825 - 834.

Dugan, P.R. (1987a) Prevention of formation of acid drainage from high-sulfur coal refuse by inhibition of iron- and sulfur-oxidizing microorganisms. I. Preliminary experiments in controlled shaken flasks. *Biotechnol. Bioeng.* **29**: 41 - 48.

Dugan, P.R. (1987b) Prevention of formation of acid drainage from high-sulfur coal refuse by inhibition of iron- and sulfur-oxidizing microorganisms. II. Inhibition in 'Run of mine' refuse under simulated field conditions. *Biotechnol. Bioeng.* **29**: 49 - 54.

Duncan D.W., Landesman, J., Walden C.C. (1967) Role of *Thiobacillus ferrooxidans* in the oxidation of sulfide minerals.
Can. J. Microbiol. **13**: 397 - 403.

Eccleston, M., Kelly, D.P., Wood, A.P. (1985) Autotrophic growth and iron oxidation and inhibition kinetics of *Leptospirillum ferrooxidans*.
In Planetary Ecology, (D.E. Caldwell, J.A. Brierley, C.L. Brierley, eds.) pp 263 - 272, Van Nostrand Reinhold Co., New York.

Ehrlich, H.L. (1963) Microorganisms in acid drainage from a copper mine.
J. Bacteriol. **86**: 350 - 352.

Ferroni, G.D., Leduc, L.G., Todd, M. (1986) Isolation and temperature characterization of psychrotrophic strains of *Thiobacillus ferrooxidans* from the environment of a uranium mine.
J. Gen. Appl. Microbiol. **32**: 169 - 175.

Fry, I.V., Lazeroff, N., Packer, L. (1986) Sulphate-dependant iron oxidation by *T.ferrooxidans*: Characterisation of a new EPR detectable electron transport component on the reducing side of rusticyanin.
Arch. Biochem. Biophys. **246**: 650 - 654.

Fukumori, Y., Takahiro, Y., Akihiko, S., Yamanaka, T. (1988) Fe(II) - oxidizing enzyme purified from *Thiobacillus ferrooxidans*. *FEMS Microbiol. Lett.* **50**: 169 - 172.

Ghauri, M.A., Johnson, D.B. (1991) Physiological diversity amongst some moderately thermophilic iron-oxidising bacteria. *FEMS Microbiol. Ecol.* **85**: 327 - 334.

Grishin, S.I., Tuovinen, O.H. (1988) Fast kinetics of Fe^{2+} oxidation in packed-bed reactors. *Appl. Environ. Microbiol.* **54**: 3092 - 3100.

Grishin, S.I., Tuovinen, O.H. (1989) Scanning electron microscopic examination of *Thiobacillus ferrooxidans* on different support matrix materials in packed bed and fluidized bed reactors. *Appl. Microbiol. Biotechnol.* **31**: 505 - 511.

Groudev, S.N. (1985) Differences between strains of *Thiobacillus ferrooxidans* with respect to their ability to oxidise sulphide minerals. *In* Biogeotechnology of Metals. (G.I. Karavaiko, S.N. Groudev eds) pp 83 - 96. Moscow: UNEP, Centre of International Projects, GKNT.

Guay, R., Silver, M. (1975) *Thiobacillus acidophilus sp. nov.*; isolation and some physiological characteristics. *Can. J. Microbiol.* **21**: 281 - 288.

Guay, R., Torma, A.E., Silver, M. (1975) Oxydation de l'ion ferreux et mise en solution de l'uranium d'minerai par *Thiobacillus ferrooxidans*. *Ann. Microbiol.* **126B**: 209 - 219.

Gupta, S.G., Agate, A.D. (1986) Preservation of *Thiobacillus ferrooxidans* and *Thiobacillus thiooxidans* with activity check. *Antonie van Leeuwenhoek.* **52**: 121 - 127.

Häfele, U., Scherer, S., Böger, P. (1988) Cytochrome aa_3 from heterocysts of the cyanobacterium *Anabaena variabilis*: isolation and spectral characterization. *Biochim. Biophys. Acta.* **934**: 186 - 190.

Harrison, A.P. Jr, (1978) Microbial succession and mineral leaching in an artificial coal spoil. *Appl. Environ. Microbiol.* **36**: 861 - 869.

Harrison, A.P. Jr, (1981) *Acidophilum cryptum, gen. nov., sp.nov.*, heterotrophic bacterium from acidic mineral environments. *Int. J. Syst. Bact.* **31**: 327 - 332.

Harrison, A.P. Jr, (1982) Genomic and physiological diversity amongst strains of *Thiobacillus ferrooxidans*, and genomic comparison with *Thiobacillus thiooxidans*. *Arch. Microbiol.* **131**: 68 - 76.

Harrison, A.P. Jr., Norris, P.R. (1985) *Leptospirillum ferrooxidans* and similar bacteria: some characteristics and genomic diversity. *FEMS Microbiol. Lett.* **30**: 99 - 102.

Hart, A., Murrell, J.C., Poole, R.K., Norris, P.R. (1991) An acid-stable cytochrome in iron-oxidizing *Leptospirillum ferrooxidans*. *FEMS Microbiol. Lett.* **81**: 89 - 94.

Huber, H., Stetter, K.O. (1990) *Thiobacillus cuprinus*, sp. nov., a novel facultatively organotrophic metal-mobilising bacterium. *Appl. Environ. Microbiol.* **56**: 315 - 322.

Hunter, D.J.B., Brown, K.R., Pettigrew, G.W. (1989) The role of cytochrome c_4 in bacterial respiration. Cellular location and removal from membranes. *Biochem. J.* **262**: 233 - 240.

Hutchins, S.R., Davidson, M.S., Brierley, J.A., Brierley, C.L. (1986) Microorganisms in reclamation of metals. *Annu. Rev. Micro.* **40**: 311 - 336.

Ingledew, W.J., Cox, J.C., Halling, P.J. (1977) A proposed mechanism for energy conservation during Fe^{2+} oxidation by *Thiobacillus ferrooxidans*: chemiosmotic coupling to net H^{+} influx.
FEMS Microbiol. Lett. **2**: 193 - 197.

Ingledew, W.J., Cobley, J.G. (1980) A potentiometric and kinetic study on the respiratory chain of ferrous iron-grown *Thiobacillus ferrooxidans*.
Biochim. Biophys. Acta. **590**: 141 - 158.

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

Johnson, D.B., McGinness, S. (1991) Ferric iron reduction by acidophilic heterotrophic bacteria.
Appl. Environ. Microbiol. **57**: 207 - 211.

Johnson, W.C. (1964) Tiron.
In Organic Reagents for Metals and for Certain Radicals, vol 2, p189 - 195.
Hopkins and Williams, Essex.

Kai, M., Takahiro, Y., Yoshiro, F., Fukumori, Y., Yamanaka, T. (1989) Cytochrome oxidase of an acidophilic iron-oxidising bacterium, *Thiobacillus ferrooxidans*, functions at pH 3.5.
Biochem. Biophys. Res. Comm. **160**: 839 - 843.

Karamanev, D.G., Nikolov, L.N. (1988) Influence of some physicochemical parameters on bacterial activity of biofilm: Ferrous iron oxidation by *Thiobacillus ferrooxidans*.
Biotechnol. Bioeng. **31**: 295 - 299.

Karavaiko, G.I., Golovacheva, T.A., Pivovarova, T.A., Tzaplina, I.A., Vartanjan, N.S. (1988) Thermophilic bacteria of the genus *Sulfobacillus*.
In Biohydrometallurgy, Proc. Int. Symp. (Norris P.R. and Kelly D.P., Eds.), pp. 29 - 41. Science and Technology Letters, Kew, U.K.

Kelly, D.P., Jones, C.A. (1978) Factors affecting metabolism and ferrous iron oxidation in suspensions and batch cultures of *Thiobacillus ferrooxidans*: relevance to ferric iron leach solution regeneration.
In Metallurgical Applications of Bacterial Leaching and Related Microbiological Phenomena, (L.E. Murr, A.E. Torma, J.A. Brierley, eds.), pp 19 - 44. Academic, New York.

Kelly, D.P., Harrison, A.P. (1989) The Thiobacilli. *In* Bergey's Manual of Systematic Bacteriology, 9th ed. (Staley, J.T., Bryant, M.P., Pfennig, N., Holt, J.G., eds.) pp 1842 - 1858. Williams and Wilkins, U.S.A.

Kim, A.G., Heisey, B.S., Kleinmann, R.L.P., Deul, M. (1982) Acid mine water: control and abatement research. U.S. Bureau of Mines information circular no. 8905. U.S. Bureau of Mines, Washington D.C.

Kino, K., Usami, S. (1982) Biological reduction of ferric iron by iron- and sulfur-oxidizing bacteria. *Agric. Biol. Chem.* **46:** 803 - 805.

Kitada, M., Krulwich, T.A. (1984) Purification and characterisation of the cytochrome oxidase from alkalophilc *Bacillus firmus* RAB. *J. Bacteriol.* **158:** 963 - 966.

Kurokawa, T., Fukumori, Y., Yamanaka, T. (1989) *Nitrobacter winogradskyi* cytochrome b_{559}: a nonheam iron-containing cytochrome related to bacterioferritin *Biochim. Biophys. Acta.* **976:** 135 - 139.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680 - 685.

Larsson, L., Olssen, G., Holst, O., Karlsson, H.T. (1990) Pyrite oxidation by thermophilic archaebacteria. *Appl. Environ. Microbiol.* **56**: 697 - 701.

Lees, H., Kwok, S.C., Suzuki, I. (1969) The thermodynamics of iron oxidation by the *Ferrobacilli*. *Can. J. Microbiol.* **15**: 43 - 46.

Lizama, H.M., Suzuki, I. (1989) Synergistic competitive inhibition of ferrous iron oxidation by *Thiobacillus ferrooxidans* by increasing concentrations of ferric iron and cells. *Appl. Environ. Microbiol.* **55**: 2588 - 2591.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 263 - 275.

Ludwig, B. (1987) Cytochrome c oxidase in prokaryotes. *FEMS Microbiol. Rev.* **46**: 41 - 56.

Lundgren, D.G. (1975) Microbiological problems in strip mine areas: relationship to the metabolism of *Thiobacillus ferrooxidans*. *Ohio J. Sci.* **75**: 280 - 287.

Lütters, S., Hanert, H.H. (1989) The ultrastructure of chemolithoautotrophic *Gallionella ferruginea* and *Thiobacillus ferrooxidans* as revealed by chemical fixation and freeze-etching.
Arch. Microbiol. **151**: 245 - 251.

Marsh, R.M., Norris, P.R. (1983a) The isolation of some thermophilic, autotrophic, iron- and sulphur-oxidizing bacteria.
FEMS Microbiol. Lett. **17**: 311 - 315.

Marsh, R.M., Norris, P.R. (1983b) Mineral sulphide oxidation by moderately thermophilic, acidophilic bacteria.
Biotechnol. Lett. **5**: 585 - 590.

MacDonald, D.G., Clark, R.H. (1970) The oxidation of aqueous ferrous sulphate by *Thiobacillus ferrooxidans*.
Can. J. Chem. Eng. **48**: 669 - 676.

Mackintosh, M.E. (1978) Nitrogen fixation by *Thiobacillus ferrooxidans*.
J. Gen. Microbiol. **105**: 215 - 218.

Metzdorf, N., Kaltwasser, H. (1988) Utilization of organic compounds as the sole source of nitrogen by *Thiobacillus thiooxidans*.
Arch. Microbiol. **150**: 85 - 88.

Mjoli, N., Kulpa Jr, C.F. (1988) Identification of a unique outer membrane protein required for iron oxidation in *Thiobacillus ferrooxidans*. *In* Biohydrometallurgy, Proc. Int. Symp. (Norris P.R. and Kelly D.P., Eds.), pp. 89 - 102. Science and Technology Letters, Kew, U.K.

Myerson, A.S., Kline, P. (1983) The adsorption of *Thiobacillus ferrooxidans* on solid particles. *Biotechnol. Bioeng.* **25**: 1669 - 1676.

Norris, P.R., Brierley, J.A., Kelly, D.P. (1980) Physiological characteristics of two facultatively thermophilic mineral-oxidising bacteria. *FEMS Microbiol. Lett.* **7**: 119 - 122.

Norris. P.R. (1983) Iron and mineral oxidation with *Leptospirillum*-like bacteria. *In* Recent Progress in Biohydrometallurgy (G. Rossi, A.E. Torma, eds.), pp 83 - 96. Iglesias: Associazione Mineraria Sarda.

Norris, P.R., Barr, D.W. (1985) Growth and iron oxidation by acidophilic moderate thermophiles. *FEMS Microbiol. Lett.* **28**: 221 - 224.

Norris, P.R., Marsh, R.M, Lindstrom, E.B. (1986) Growth of mesophilic and thermophilic acidophilic bacteria on sulfur and tetrathionate.
Biotech. Appl. Biochem. **8**: 318 - 329.

Norris, P.R. (1988) Bacterial diversity in reactor mineral leaching.
8th International Biotechnology Symposium Proceedings (Durard, G., Bobichan, L., Florent, J. eds.) pp 1119 - 1130. Societe Francais de Microbiologie, Paris, France.

Norris, P.R., Barr, D.W. (1988) Bacterial oxidation of pyrite in high temperature reactors.
In Biohydrometallurgy, Proc. Int. Symp. (Norris P.R. and Kelly D.P., Eds.), pp. 532 - 536. Science and Technology Letters, Kew, U.K.

Norris, P.R., Barr, D.W., Hinson, D. (1988) Iron and mineral oxidation by acidophilic bacteria: affinities for iron and attachment to pyrite.
In Biohydrometallurgy, Proc. Int. Symp. (Norris P.R. and Kelly D.P., Eds.), pp. 43 - 60. Science and Technology Letters, Kew, U.K.

Okereke, A., Stevens, S.E. Jr. (1991) Kinetics of iron oxidation by *Thiobacillus ferrooxidans*.
Appl. Environ. Microbiol. **57**: 1052 - 1056.

Oliver, D.J., Van Slyke, J.K. (1988) Effect of sulphur on metabolism by iron-grown *Thiobacillus ferrooxidans*. *In* Biohydrometallurgy, Proc. Int. Symp. (Norris P.R. and Kelly D.P., Eds.), pp. 119 - 126. Science and Technology Letters, Kew, U.K.

Olsen, G.J. (1991) Rate of pyrite bioleaching by *Thiobacillus ferrooxidans*: results of an interlaboratory comparison. *Appl. Environ. Microbiol.* **57**: 642 - 644.

Ongcharit, C., Sublette, K.L., Shah, Y.T. (1991) Oxidation of hydrogen sulfide by flocculated *Thiobacillus denitrificans* in a continuous culture. *Biotechnol. Bioeng.* **37**: 497 - 504.

Paknikar, K.M., Agate, A.D. (1988) Occurrence of a *Thiobacillus ferrooxidans* strain tolerating unusually high concentrations of metals and an associated metal tolerant acidophilic heterotrophic bacterium. *In* Biohydrometallurgy, Proc. Int. Symp. (Norris P.R. and Kelly D.P., Eds.), pp. 558 - 560. Science and Technology Letters, Kew, U.K.

Pesic, B., Oliver, D.J., Wichlacz, P. (1989) An electrochemical method of measuring the oxidation rate of ferrous to ferric iron with oxygen in the presence of *Thiobacillus ferrooxidans*.
Biotechnol. Bioeng. **33**: 428 - 439.

Pronk, J.T., Meesters, P.J.W., van Dijken, J.P., Bos, P., Kuenen, J.G. (1990) Heterotrophic growth of *Thiobacillus acidophilus* in batch and chemostat cultures.
Arch. Microbiol. **153**: 392 - 398.

Radway, J.C., Tuttle, J.H., Fendinger, N.J., Means, J.C. (1987) Microbially mediated leaching of low-sulfur coal in experimental coal columns.
Appl. Environ. Microbiol. **53**: 1056 - 1063.

Sato, A., Fukumori, Y., Yano, T., Kai, M., Yamanaka, T. (1989) *Thiobacillus ferrooxidans* cytochrome c_{552}: purification and some of its molecular features.
Biochim. Biophys. Acta. **976**: 129 - 134.

Schaeffer, W.I., Umbreit, W.W. (1962) Phosphotidylinositol as a wetting agent in sulfur oxidation by *Thiobacillus ferrooxidans*.
J. Bacteriol. **85**: 492 - 493.

Shafia, F., Wilkinson, R.F. Jr. (1969) Growth of *Ferrobacillus ferrooxidans* on organic matter. *J. Bacteriol.* **97:** 256 - 260.

Shewmon, P.G. Diffusion in Solids. (McGraw-Hill, New York, 1963)

Silverman, M.P., Ehrlich, H.L. (1964) Microbial formation and degradation of minerals. *Advan. Appl. Microbiol.* **6:** 153 - 206.

Silverman, M.P. (1967) Mechanism of bacterial pyrite oxidation. *J. Bacteriol.* **94:** 1046 - 1051.

Sone, N., Ohyama, T., Kagawa, Y. (1979) Thermostable, single band cytochrome oxidase. *FEBS Lett.* **106:** 39 - 42.

Sone, N., Kutoh, E., Yanagita, Y. (1989) Cytochrome c_{551} from the thermophilic bacterium PS3 grown under air-limited conditions *Biochim. Biophys. Acta.* **977:** 329 - 334.

Stokes, H.N. (1901) On pyrite and marcasite. U.S. Geological Survey bulletin 186.

Sugio, T.,Chitoshi, D., Munakata, O., Tano, T., Kazutami, I. (1985) Role of a ferric ion-reducing system in sulfur oxidation in *Thiobacillus ferrooxidans*. *Appl. Environ. Microbiol.* **49:** 1401 - 1406.

Sugio, T., Mizunashi, T., Tano, T., Kazutami, I. (1986) Production of ferrous ions as intermediates during aerobic sulfur oxidation in *Thiobacillus ferrooxidans*. *Agric. Biol. Chem.* **50:** 2755 - 2761.

Sugio, T., Mizunashi, W., Inagaki, K., Tano, T. (1987) Purification and some properties of sulfur:ferric ion oxidoreductase from *Thiobacillus ferrooxidans*. *J. Bacteriol.* **169:** 4916 - 4922.

Sugio, T., Wada, K., Mori, M., Inagaki, K., Tano, T. (1988a) Synthesis of an iron-oxidising system during growth of *Thiobacillus ferrooxidans* on sulfur-basal salts medium. *Appl. Environ. Microbiol.* **54:** 150 -152.

Sugio, T., Katagiri, T., Moriyama, M., Zhen, Y.L., Inagaki, K., Tano,T. (1988b) Existence of a new type of sulfite oxidase which utilizes ferric ion as an electron acceptor in *Thiobacillus ferrooxidans*. *Appl. Environ. Microbiol.* **54:** 153 - 157.

Sugio,T., Katagiri, T., Inagaki, K., Tano, T. (1989) Actual substrate for elemental sulfur oxidation by sulfur: ferric ion oxidoreductase purified from *Thiobacillus ferrooxidans*.
Biochim. Biophys. Acta. **973**: 250 - 256.

Suzuki, I., Lizama, H.M., Tackaberry, P.D. (1989) Competitive inhibition of ferrous iron oxidation by *Thiobacillus ferrooxidans* by increasing concentration of cells.
Appl. Environ. Microbiol. **55**: 1117 - 1121.

Takakuwa, S. (1975) Purification and some properties of c_{552} from a sulfur-oxidizing bacterium *Thiobacillus thiooxidans*.
J. Biochem. **78**: 181 - 185.

Takakuwa, S., Fujimori, T., Iwasaki, H. (1979) Some properties of cell-sulfur adhesion in *Thiobacillus thiooxidans*.
J. Gen. Appl. Microbiol. **25**: 21 - 29.

Temple, K.L., Colmer, A.R. (1951) The autotrophic oxidation of iron by a new bacterium: *Thiobacillus ferrooxidans*.
J. Bacteriol. **62**: 605 - 611.

Tikhonova, G.V., Lisenkova, L.L., Doman, N.G., Skulachev, V.P. (1967) Electron transport pathways in *Thiobacillus ferrooxidans*.
Biokhimiya. **32**: 725 - 734.

Tillet, D.M., Myerson, A.S. (1987) The removal of pyritic sulfur from coal employing *Thiobacillus ferrooxidans* in a packed column reactor.
Biotechnol. Bioeng. **29**: 146 - 150.

Torma, A.E. (1977) The role of *Thiobacillus ferrooxidans* in hydrometallurgical processes.
Adv. Biochem. Eng. **6**: 1 - 37.

Tsuchiya, H.M., Trivedi, N.C., Schuler, M.L. (1974) Microbial mutualism in ore leaching.
Biotechnol. Bioeng. **16**: 991 - 995.

Tuovinen, O.H., Niemelä, S.I., Gyllenburg, H.G. (1971) Tolerance of *Thiobacillus ferrooxidans* to some metals.
Antonie van Leeuwenhoek. **37**: 489 - 496.

Tuovinen, O.H., Kelly, D.P. (1972) Biology of *Thiobacillus ferrooxidans* in relation to the microbiological leaching of sulfide ores.
Z. Allg. Mikrobiol. **12**: 311 - 346.

Tuttle, J.H., Randles, C.I., Dugan, P.R. (1968) Activity of microorganisms in acid mine water. I. Influence of acid water on aerobic heterotrophs of a normal stream. *J. Bacteriol.* **95**: 1495 - 1503.

Tuttle, J.H., Dugan, P.R., Apel, W.A. (1981) Leakage of cellular material from *Thiobacillus ferrooxidans* in the presence of organic acids. *Appl. Environ. Microbiol.* **33**: 459 - 465.

Van Aswegan, P.C., Haines, A.K., Marais, H.J. (1988) Design and operation of a commercial bacterial oxidation plant at Fairfax. *Randol. Perth Gold* **88**: 144 - 147.

Vernon, L.P., Mangum, J.H., Beck, J.B., Shafia, F.M. (1960) Studies on a ferrous ion-oxidizing bacterium. II. Cytochrome composition. *Arch. Biochem. Biophys.* **88**: 227 - 231.

Vuorinen, A., Tuovinen, O.H. (1987) Analysis of soluble iron compounds in the bacterial oxidation of pyrite. *J. Ferment. Technol.* **65**: 37 - 42.

Wakagi, T., Yamauchi, T., Oshima, T., Muller, M., Azzi, A., Sone, N. (1989) A novel a-type terminal oxidase from *Sulfolobus acidocaldarius* with cytochrome c oxidase activity.
Biochem. Biophys. Res. Comm. **165:** 1110 - 1114.

Wood, A.P., Kelly, D.P. (1983) Autotrophic and mixotrophic growth of three thermoacidophilic iron-oxidising bacteria.
FEMS Microbiol. Lett. **20:** 107 - 112.

Wood, A.P., Kelly, D.P. (1988) Isolation and physiological characterisation of *Thiobacillus aquasulis*, sp. nov., a novel facultatively autotrophic moderate thermophile.
Arch. Microbiol. **149:** 339 - 343.

Yamanaka, T., Fujii, K. (1980) Cytochrome a-type terminal oxidase derived from *Thiobacillus novellus*.
Biochim. Biophys. Acta. **591:** 53 - 62.

Yoshida, T., Lorence, R.M., Choc, M.G., Tarr, G.E., Findling, K.L., Fee, J.A. (1984) Respiratory proteins from the extremely thermophilic bacterium *Thermus thermophilus*. Purification procedures for cytochromes c_{552}, c_{549} and c_1aa_3 and chemical evidence for a single subunit cytochrome aa_3.
J. Biol. Chem. **259:** 112 - 123.

Zimmerman, B.H., Nitsche, C.I., Fee, J.A, Rusnak, F., Münck, E. (1988) Properties of a copper-containing cytochrome ba_3: A second terminal oxidase from the extreme thermophile *Thermus thermophilus*. *Proc. Natl. Acad. Sci. U.S.A.* **85**: 5779- 5783.