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Physiological and phylogenetic studies of some novel acidophilic mineral-oxidising bacteria

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PHYSIOLOGICAL AND PHYLOGENETIC STUDIES OF SOME NOVEL ACIDOPHILIC MINERAL-OXIDISING BACTERIA

A Thesis submitted to the University of Wales in candidature for the degree of Philosophiae Doctor

by

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Abstract

Physiological and biomolecular studies were carried out to characterise five novel mineral-oxidising microorganisms. All were obligately acidophilic, iron- and sulphur-oxidising eubacteria, which grew aerobically or anaerobically (using ferric iron as terminal electron acceptor). They were grouped as: (i) mesophilic Gram-negative bacteria (isolate S-10 and M-12); (ii) mesophilic Gram-positive bacteria (isolate RIV-14 and L-15); and (iii) moderately thermophilic Gram-positive bacteria (isolate GSM). The four mesophilic bacteria were isolated from geothermal sites on the island of Montserrat (W.I) and the moderately thermophile from waste rock from the Golden Sunlight Mine, Montana, U.S.A.. A variety of techniques were used to establish the taxonomic relationship of these isolates to known acidophilic, metal-mobilising acidophiles. Isolates S-10 and M-12 shared some morphological and physiological traits in common with *Thiobacillus ferrooxidans*, though they differed from *T. ferrooxidans* in being able to grow mixotrophically in ferrous iron/yeast extract liquid medium, greater tolerance to hydrogen ions, and in some other areas. In addition, these isolates had a greater (65%) G + C content than the type strain *T. ferrooxidans* (58 – 59%), suggesting that they may represent a novel species. Isolates RIV-14 and L-15 were, in contrast, Gram-positive spore-forming rods. These isolates displayed considerable metabolic flexibility, similar to moderately thermophilic *Sulfobacillus* spp.. Comparative analysis of 16S rRNA gene sequences confirmed that RIV-14 and L-15 had a close phylogenetic affiliation with iron-oxidising *Sulfobacillus* spp.. However, they differed from the latter in being mesophilic and much tolerant to extreme acidity (growth at pH <1, the lowest values yet recorded for any iron-oxidising prokaryote). Isolate RIV-14 and L-15 differed from each other in some physiological traits, and the binary level comparison of their 16S rRNA gene sequences was low enough (97% sequence identity) to consider them as two novel and distinct species of the *Sulfobacillus*. Isolate GSM was readily distinguished from the four Montserrat isolates by its thermotolerant nature and its greater capacity for heterotrophic growth. Whilst isolate GSM was found to share some morphological characteristics with the thermotolerant endospore-forming acidophiles *Sulfobacillus* spp. and *Alicyclobacillus* spp., biomolecular analysis showed that isolate GSM is phylogenetically distinct from both these and appears to represent a novel bacterial genus. Pure cultures of all five isolates were found to be capable of oxidising pyrite (and the four Montserrat isolates of oxidising chalcopyrite). The Gram-positive Montserrat isolates leached sulphide minerals at much lower pH values and redox potentials than the more well-characterised mineral-oxidising bacteria (*T. ferrooxidans* and *Leptospirillum ferrooxidans*) suggesting that they may have a useful role in bioleaching of some important mineral ores.

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Abbreviations

%	percent
°C	degree Celsius
µm	micrometer
µ	specific growth rates
µg	microgram
µl	microlitre
AAS	atomic absorbance spectroscopy
AMD	acid mine drainage
ANOVA	analysis of variance
AODCs	acridine orange direct counts
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
CO_2	carbon dioxide
CoA	coenzyme A
d	day
DNA	deoxyribonucleic acid
dpm	disintegration per minute
DSMZ	Deutsche Sammlung von Mikroorganismen und Zelkulturen
EDTA	ethylenediaminetetraacetic acid
E_h	redox potential
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
Feo	ferrous iron overlay
FeSo	ferrous iron/tetrathionate overlay
FS°	flowable elemental sulphur
g	gram
G	guanine
$\Delta G°$	standard-free-energy (Gibbs) change

HCl	hydrochloric acid
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
l	litre
ln	natural logarithm
log	logarithm to the base 10
M	molar
ml	millilitre
mm	millimeter
n	replicate
nmole	nanomole
OD600	optical density at 600 nm
r.p.m.	revolutions per minute
RFLP	restriction fragment length polymorphism
RISC	reduced inorganic sulphur compound
RNA	ribonucleic acid
SEM	scanning electron microscope
TEM	transmission electron microscope
Tris	tris[hydroxymethyl]aminomethane
UV	ultraviolet light
UWB	University of Wales Bangor
v	volume
w	weight
YE	yeast extract
PCR	polymerase chain reaction
t_d	culture doubling time
BLAST	Basic Local Alignment Search Tool
PHYLIP	Phylogeny Inference Package

This is dedicated to my father, Yahya and my mother, Ramlah with love for their neverending support, advice as well as understanding with my absent- minded preoccupation with the writing of this thesis.

(1)

General Introduction

1.1 Life in acidic environments

Acidic, and often metal-rich, ecosystems are examples of extreme environments. They are frequently associated with the production of acid mine drainage (AMD), a severe environmental pollution problem resulting from the oxidation of sulphide minerals in mine wastes, ore tailings and overburden, at active or abandoned mines sites. However, this type of ecosystem also harbours a unique microbiology that constitutes a valuable scientific research resource. Extremely acidic environments can also originate from the oxidation of sulphur in volcanic areas, formed from the condensation of hydrogen sulphide and sulphur dioxide, two common volcanic gases.

It has long been recognised that life is also not necessarily excluded from natural and anthropogenic environments that are regarded as 'extreme'. Some 'extremophiles' grow in either very alkaline environments (alkaliphiles) or in acidic environments (acidophiles). The term 'acidophile' generally refers to those organisms which grow in extremely acidic conditions (defined in this context as being less than pH 3.0), and are not capable of growth at neutral pH. A variety of acidophilic microorganisms have been isolated from extremely acidic environments, including fungi, algae, yeasts, protozoa, bacteria and archaea (Norris and Johnson, 1998; Johnson, 1998a). Classification of acidophilic microorganism has sometimes been based in their temperature range (Wiegel, 1990; Norris, 1990). Further subdivision may be made on the basis of their mode of respiration, nutrition and energetics, and more recently on details of their molecular biology (e.g. Hallberg *et al.*, 1994; Clark and Norris, 1996; Hugenholtz *et al.*, 1998)

Some microorganisms have evolved to live, thrive and survive under 'extreme' conditions, and are equipped to withstand the physicochemical stresses imposed by extreme conditions such as high or low temperature. The chemical compositions of their environments may be very different from their microbial cytoplasm, which accentuates the need for strict maintenance of proton and solute gradients across the cytoplasmic membrane.

Acidophilic microorganisms have been the focus of a considerable amount of research. The most studied acidophiles are those which are capable of oxidising ferrous iron and/or sulphur compounds (mesophiles and moderate thermophiles), chiefly through the importance of these prokaryotes in extracting metals from sulphide ores (Rawlings, 1997; Norris, 1990; Barrett *et al.*, 1993). The utilisation of acidophiles in coal desulphurisation has also attracted interest (Bos and Kuenen, 1990) but its application is not as widely operated as that of microbial leaching of metal sulphides. In recent years, extremely thermophilic acidophiles have also been studied. These are archaea, rather than eubacteria, and are of considerable interest in the study of possible early life forms on our planet.

1.2 Taxonomy of acidophilic eubacteria

Taxonomy is the science of classification and consists of two major subdisciplines, identification and nomenclature. Bacterial taxonomy has traditionally relied on phenotypic analyses as the basis of classification but, because bacteria are so small and contain relatively few structural clues to their evolutionary roots, phylogenetic relationships between prokaryotes have emerged only from genotypic analyses. This includes three molecular-based analyses: (i) DNA base composition; guanine plus cyctosine mol% (G + C) content (Mandel and Marmur, 1968); (ii) DNA-DNA

hybridisation (e.g. Harrison, 1982; Werman *et al.*, 1996) and (iii) comparison of 16S rRNA gene sequences (e.g. Woese, 1987; Palumbi, 1996; Hillis *et al.*, 1996). The first two methods offer limited information, with e.g. DNA-DNA hybridisation studies being suitable only to establish proximal phylogenies. Analyses of ribosomal gene sequences have the advantage of establishing a 'finger-print' for each organism tested and establishing distal as well as proximal phylogenies, though a 99% 16S rRNA similarity typically corresponds to 50% DNA-DNA pairing, i.e. the nearest proximal phylogenic relationship established by ribosomal base sequences analyses is at the genus level (Amann *et al.*, 1995; Hugenholtz *et al.*, 1998). The use of PCR-based analyses techniques, e.g. restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and amplified ribosomal DNA restriction enzyme analyses (ARDRA), have been reported to be highly reliable procedures for fast identification and taxonomical categorisation of species of the genus *Thiobacillus* (Selenska-Pobell *et al.*, 1998).

A practical classification in terms of growth temperature ranges has often been used by researchers to differentiate acidophilic prokaryotes. As such, acidophilic microorganisms may be divided into mesophiles, moderate thermophiles and extreme thermophiles. Bacterial taxonomy was, until the middle of the twentieth century, based on classical taxonomical studies which involved mostly morphological, physiological, biochemical and nutritional characteriasation. Chemotaxonomical approaches (using biochemical analytical techniques such as chromatography and electrophoresis) and characterisation of biomolecular characteristics both developed during the second half of the century, and contributed to the development of more meaningful classification systems. For example, composition and distribution of cellular lipids (Katayama-Fugimura *et al.*, 1982; Guckert *et al.*, 1991; Langworthy *et al.*, 1974), whole cell protein

patterns or specific enzymatic activities (Jackman, 1987; Bascomb, 1987) and respiratory chain components (redox-active biomolecules and isoprenoid quinones; Blake *et al.*, 1993; Katayama-Fugimura *et al.*, 1982) have been frequently used in bacterial taxonomy. Even though invaluable taxonomic information is obtained from such studies, classification based on phenotypic characteristics does not always reflect the genetic relationships between microorganisms and, in this particular case, acidophilic microorganisms (Harrison, 1982; 1986). Moreover, from a practical point of view, a taxonomic system based on genetic similarities might facilitate genetic manipulation which, in the case of acidophilic microorganisms, would tend to focus on improving mineral leaching.

The analyses of ribosomal gene sequences was initially developed with 5S rRNA subunits as a technique for identification of microorganisms in environmental samples (Stahl *et al.*, 1985) and has been now standardised using 16S rRNA, predominantly due to the greater size of the molecule (about a ten times greater number of base pairs), and allows more reliable genetical analyses (Woese, 1987; Amann *et al.*, 1995). Analyses of 16S rRNA gene sequences has been used successfully in ecological studies for monitoring the development of mixed populations in natural and laboratory cultures (Kane *et al.*, 1993; Goebel and Stackebrandt, 1994; Amann *et al.*, 1992; Durand *et al*, 1997). It is now well established that the analyses of 16S rRNA gene sequences also facilitate phylogenetic classification of the microorganisms thriving in acidic, as well as other environments (Woese, 1987; Lane *et al.*, 1992) and a phylogenetic taxonomic system has been proposed composed of three domains (Fig 1.1): *Bacteria* (formerly eubacteria), *Archaea* (formerly archaebacteria) and *Eucarya* (formally eucaryotae). In this taxonomic system, acidophilic prokaryotes are included in both the *Archaea* and *Bacteria* domains (Fig. 1.2 and 1.3). Phylogenies derived from such data should not,

however, be over interpreted and a polyphasic taxonomy approach, integrating both phylogenetic and phenotypic characteristics should be employed to test evolutionary relationships (Woese, 1987).

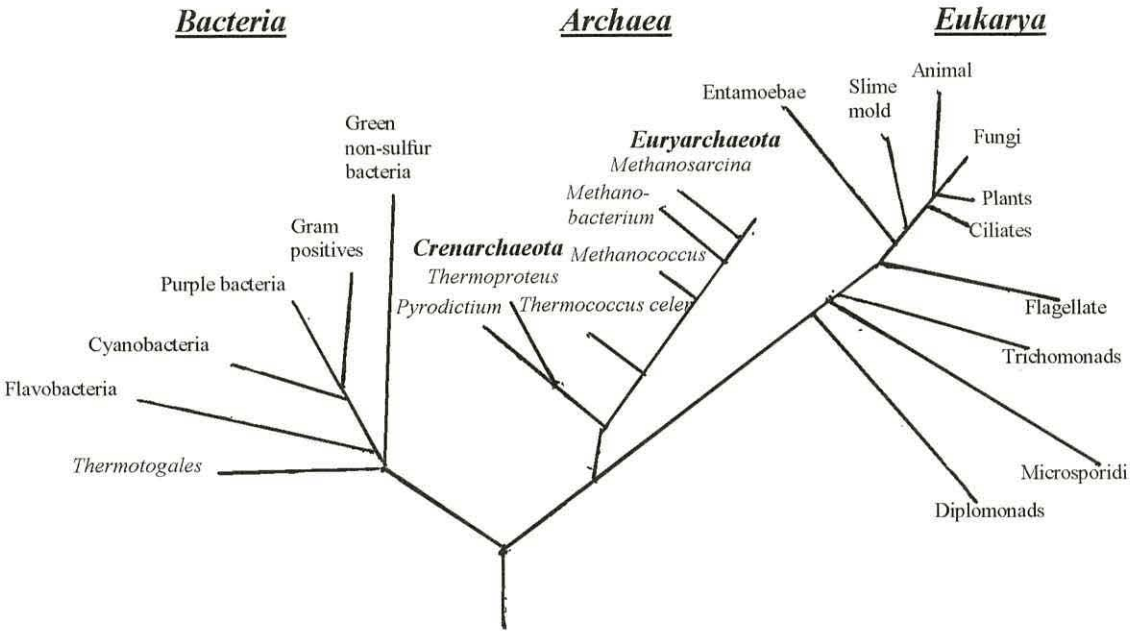


Fig 1.1: General phylogenetic tree derived from comparative sequencing of 16S or 18S ribosomal RNA showing the three major domains of living organisms: the *Bacteria*, the *Archaea* and the *Eukarya*. Branch lengths are proportional to evolutionary distances (from Logan, 1994).

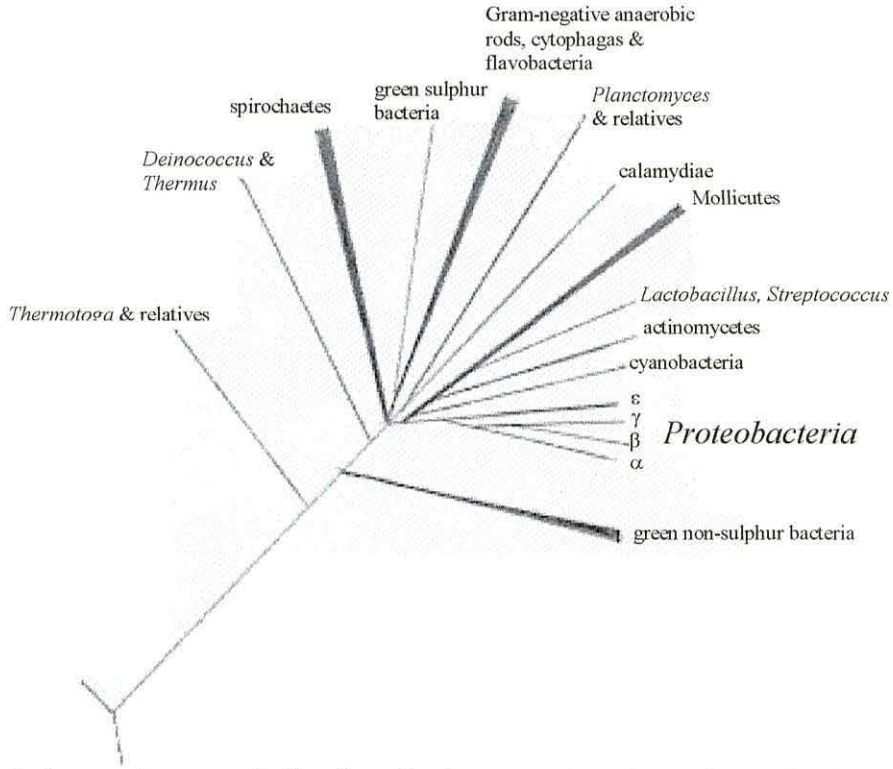


Fig 1.2: Phylogenetic tree of the domain *Bacteria* based on the 16S rRNA sequence comparisons. Branch lengths are proportional to evolutionary distances (from Logan, 1994)

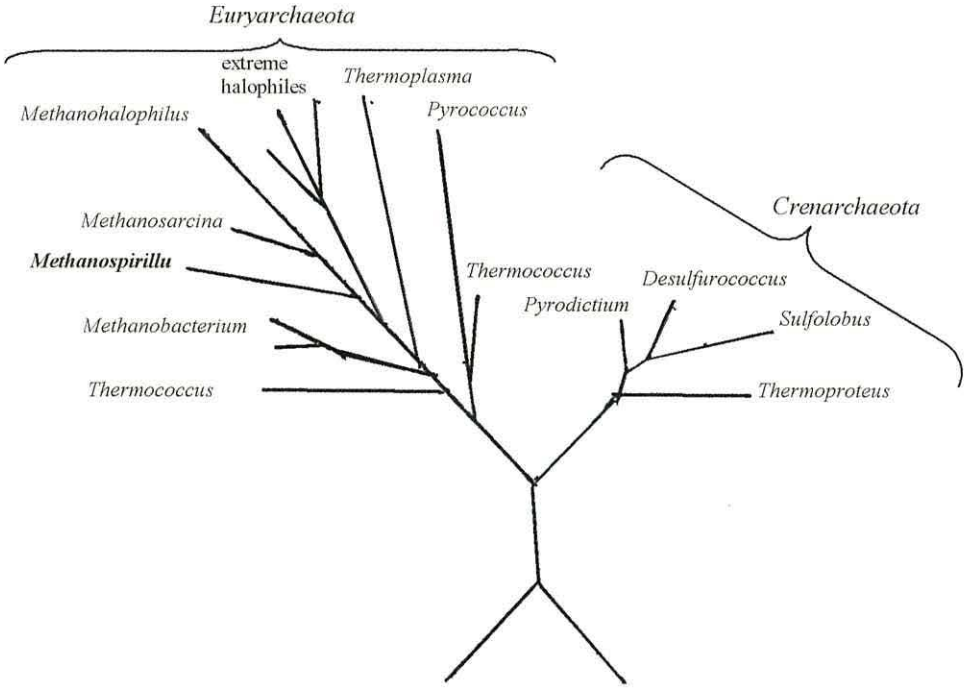


Fig: 1.3 Phylogenetic tree of the domain *Archaea* based on the 16S rRNA sequence comparisons. Branch lengths are proportional to evolutionary distances (from Logan, 1994)

1.3 Energetics and carbon metabolisms in acidophilic bacteria

Microorganisms depend upon external energy sources to drive processes within cells, which require a net input of energy. Depending on the type of microorganism, energy may be derived from light (phototrophic) or from the conversion of a variety of organic or inorganic substrates (chemotrophic growth). The implications of acidophily for chemiosmotic energy transduction have been examined with mesophilic and thermophilic prokaryotes. The subject of a considerable body of research on the bioenergetics of acidophiles is *Thiobacillus ferrooxidans* (Ingledew, 1982). Subsequent studies have involved the moderate thermophiles *Bacillus* (*Alicyclobacillus*) *acidocaldarius* (bacteria) and *Thermoplasma acidophilum* (archaea) (Michels and Bakker, 1985), and the thermophilic archaeon *Sulfolobus acidocaldarius* (Lübben and Schäfer, 1989) and *Metallosphaera sedula* (Peeples and Kelly, 1995).

The impact of metabolic diversity in microorganisms and its evolution has widened the concept of bacterial autotrophy and heterotrophy. The terms chemolithoautotrophy and chemolithoheterotrophy have been widely used in the literature to describe acidophilic microorganisms, based on their carbon and energy metabolisms. Chemolithoautotrophs derive energy from the oxidation of inorganic compounds such as ferrous iron, sulphur, ammonium or hydrogen (H_2), and characteristically build up cellular organic substances by assimilating carbon exclusively from atmospheric carbon dioxide (CO_2), and often have no potential to degrade organic compounds as carbon or as energy sources. However, in addition to obligate chemolithotrophs, some acidophiles grow as facultative chemlithoautotrophs and chemolithoheterotrophs, when appropriate organic energy and carbon sources are present. In some cases, the growth of bacteria on organic substrates has been reported to depend on the oxidation of inorganic compounds, particularly iron and sulphur, with

carbon dioxide still being an important source of carbon. This phenomenon (mixotrophic metabolism) has been reported for the neutrophilic ferrous iron oxidiser *Gallionella ferruginea* (Hallbeck and Pedersen, 1991), the acidophilic heterotroph *Acidiphilium acidophilum* (Mason and Kelly, 1988), and several strains of themoacidophilic sulfobacilli (Wood and Kelly, 1986; Zakharchuk *et al.*, 1994).

Energy metabolism in bacteria is characterised by a great diversity of different pathways for the breakdown of organic compounds, reflected in the diversity of the chemical structures which microbes can degrade. In addition, bacteria may grow at various concentrations of oxygen or in its absence, i.e. aerobes, anaerobes and microaerobes as well as facultative anaerobes. To date, four types of biological CO_2 fixation pathways have been described: (i) the reductive pentose phosphate cycle (Calvin-Benson cycle; Calvin, 1962), (ii) the acetyl-CoA pathway (Pezacka and Wood, 1986), (iii) the reductive tricarboxylic acid (TCA) cycle (Antranikian *et al.*, 1982; Shiba *et al.*, 1985), and (iv) the 3-hydroxypropionate cycle (Strauss and Fuchs, 1993).

The best known pathway is the Calvin-Benson cycle, which has been reported for the chemolithoautotrophic growth of *T. ferrooxidans*, using ATP and reducing power (NADPH) generated during the oxidation of iron or sulphur compounds (Gale and Beck, 1967; Katayama-Fujimura *et al.*, 1984, Leduc and Ferroni, 1994). The key enzymes of the Calvin cycle are ribulose bisphosphate carboxylase (RubisCO) and phosphoribulokinase (McFadden, 1989). The presence of these enzymes has also been reported in the mixotrophic growth of *A. acidophilum* though at lower levels than in autotrophic-grown cells (Mason and Kelly, 1988; Pronk *et al.*, 1990a). Assimilation of carbon dioxide by Gram-positive moderate thermophiles was also found to be via the Calvin cycle (Wood and Kelly, 1986) though this ability is relatively limited when compared with those of the mesophilic chemolithoautotropic bacteria, and some

moderate thermophiles require carbon dioxide-enriched atmospheres for optimum autotrophic growth (Wood and Kelly, 1983; Norris *et al.*, 1996).

In several anaerobic eubacteria (strictly anaerobic or microaerophilic) and archaea, two alternatives have been reported, the reductive TCA cycle and the reductive acetyl-CoA pathway, both of which bring about the synthesis of acetyl-CoA from carbon dioxide ($2CO_2 + 8H \rightarrow (CH_2O)_2 + 2H_2O$) (Fuchs, 1999). The key enzymes are for TCA cycle are 2-oxoglutarate synthase and ATP citrate lyase. The reductive acetyl-CoA pathway is non-cyclic and the key enzyme of this pathway is the nickel enzyme acetyl-CoA synthase/CO dehydrogenase. The most remarkable trait of these two pathways is the reversibility of the reactions in one organism: autotrophic CO_2 fixation as well as acetyl CoA oxidation to $2CO_2$ under heterotrophic conditions. The 3-hydroxypropionate cycle has been reported in phototrophic green non-sulphur bacteria (*Chloroflexus aurantiacus*) and in archaea which bring about the synthesis of glyoxylate (precursor of cell carbon) from bicarbonate ($2HCO_3^- + 2NADPH + 3H^+ + 3ATP \rightarrow CHO\text{-}COO^- + 2NADP^+ + 3ADP + 3Pi$) (Fuch, 1999). The key enzymes of the 3-hydroxypropionate cycle are propionyl-CoA carboxylase and acetyl-CoA carboxylase. *Acidianus brierleyi*, a facultatively anaerobic thermoacidophilic archaeon, has been reported to use the 3-hydroxypropionate pathway for its autotrophic CO_2 fixation (Ishii *et al.*, 1997)

1.4 Diversity of acidophilic prokaryotes

Acidophilic bacteria are widespread in nature and their biodiversity is also fairly broad. The increasing numbers of novel species of acidophilic prokaryotes has been highlighted in recent reviews (e.g. Johnson, 1998a) together with the emerging picture of complex interactions between acidophiles in natural and synthetic environments. Acidophilic microorganisms display, as would be expected by the extreme characteristics

of their natural environment, special physiological and morphological adaptations. These include adaptation or tolerance mechanisms to high concentrations of heavy metals and protons, and high temperatures (in the case of thermophilic microorganisms) (Norris and Ingledew, 1992; Norris, 1989; Cobley and Cox, 1983).

1.4.1 Mesophilic acidophilic bacteria

1.4.1.1 Chemolithotrophic acidophilic bacteria

Mesophilic chemolithotrophic bacteria have been the most widely studied of acidophilic organisms. This is partly due to the fact that they are readily isolated and cultured and also because of their direct role in mineral leaching processes. Acidophilic chemolithotrophic bacteria have optimum growth temperatures generally between 25 and 37°C, and derive energy for growth from the oxidation of various inorganic compounds, particularly iron and reduced sulphur compounds. As a carbon source, these organisms use exclusively carbon dioxide, which they assimilate by chemosynthesis (Kelly, 1989). Besides the genera and the type species reviewed below, many other mesophilic chemolithotrophic strains have been isolated, though remain to be classified.

1.4.1.1.1 Genus *Thiobacillus*

The genus *Thiobacillus* consists of Gram-negative bacteria, which all have the ability to grow autotrophically and derive energy from the oxidation of reduced inorganic sulphur compounds. Various species fall into the α-, β-, and γ-subclasses of the *Proteobacteria* (Woese *et al.*, 1987; Lane *et al.*, 1992 and McDonald *et al.*, 1997). The genus is extremely heterogeneous, as evident from their G + C contents, DNA homologies, optimal growth conditions and other physiological characteristics (Kelly and

Harrison, 1989). Table 1.1 summarises some general characteristics of several selected species of *Thiobacillus* described in the literature.

In the latest edition of Bergey's Manual of Systematic Bacteriology (due to be published in 2001), some species of the genus *Thiobacillus* will be reclassified to the newly designated genera *Acidithiobacillus* gen. nov. (containing *T. thiooxidans*, *T. ferrooxidans*, *T. caldus* and *T. albertensis*), *Halothiobacillus* gen. nov. (containing *T. neapolitanus*, *T. halophilus* and *T. hydrothermalis*) and *Thermithiobacillus* gen. nov. (containing *T. tepidarius*) (Kelly and Wood, 2000). However, the original genus and species name is retained for the type species *Thiobacillus thioparus*, a member of the β-subclass of *Proteobacteria*. In addition a member of the α-subclass, *Thiobacillus novellus* and of the γ-subclass, *Thiobacillus prosperus* are also retained in the genus *Thiobacillus* due to insufficient data to assign them to existing or new genera (Kelly and Wood, 2000). Despite the proposal of these new genera, the genus *Thiobacillus* is still in common usage and will be referred to throughout this thesis.

Thiobacillus thiooxidans was the first acidophilic *Thiobacillus* species to be isolated (Walksman and Joffe, 1922), and is motile by mean of a polar flagellum and commonly occurs as single and paired rods (0.5 μm x 1.0-2.0 μm). The optimum growth temperature and pH are 28-30°C and 2.0-3.0, respectively (Kelly, 1989), although it has been reported that this species can tolerate to extremely low pH values (pH 0.5 to 1.0; Tuovinen *et al.*, 1991). The G + C content of *T. thiooxidans* is about 52 mol% (Harrison and Norris, 1984). *T. thiooxidans* is a strict chemolithotroph that obtains energy for growth from the oxidation of variety of reduced sulphur compounds (Unz and Lundgren, 1961) and is not capable of iron or pyrite oxidation, though it has been shown to grow on sulphur produced from pyrite when grown in co-culture with *Leptospirillum ferrooxidans* (Helle and Onken, 1988; Sand *et al.*, 1992). Ferric iron reduction has been observed with

T. thiooxidans but it is not known if iron reduction can support its growth under anaerobic conditions (Brock and Gustafson, 1976).

Thiobacillus ferrooxidans was first isolated from acidic mine drainage water (Temple and Colmer, 1951) and has since been isolated from a variety of acidic environments. It is the most studied and best known acidophilic bacterium (Rawlings and Kusano. 1994; Leduc and Ferroni, 1994; Ingledew, 1982). Many strains of *T. ferroxidans* have been described in the literature, all sharing the more or less uniform phenotype of being acidophilic, obligately chemolithoautotrophic, Gram-negative rods (0.5 μm wide by 1-1.0 μm long) that use the oxidation of ferrous iron in addition to sulphur for energy generation. They do, however, exhibit considerable genetic variation (Harrison, 1982; Kelly and Harrison, 1989).

Due to the ability to oxidise both sulphur and ferrous iron in pure culture, *T. ferrooxidans* has been assumed to be the most important microorganism in the biological leaching of copper, uranium and gold (Cwalina *et al.*, 1988; diSpirito *et al.*, 1983; Credá *et al.*, 1993; Leduc and Ferroni, 1994; Schipper *et al.*, 1995). *T. ferrooxidans* was long considered to be an organism of limited nutritional versatility. However, there are reports that *T. ferrooxidans* can also grow aerobically by the oxidation of hydrogen (Drobner *et al.*, 1990), formic acid (Pronk *et al.*, 1991a) and anaerobically by coupling the oxidation of sulphur or formic acid to the reduction of ferric iron (Pronk, *et al.*, 1991b). In the past, there were a number of reports claiming heterotrophic or mixotrophic growth of putative strains of *T. ferrooxidans* (Shafia and Wilkinson, 1969; Shafia *et al.*, 1972; Tabita and Lundgren 1971a,b). However these claims have not been substantiated and many have probably been caused by contamination of the *T. ferrooxidans* cultures with heterotrophic acidophiles (Kelly and Harrison, 1989).

Mixotrophic growth has been claimed for one strain, *T. ferrooxidans* FDI, which appeared to concomitantly utilise glucose and ferrous iron (Barros *et al*, 1984). Other metal mobilising thiobacilli include *T. prosperus* (Huber and Stetter, 1989) and '*T. ferrooxidans*' strain T3.2 (de Silóniz *et al.*, 1993). Both are capable of chemolithotrophic growth on pyrite and elemental sulphur, though the latter is unable to grow on ferrous iron as the sole source of energy unless glucose or yeast extract or some reduced sulphur source (tetrathionate or thiosulphate) is included. *T. prosperus* is a halotolerant iron-oxidising *Thiobacillus* sp. capable of growing in up to 6% (w/v) sodium cloride. *Thiobacillus kabobis* (Reynolds *et al.*, 1981) and *Thiobacillus albertis* (Bryant *et al.*, 1983) are acidophilic chemolithotropic thiobacilli, which are capable of autotrophic growth on reduced sulphur compounds.

The optimum temperature for growth of *T. ferrooxidans* strains ranges from 30° to 35°C, and temperature limits vary to some extent between strains and growth conditions (Norris and Johnson, 1998). The range of pH for growth is dependent on substrate. The optimum pH for growth on ferrous iron is *ca* 2.0, on elemental sulphur 1.75 to 5.0, and on pyrite and chalcopyrite between pH 1.8 and 3.5 (McGoran *et al.*, 1969). The type strain of *T. ferrooxidans* (ATCC 23270) has been reported to be capable of growth on ferrous iron at pH as low as 1.25 (culture doubling time of ~100 h); however, it appears that the culture viability declines rapidly during incubation (Gomez *et al.*, 1999).

The G + C content of '*T. ferrooxidans*' strains was initially described to be in the range of 55 – 65 mol% (Harrison 1982), though has since been redefined to be in the range of 57 – 59 mol% (Harrison, 1986a). Based on the 16S rRNA gene sequences analyses, two strains of *T. thiooxidans* and six strains of *T. ferrooxidans* were found to be closely clustered in the β-subclass of the *Proteobacteria* phylum (Lane *et al.*, 1992). The

uncertainty of the taxonomic position of *T. ferrooxidans* and *T. thiooxidans* was reported (Geobel and Stackebrandt, 1994) though it was recently concluded that *T. thiooxidans* is more closely clustered with *T. ferrooxidans* than other species of the genus *Thiobacillus* (*Acidithiobacillus*) (Kelly and Wood, 2000).

Table 1.1: Some characteristics of the Gram-negative, mesophilic acidophilic species of the genus *Thiobacillus* and *Thiomonas*.

Organisms / Characteristics	PH range[a]	Temperature range[a] (°C)	Mol% G + C	Substrates	References
T. ferrooxidans	1.4 – 6.0 (2.5 – 2.8)	20 – 40 (30 – 35)	57 – 59	Fe^{2+}, S°, $S_2O_3^{2-}$, $S_4O_6^{2-}$, sulphide minerals	e.g.: Leduc and Ferroni (1994); Kelly (1989)
T. thiooxidans	0.5 – 6.0 (2.0 – 3.5)	10 – 37 (28 – 30)	52 – 53	S°, $S_2O_3^{2-}$, $S_4O_6^{2-}$	Harrison (1982; 1984); Kelly (1989)
T. prosperus	n.d. (2.0)	20 – 45 (33 – 37)	64	S°, Fe^{2+}, sulphide minerals	Huber and Stetter (1989)
T. albertis	2.0 – 4.0 (3.5 – 4.0)	n.d. (28 – 30)	61 – 62	S°, $S_2O_3^{2-}$, $S_4O_6^{2-}$	Bryant *et al.*, (1983)
Th. cuprinus	n.d. (3.0 – 4.0)	20 – 45 (30 – 36)	66 – 69	S°, sulphide minerals, organic nutrients	Huber and Stetter (1989); Moreira and Amils (1997)

([a]): values in brackets refer to optima pH and temperature for growth; n.d.: not determined

1.4.1.1.2 Genus *Leptospirillum*

Leptospirillum ferrooxidans was first isolated from a copper deposit in Armenia (Markosyan, 1972), and is a Gram-negative, obligately chemolithotrophic iron-oxidising bacterium (Norris, 1990). *L. ferrooxidans* strains show some morphological variation but are readily distinguished from the rod-shaped *T. ferrooxidans* in being vibriod cells that often develop into spiral forms of varying length (Pivovarova *et al.*, 1981), and which are slightly thinner than *T. ferrooxidans* and more frequently motile with a single long polar flagellum. In addition, exopolymer production and flocculation often accompany its growth, particularly at low temperatures; e.g. 15 to 20°C (Norris, 1990). About half of the iron-oxidising bacteria in samples from a copper-zinc mine appeared to be *L. ferrooxidans* (Sand *et al.*, 1992) and it can outnumber *T. ferrooxidans* in acidic mine drainage water and mineral processing bioreactors (Rawlings *et al.*, 1999).

Unlike *T. ferrooxidans*, *L.ferrooxidans* is not capable of oxidising hydrogen (Drobner *et al.*, 1990) and or reduced sulphur (Norris, 1990). Somewhat paradoxically, Sugio *et al.* (1992a,b) reported that some strains of *L. ferrooxidans* displayed hydrogen sulphide:ferric ion oxidoreductase (SFORase) activity, i.e. were in principle able to oxidise sulphur, utilising ferric ions as electron acceptor. The optimum growth temperature for *L. ferrooxidans* tends to be higher than that of *T. ferrooxidans*, ranging from 30 to 37°C (Harrison and Norris, 1985; Norris and Johnson, 1998). In addition, some strains of *Leptospirillum*-like bacteria have been reported to grow on pyrite at temperatures between 40 to 45°C (Marsh and Norris, 1983; Norris, 1983). An isolate that grows optimally at 45 to 50°C has also been described as a new species, *Leptospirillum thermoferrooxidans* (Golovacheva *et al.*, 1992), but this has since been lost.

The lack of sulphur oxidising capacity restricts growth of *L. ferrooxidans* on some mineral sulphides, including chalcopyrite, in pure culture (e.g. Hutchins *et al.*,

1986; Norris, 1990). The presence and importance of *L. ferrooxidans* in bioleaching processes was overlooked for a considerable time. This might be explained by the fact that this bacterium is outcompeted by the faster growing *T. ferrooxidans* in ferrous iron-containing enrichment cultures (e.g. Norris and Kelly, 1982). However, pure cultures of *L. ferrooxidans* can grow on, and efficiently degrade, pyrite (Norris and Kelly, 1982; Sand *et al.*, 1992) and pyritic coal (Merretig *et al.*, 1989) in pure culture. In mixed cultures with *T. ferrooxidans* growing on pyrite, *L. ferrooxidans* was found to gradually dominate and to support more extensive leaching than *T. ferrooxidans* (Norris and Kelly, 1982) particularly at low pH values (Helle and Onken, 1988; Norris *et al*, 1988) or at higher temperatures (Sand, *et al.*, 1992). Studies based on the 16S rRNA gene sequence analyses of natural bacterial populations present in bioleaching operations sites have recently showed that *L. ferrooxidans* also outnumbers *T. ferrooxidans* in these environments (Espejo *et al.*, 1995; Rawlings, 1995; Durand *et al.*, 1997). The reason why *Leptospirillum*-like species rather than *T. ferrooxidans* are the dominant iron-oxidising bacteria in many of such environments is due to four criteria: (1) *L. ferrooxidans* is more tolerant to very low pH values than *T. ferrooxidans* (Sand *et al.*, 1992); (2) its thermotolerant features contribute to it being more active at elevated temperatures commonly found in leaching processes (Rawlings, 1997, 1999); (3) K_m values for ferrous iron oxidation by *L. ferrooxidans* are lower than those of *T. ferrooxidans* (Norris *et al.*, 1988); (4) K_i values for ferric iron are higher than those of *T. ferrooxidans* (Norris *et al.*, 1988).

A degree of diversity in *Leptospirillum*-like bacteria has been indicated by some differences on electrophoretic patterns of whole cell proteins, limited DNA homology studies in some isolates (Harrison and Norris, 1985) and relatively wide range of G + C content of the chromosomal DNA, (51 to 56 mol%; Eccleston *et al.*, 1985; Harrison and

Norris, 1985). In view of phylogenetic analyses (based on the 16S rRNA gene sequences), *Leptospirillum*-like bacteria were initially grouped in a separate cluster within the *Bacteria* domain, with no phylogenetical relationship to other sulphur- and iron-oxidising bacteria (Lane *et al.*, 1992) though, more recently *L. ferrooxidans* has been reported to be closely related with the magnetotactic bacterium *Magnetobacterium bavaricum*, and possibly forms a new phylum (Amann *et al.*, 1995; Durand *et al.*, 1997). This observation was also supported by chemotaxonomic studies that showed the presence of distinct chain of electron transporters in this bacterium (Barr *et al.*, 1990), menaquinone as respiratory quinones (in Goebel and Stackebrandt, 1995) and G + C contents. Molecular analyses of 16S rRNA gene sequences in a natural acidic environment showed that, from a total of 120 clones of 16S rRNA recovered, 70 were from *L. ferrooxidans*-like bacteria, while 37 were from *T. ferrooxidans*, suggesting a greater abundance of the former (Goebel and Stackebrandt, 1995; Durand *et al.*, 1997). Based on the ribosomal DNA sequence similarities and chemotaxonomic characteristics, it was suggested that bacteria describes as '*L. ferrooxidans*' is likely to consist of at least two species or even genera (Goebel and Stackebrandt, 1995).

1.4.1.2 Facultative chemolithotrophic acidophilic bacteria

Two mesophilic acidophilic facultative chemolithotrophs have been described, belong to the genera *Acidiphilium* and *Thiomonas*. These are *Thiomonas cuprinus* (Huber and Stetter, 1990; Moreira and Amils, 1997) and *Acidiphilium acidophilum* (Guay and Silver, 1975; Lane *et al.*, 1992). Other facultative chemolithotrophic thiobacilli have also been described, but are neutrophilic (e.g. *T. novellas* and *T. perometabolis*; Kelly, 1989).

A. acidophilum (formerly *Thiobacillus acidophilus*) was originally isolated from an apparently heterotrophic culture of *T. ferrooxidans* (Guay and Silver, 1975; Arkensteyn and de Bont, 1980). Reclassification of this bacterium from the genus *Thiobacillus* (β-subclass of *Proteobacteria*) into the genus *Acidiphilium* (α-subclass of *Proteobacteria*) is based on 16S rRNA gene sequence analyses by Lane *et al.* (1992). It is a Gram-negative, non-sporulating motile rod-shaped eubacterium. It grows autotrophically on reduced sulphur compounds and formate, heterotrophically on a variety of single organic compounds, and mixotrophically on glucose and various inorganic compounds (Meulenberg *et al.*, 1992; Pronk, *et al.*, 1990a, 1991b; Mason and Kelly, 1988). This bacterium is not capable of oxidising ferrous iron, but was reported to reduce ferric iron under microaerobic and anaerobic conditions (Bridge, 1995).

Thiomonas cuprinus was isolated from solfatara fields in Iceland and a uranium mine in Federal Republic Germany (Huber and Stetter, 1990) and was described as the first facultatively organotrophic, mineral sulphide ore-leaching, mesophilic acidophile. It was originally classified as *Thiobacillus cuprinus* but has been reclassified into a new genus *Thiomonas*, based on 16S rDNA sequences analyses as well as physiological differences from *Thiobacillus* spp. (Moreira and Amils, 1997), and has a G + C base content of the chromosomal DNA of 66 to 69 mol% (Huber and Stetter, 1990). *Th. cuprinus* is an aerobic, Gram-negative, motile, rod-shaped bacterium, which is able to grow heterotrophically on complex organic substrates and pyruvate, and autotrophically on elemental sulphur and various sulphidic ores. Autotrophic growth was, however, less efficient on elemental sulphur and single sulphidic ore than in ores mixtures and arsenopyrite, and no growth was obtained on pyrite (Huber and Stetter, 1990).

1.4.1.3 Heterotrophic acidophilic bacteria

Heterotrophic, ferrous iron-oxidising acidophilic bacteria appear to be widespread in acidic mine-drainage waters (Johnson *et al.*, 1995b). Some novel strains (the 'T-series' bacteria; Bacelar-Nicolau, 1996) display the ability to conserve energy for growth from the oxidation and reduction of ferrous and ferric iron, respectively. This includes the novel iron-oxidising obligate heterotroph '*Ferromicrobium acidophilus*' (DSM 11138; strain T23) which is capable of growth on ferrous iron only in the presence of yeast extract (Bacelar-Nicolau, 1996). Phylogenetically, this bacterium is located in between the cluster of Gram-negative and Gram-positive bacteria and different strains (or species) contain 51 – 55 mol% G + C in chromosomal DNA.

Acidophiles similar to heterotrophic contaminants of *T. ferrooxidans* cultures were isolated directly from acidic coal refuse and mine drainage (Harrison *et al.*, 1980; Wichlacz and Unz, 1981; Johnson and Kelso, 1983). Most isolates were found to be mesophilic, Gram-negative, non spore-forming rods, usually motile and were mostly located in the genus *Acidiphilium* (Table 1.2). These bacteria grow at pH values ranging from 2.0 to 6.0, which appears to be strain/species-dependent, and optimum at the pH 3.0 – 4.0. In contrast to the chemolithotrophic acidophiles, they are incapable of autotrophic growth on ferrous iron or reduced sulphur compounds. *Acidiphilium* SJH grows on low concentrations of yeast extract over a wide range of pH (between pH 1.5 to at least 7.0) while growth on higher concentration of some organic compounds (e.g. 10 mM glycerol) appears restricted between pH 2 and 4.5 (McGinness and Johnson, 1993). The base composition of *Acidiphilium* spp. has been found to be within the range 60 to 70 mol% G + C.

Acidiphilium cryptum, the first *Acidiphilium* sp. to be described, is an adept scavenger capable of surviving multiple serial subcultures in apparently organic-free

acidified basal salts media. It has also been shown to live on the trace amounts of organic compounds leaked by *T. ferrooxidans* and *L. ferrooxidans* (Harrison, 1984). They grow on a variety of single organic compounds as carbon sources, though this is species-dependent. Yeast extract was found to stimulate growth of some *Acidiphilium* spp. when added at 0.05 – 0.1% (w/v), and in some cases is a requirement for growth on single organic compounds (Bhattacharyya *et al.*, 1991). Higher concentrations of yeast extract (0.3 – 0.4%) were, however, found to inhibit growth of *Acidiphilium* spp. (Kishimoto *et al.*, 1990). Growth of *A. cryptum* is inhibited by organic substrates at about 1% (w/v) or greater and, in common with many other acidophilic bacteria, it is sensitive to organic acids.

Acidocella aminolytica and *Acidocella facilis*, both initially described as *Acidiphilium* spp. (Kishimoto *et al.*, 1993; Wichlacz *et al.*, 1986), were reclassified into the new genus, *Acidocella*, based on the comparative analyses of 16S rRNA gene sequences (Kishimoto *et al.*, 1995). This was also supported by G + C contents of the chromosomal DNA composition, which are significantly different to each other (Table 1.2).

From 16S rRNA gene sequence analyses, both *Acidiphilium* spp. and *Acidocella* spp. are phylogenetically located in the α1-subgroup of the α-subclass of the *Proteobacteria* phylum (Lane, *et al.*, 1992; Kishimoto *et al.*, 1995).

Acidomonas methanolica (Urakami *et al.*, 1989) and *Acidobacterium capsulatum* are also acidophilic mesophiles (Kishimoto *et al.*, 1990) but differ from *Acidiphilium* spp. and *Acidocella* spp. in both chemotaxic and phenotypical characteristics such as the presence of an exocellular capsule in *Acidobacterium capsulatum* and the fact that *Acidomonas methanolica* is facultatively methylotrophic. *A. angustum*, *A. cryptum* and *A. rubrum* were recently reported to produce bacteriochlorophyll-a and have limited

capacity for light-enhanced carbon dioxide incorporation (Kishimoto *et al.*, 1995). Bacteriochlorophyll-a is also present in *A. multivorum* (Wakao *et al.*, 1994).

Other mesophilic acidophilic heterotrophic bacteria which have the ability to oxidise ferrous iron have also been described, including filamentous and non-filamentous forms. Isolate CCH7 was described by Johnson *et al.* (1992) as a Gram-negative heterotrophic filamentous bacteria which is capable of ferrous iron oxidation, but cannot grow autotrophically on ferrous iron (and did not fix carbon dioxide during ferrous iron oxidation). The bacterium was unable to oxidise reduced forms of sulphur and pyrite, in either the presence or absence of yeast extract.

The major role of heterotrophic acidophilic bacteria in leaching process appears to be their consumption of organic compounds, which can inhibit the growth of obligately chemolithoautotrophic acidophiles (e.g. Harrison, 1984; Wichlacz and Thompson, 1988). Recent reports of acidophilic heterotrophic isolates which are capable of oxidising ferrous iron, and the finding that 40% of a total of fifty *Acidiphilium*-like isolates were capable of ferric iron reduction under aerobic and microaerobic conditions (Johnson and McGinness, 1991) might, however, imply that heterotrophic acidophilic bacteria have a more prominent and direct role in bioleaching processes.

Table 1.2: Some characteristics of mesophilic acidophilic heterotrophic bacteria

Organisms / Characteristics	pH range[1]	Temperature range[1] (°C)	Mol% G + C	Iron oxido-reduction	References
Acidiphilium spp.					
A. cryptum	1.9 – 5.9 (3.0)	35 41	68 – 70([2])	reducer	Harrison, 1984
A. organovoum	2.5 5.0	37	64	reducer	Lobos *et al.*, 1986
A. rubrum	2.6 – 6.0	n.d.	63	-	Wichlacz *et al.*, 1986
A. angustum	2.5 – 6.0	n.d.	67	-	Wichlacz *et al.*, 1986
A. symbioticum	1.5 – 6.0 (3.0 – 4.0)	37	59.5	n.d.	Battachrayya *et al.*, 1991
Acidiphilium SJH	1.8 – 6.0	n.d	64	reducer	Johnson and McGinness, 1991
Acidocella spp.					
Ac. aminolytica	3.0 – 6.0	n.d.	59	n.d.	Kishimoto *et al.*, 1995
Ac. facilis	2.5 – 6.0	n.d.	65	-	Kishimoto *et al.*, 1995
Acidobacterium capsulatum	3.0 – 6.0	20 - 37	60	n.d.	Kishimoto *et al.*, 1991
Acidomonas methanolica	2.0 – 5.5	<30 – 42	63 – 65	n.d.	Urakami *et al.*, 1989
Isolate CCH7	2.4 – 4.0 (3.0)	n.d.	62	oxidiser	Johnson *et al.*, 1992

([1]): data from Johnson and McGinness (1991); ([2]): Lobos *et al.*, (1986) described a G + C content of 64 mol%; n.d.: not determined; (-): not an iron oxidiser or reducer

1.4.2 Moderately thermophilic acidophilic bacteria

Moderately thermophilic bacteria are a diverse group of microorganisms which have been isolated from various geothermal, self-heating coal spoils, leach dumps and

soils, and generally grow optimally at 45 to 55°C. The main groups of acidophiles that are active at about 50°C have been characterised, as mostly Gram-positive, spore-forming *Bacillus*-like but also include Gram-negative sulphur-oxidising autotrophs (Table 1.3) and three genera of archaea (Table 1.4). Two sub-groups of Gram-positive, moderately thermophilic acidophilic bacteria have been cited, those which tend to have highly versatile metabolisms, being able to grow as autotrophs, mixotrophs, chemolithoheterotrophs or heterotrophs, and the obligate heterotrophs which are not capable of iron or sulphur oxidation (Norris, 1990). At the elevated temperatures at which these organisms grow, the solubility of gases such as carbon dioxide is lowered and alternative metabolisms may have developed for carbon assimilation (section 1.3) which contribute to the metabolic flexibility of the bacteria that assimilate carbon alternatively and/or simultaneously from both carbon dioxide and organic compounds.

1.4.2.1 Genus *Sulfobacillus*

Sulfobacillus is the genus most readily isolated from hot-spring run-off, coal spoil heaps and acidic water at mine sites. These bacteria are metabolically versatile, capable of chemolithoautotrophic growth on ferrous iron or sulphur compounds, and chemolithoheterotrophic or mixotrophic growth on ferrous iron in the presence of organic compounds. The genus *Sulfobacillus* currently contains three species: *Sulfobacillus thermosulfidooxidans* (Golovacheva and Karavaiko, 1979), *Sulfobacillus acidophilus* (Norris *et al.*, 1996) and *Sulfobacillus disulfidooxidans* (Dufresne *et al.*, 1996). Several strains of Gram-positive bacteria have been isolated and reported e.g. THWX, YTF-1, HPTH and LM2 (Ghauri and Johnson, 1991; Norris, 1990) though no firm phylogenetical characterisation has been made for these. In a recent report, several strains of *Sulfobacillus*-like bacteria (coded: YTF-1, YTF-5, YTF-17 and YS5-F1)

isolated from the Yellowstone National Park were identified; sequence analyses of 16S rDNA indicated that two of these isolates (YTF-1 and YS5-F1) are closely related to *S. acidophilus* strain ALV (Johnson *et al.*, 2000). These bacteria have been generally described as non-motile spore-forming rods, aerobic obligately acidophilic iron-oxidising bacteria, though some strains have been demonstrated to grow anaerobically. *S. disulfidooxidans* was once claimed to be capable of iron oxidation, though the validity of this remains unclear.

Autotrophic growth on ferrous iron and pyrite has been described for both *S. acidophilus* and *S. thermosulfidooxidans*, while autotrophic growth on elemental sulphur was only observed with *S. acidophilus*, and *S. thermosulfidooxidans* appears to be only capable of sulphur oxidation in the presence of yeast extract (Norris *et al.*, 1996). Both species are also capable of heterotrophic growth on yeast extract, but strains of *S. acidophilus* are more readily maintained and display higher biomass yields than *S. thermosulfidooxidans* (Norris *et al.*, 1996). Mixotrophic growth was demonstrated for several strains of sulfobacilli by simultaneous assimilation of carbon from carbon dioxide and organic compounds during ferrous iron oxidation (Wood and Kelly, 1983). When organic compounds (yeast extract or glucose) are added to ferrous iron liquid medium, this tends to result in a decrease in carbon dioxide fixation by the bacteria, and increased biomass yields (e.g. Ghauri and Johnson, 1991; Wood and Kelly, 1985). Anaerobic heterotrophic growth in the presence of organic compounds and ferric iron was recently reported in some iron-oxidising moderately thermophilic strains (ALV, TH1 and THWX). Anaerobic autotrophic growth on tetrathionate coupled to ferric iron reduction was also reported by isolates ALV, TH1 and THWX (Bridge, 1995; Bridge and Johnson, 1998).

A requirement for yeast extract was initially reported for many moderately thermophilic iron-oxidising bacteria grown on ferrous iron and sulphide minerals (Kelly *et al.*, 1979). It was later found that yeast extract could be replaced by a defined organic carbon source or by a reduced inorganic sulphur compounds (Norris and Barr, 1985). However, autotrophic growth in the absence of reduced sulphur supplements has recently been reported for both *S. thermosulfidooxidans* and *S. acidophilus* (Norris *et al.*, 1996).

1.4.2.1.1 Other moderately thermophilic acidophilic bacteria

Other than *Sulfobacillus*, one other genus of a Gram-positive moderately thermophilic bacteria has been described. *Acidimicrobium* currently contains a single species (*Acidimicrobium ferrooxidans*) which includes strains TH3 (Brierley, 1978; Norris and Barr, 1985) and ICP (Clark and Norris, 1996). As with *Sulfobacillus* spp., *Ad. ferrooxidans* is also able to grow heterotrophically, mixotrophically, or autotrophically via the oxidation of ferrous iron, though it has greater capacity for carbon dioxide fixation than species of *Sulfobacillus*. Unlike *Sulfobacillus* spp., *Ad. ferrooxidans* has not been shown to grow successfully on ferrous iron without the additional of reduced inorganic sulphur compound (Clark and Norris, 1996). However, sulphur and pyrite oxidation appear to be very limited with the latter bacterium. Anaerobic growth has been reported with isolate TH3, grown on glycerol in the presence of ferric iron (Bridge, 1995). An isolate YTF-3, isolated from Sylvan Springs site in the Yellowstone National Park is possibly another novel strain of *Ad. ferrooxidans* which shares similar morphological traits with strain TH3 (Johnson *et al.*, 2000).

Two Gram-negative moderately thermophilic species have also been described. These are *Thiobacillus caldus* (Hallberg and Lindström, 1994) and *Leptospirillum*

thermoferrooxidans (Golovacheva *et al.*, 1992). *T. caldus* (strains KU and BC13) are motile rods, and are the first acidophilic thermophilic thiobacilli to be described (based on the 16S rRNA gene sequence analyses). *T. caldus* grows autotrophically on a range of reduced inorganic sulphur compounds and molecular hydrogen, though there is no evidence of ferrous iron oxidation by this bacterium. Mixotrophic growth has also reported in reduced sulphur media containing organic substrates (yeast extract or glucose). *L. thermoferrooxidans* differs from other *Leptospirillum*-like bacteria (section 1.4.1.1.2) by its higher temperature range for growth (30 - 60°C), which optima at 45 - 50°C and the chromosomal DNA base composition (56.2 mol% G + C).

Table 1.3: Some characteristics of acidophilic moderately thermophilic eubacteria

Organisms / Characteristics	pH range[1]	Temperature range[1] (°C)	Mol% G + C	Substrates ([2])	References
Thiobacillus caldus	1.0 – 4.0 (2.0 – 2.5)	<30 – 55 (45)	63.1 – 63.9	S°, S + YE, H_2	Hallberg and Lindstrőm, 1994
Leptospirillum thermoferrooxidans	1.3 >1.65	<20 – 60	56	Fe^{2+}	Golovacheva *et al.*, 1992
Sulfobacillus thermosulfidooxidans	1.5 – 5.5	<30 – 60	48 – 50	Fe^{2+}, pyrite, YE, Fe^{2+}+YE, S°+YE	Golovacheva and Karavaiko, 1979
Sulfobacillus acidophilus	(2.0)	<30 – 55	55 – 57	Fe^{2+}, S°, pyrite, YE, Fe^{2+}+YE	Norris *et al.*, 1996
Acidimicrobium ferrooxidans	(2.0)	<30 – 55	67 – 68	S° ([1]), YE, pyrite, Fe^{2+}+YE	Clark and Norris, 1996

([1]): data in bracket indicates the optima pH or temperature for growth; ([2]): S°= elemental sulphur, Fe^{2+}= ferrous iron; YE= yeast extract

1.4.2.1.2 Moderately thermophilic acidophilic archaea

Three genera of moderately thermophilic acidophilic heterotrophic archaea currently recognised are *Thermoplasma*, *Picrophilus* and *Ferroplasma*. They are phylogenetically closely related to each other, and are members of the order *Thermoplasmales* (Schleper *et al.*, 1995). The genus *Thermoplasma* contains two species: *Thermoplasma acidophilum* (Darland *et al.*, 1970) and *Thermoplasma volcanium* (Segerer *et al.*, 1988); both are pleomorphic cocci which grow in the pH range 0.8 to 4.0 and temperature of about 33° to 67°C. They are facultative anaerobic heterotrophs that grown anaerobically by sulphur respiration (Segerer *et al.*, 1988). The hyperacidophilic genus *Picrophilus* was described with two species; *Picrophilus oshimae* and *Picrophilus torridus*, as being obligately aerobic (in contrast to *Thermoplasma* spp.) heterotrophic archaea with growth at temperatures ranging from 45° to 65°C and a pH range of 0.0 to 3.5 (Schleper *et al.*, 1995). It appears that *Picrophilus* spp. surpass all hyperacidophilic prokaryotes known so far in their ability to live in extremely acidic environments (Schleper *et al.*, 1995). More recently, a moderate thermophilic iron-oxidising archaeon within the order *Thermoplasmales* has been reported, *Ferroplasma acidarmanus* (Edwards *et al.*, 2000) which is closely related to *Ferroplasma acidiphilum* (Golyshina *et al.*, 2000) though, the former is more acidophilic than the latter with pH optima of 1.2 and pH 1.7, respectively.

Table 1.4: Some characteristics of acidophilic moderately thermophilic archaea

Organisms / Characteristics	pH range[1]	Temperature range[1] (°C)	Mol % G + C	Substrates[2]	References
Heterotrophs and sulphur reducers					
Thermoplasma acidophilum	0.8 - 4.0 (2.0)	45 – 63 (50 – 64)	46	Various organic compounds	Darland *et al.*, 1970
Thermoplasma volcanium	0.8 – 4.0	33 – 67	38	YE	Segerer *et al.*, 1986
Heterotrophs					
Picrophilus oshimae	0.06 – 3.5 (0.7)	45 – 65 (60)	36	YE	Schleper *et al.*, 1995
Picrophilus torridus	(0.7)	45 – 65 (60)	36	YE	Schleper *et al.*, 1995
Iron oxidiser					
Ferroplasma acidarmanus	0 – 2.5 (1.2)	40	n.d.	Fe^{2+}, pyrite	Edwards *et al.*, 2000
Ferroplasma acidophilus	1.3 – 2.2 (1.7)	20 – 45 (35)	36.5	Fe^{2+}, pyrite, Mn^{2+}	Golyshina *et al.*, 2000

([1]): data in brackets indicates the optima pH or temperature for growth; ([2]): YE= yeast extract, Fe^{2+}= ferrous iron; n.d.: not determined

1.4.2.2 Genus *Alicyclobacillus*

The genus *Alicyclobacillus* was proposed by Wisotzkey (1992) and currently contains four species: *Alicyclobacillus acidocaldarius* (Darland and Brock, 1971), *Alicyclobacillus acidoterrestris* (Deinhard *et al.*, 1987a), *Alicyclobacillus cycloheptanicus* (Deinhard *et al.*, 1987b) and *Alicyclobacillus hesperidum* (Albuquerque *et al.*, 2000).

These bacteria are obligately acidophilic, Gram-positive (or Gram variable), spore-forming, rod-shaped bacteria which grow at temperature of less then 40°C to up to

70°C (in Wisotzkey *et al.*, 1992). Some characteristics of these bacteria have been previously reported and are summarised in Table 1.5. They were formerly classified as *Bacillus* spp. and were later differentiated on the basis of comparative 16S rRNA gene sequence analyses and by the unique characteristic of possessing ω-alicyclic fatty acids as major lipids of the cell membrane, which is not found in any other *Bacillus* species. *Al. cycloheptanicus* differs from other *Alicyclobacillus* spp. by the fatty acid in the cell membrane which are by ω-cycloheptane, rather than ω-cyclohexane fatty acids, that are commonly found in other species (Deinhard *et al.*, 1987b). Phylogentically, *Sulfobacillus* is the closest related genus to *Alicyclobacillus*, though the latter was distinguished from the former by being an obligate heterotroph, and no evidence of inorganic compounds utilisation has been reported for *Alicyclobacillus* spp.. The G + C content of the chromosomal DNA is ranges from 51.6 to 60.3 mol% (Wisotzkey *et al.*, 1992). Several *Alicyclobacillus*-like isolates have also been reported. These include two strains, YTH1 and YTH2, isolated from Frying Pan Hot Spring in Yellowstone National Park, USA (Johnson *et al.*, 2000).

Table 1.5: Some characteristics of moderately thermophilic acidophilic bacteria of the genus *Alicyclobacillus*.

Organisms / Characteristics	pH range[1]	Temperature range[1] (°C)	Mol % G + C	Substrates	References
Al. acidocaldarius	2.0 – 6.0	45 – 70	61 – 62		Darland and Brock., 1971
Al. acidoterrestris	2.2 – 5.8	<35 - >55 (42 - 53)	61 – 53	Various organic	Deinhard *et al.*, 1987a
Al. cycloheptanicus	3.0 – 5.5	40 – 58 (48)	54 - 57	compounds	Deinhard *et al.*, 1987b
Al. hesperidum	(3.5 – 4.0)	35 - <60 (50 - 53)	53.3		Albuquerque *et al.*, 2000

([1]): data in brackets indicates the optima pH or temperature for growth

1.4.3 Extremely thermophilic acidophiles

Brock *et al.* (1972) were the first to isolate prokaryotes from boiling springs of Yellowstone National Park, U.S.A.; the first extremely thermophilic organism *Sulfolobus acidocaldarius* was isolated which can grow at temperature up to 92°C and with an optimum at 80°C. Since then, various extremely thermophilic organisms have been isolated from continental and submarine volcanic areas, such as solfatara fields, geothermal and hydrothermal power plants or geothermally-heated sea sediments and hydrothermal vents (Stetter, 1989). Marsh and Norris (1983) also found such prokaryotes in samples of a drainage channel emanating from a coal pile in Warwickshire, U.K., suggesting that they may be present in metal-rich habitats, and have potential use in leaching operations.

All acidophilic extreme thermophiles of interest in mineral leaching processes belong to the domain *Archaea*. Many were originally isolated from hot springs and

thermal acidic soils or sediment samples, live optimally at 70°C or above, and consist mostly of coccoid cells which are facultatively or obligately chemolithotrophic on sulphur oxidation. Four genera have been described, all belonging to the order *Sulfolobales*: *Sulfolobus*, *Acidianus*, *Metallosphaera* and *Sulfurococcus* (Table 1.6). The chromosomal DNA base (guanine + cytosine) composition varies depending on species between 30 and 45 mol%.

Members of the genus *Sulfolobus* are strictly aerobic sulphur-oxidisers, though most strains are facultative heterotrophs and some can also grow autotrophically by oxidation of ferrous iron (Stetter, 1989). Two species of the genus, *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus* were reported to grow optimally at temperature between 85° and 87°C and have a pH range of about 1.0 to 6.0 (in Stetter, 1989). Some *Sulfolobus*-like isolates (BC, LM and BC65) have been reported to grow autotrophically on mineral sulphides at temperatures near 70°C (Norris, 1990; Barr *et al.*, 1992). However, neither *S. acidocaldarius* nor *S. solfataricus* are able to oxidise pyrite (Larsson *et al.*, 1990).

The genus *Acidianus* was first described by Segerer *et al.* (1986) and has an optimum temperature and pH at around 85°C and 2.0, respectively. These organisms grow aerobically with oxidation of elemental sulphur to sulphate and anaerobically with molecular hydrogen via elemental sulphur reduction to hydrogen sulphide (Segerer *et al.*, 1986). Two species have been described, *Acidianus infernus* and *Acidianus brierleyi* (previously *Sulfolobus brierleyi*) though only the latter is able to oxidise ferrous iron in the presence of yeast extract and to grow autotrophically on pyrite (Larsson *et al.*, 1990). Another isolate, named *Desulfurolobus ambivalens* (previously *Sulfolobus ambivalens*; Zillig *et al.*, 1985; 1987) has been suggested to be a species of *Acidianus*, as it showed 60% DNA homology with *Acidianus infernus* (in Tuovinen *et al.*, 1991).

The genus *Metallosphaera* consists of only one species, *Metallosphaera sedula*, which is an aerobic, facultative autotrophic sulphur- and mineral sulphide-oxidising archaeon that can grow heterotrophically on complex organic compounds (Huber *et al.*, 1989). Growth is optimum at a temperature of 75°C and pH 1.0 to 4.5.

The members of the genus *Sulfuroccocus* are aerobic, facultative autotrophic sulphur-oxidising microorganisms, which can grow heterotrophically and mixotrophically on organic compounds. Growth occurs at temperatures ranging from about 50 to 86°C and pH 1.0 to 5.6. *S. yellowstonii* has been also reported to grow autotrophically by ferrous iron oxidation (Karavaiko *et al.*, 1994).

Table 1.6: Some characteristics of extremely thermophilic acidophiles.

Organisms / Characteristics	Growth pH (optimum)	Temperature range (optimum; °C)	Mol% G + C	Substrates	References
Sulfolobus spp.	aerobic facultative autotrophs				
S. acidocaldarius	60 – 85 (70 - 75)	1.5 – 5.5 (3.0 – 3.5)	37	sulphur compounds;	Stetter, 1989
S. solfataricus	50 – 87 (87)	2.0 – 5.0 (4.0)	34 – 36	Fe^{2+}, organic compounds	Zillig *et al.*, 1980
Acidianus spp	aerobically oxidise and anaerobically reduce sulphur				
A. brierleyi	45 – 75 (70)	1.0 – 6.0 (1.5 – 2.0)	31	S°, Fe^{2+}, sulphide minerals, organic compounds	Segerer *et al.*, 1986
A. infernus	65 – 96 (90)	1.0 –5.5 (2.0)	31	S°, organic compounds	Segerer *et al.*, 1986
Desulfurolobus ambivalens	55 – 86 (81)	0.8 – 4.0 (2.5)	32.7	S°	Zillig *et al.*, 1987
Metallosphaera sedula	50 – 80 (75)	1.0 – 4.5	45	S°, sulphide minerals, organic compounds	Huber *et al.*, 1989
Sulfuroccocus spp.	aerobic facultative sulphur oxidisers				
S. mirabilis	50 – 86 (70 - 75)	1.0 – 5.6 (2.0 – 2.6)	43 – 44	S°, organic compounds	in Karavaiko *et al.*, 1994
S. yellowstonii	40 – 80 (60 – 65)	1.0 – 5.5 (2.0 – 2.5)	44.6	S°, Fe^{2+}, sulphide minerals, organic compounds	Karavaiko *et al.*, 1994

1.5 Growth of acidophilic bacteria that respire on iron

Many iron-oxidising bacteria also oxidise sulphur and are obligate acidophiles. This is in part because spontaneous oxidation of ferrous iron in oxic environment at neutral pH is very rapid, and therefore significant amounts of ferrous iron accumulate only in the absence of oxygen. Ferric iron is only found in soluble form under acidic

conditions and precipitates in non-acidic pH liquors as insoluble ferric oxides and hydroxides. Ferrous iron is, however, relatively stable in acidic solutions. Anaerobic conditions favour the accumulation of ferrous iron and, under some anaerobic conditions, e.g. in the presence of hydrogen sulphide (H_2S), ferrous iron may precipitate as ferrous sulphide (Atlas and Bartha, 1993).

1.5.1 Energy from ferrous iron oxidation

The aerobic oxidation of iron from the ferrous (Fe^{2+}) to the ferric (Fe^{3+}) state is an energy-yielding reaction for some bacteria. However, only a small amount of energy is available from this oxidation (-30 kJ/mol at pH 2) and for this reason, iron-oxidising bacteria must oxidise large amounts of iron in order to grow. The microbial mechanism of iron oxidation has been thoroughly and predominantly studied in the acidophilic chemoautotroph *T. ferrooxidans* as shown in Fig 1.4 (Blake *et al.*, 1989; Ingledew, 1982; Mansch and Sand, 1992). Ferrous ions are oxidised at the cell surface on the outer membrane (Hooper and DiSpirito, 1985) and the electrons transferred, through a chain of transporters, to a terminal oxidase in the cytoplasmic membrane, during which process energy is conserved. The bioenergetics of iron oxidation by *T. ferrooxidans* is of biochemical interest because of the very electropositive reduction potential of the Fe^{3+}/Fe^{2+} couple (+770 mV at pH 2). Because the reduction of potential of Fe^{3+}/Fe^{2+} couple is so high, the route of electron transport to oxygen is very short ($½O_2/H_2O$ = 820 mV).

The respiratory components of *T. ferrooxidans* involved in the oxidation of ferrous iron consist of a small number of molecules, present at high concentrations, which operate between ferrous iron and oxygen (Blake *et al.*, 1992). These are rusticyanin (Cox and Boxer, 1978), various cytochromes c (Ingledew and Cobley, 1980;

Mansch and Sand, 1992; Yamanaka *et al.*, 1993; Cavazza and Bruschi, 1995; Blake and Shute, 1994), a cytochrome a-type oxidase (Ingledew and Cobley, 1980; Yamanaka et al., 1993), iron-sulphur proteins (Fry *et al.*, 1986; Fukumori *et al.*, 1988), a glycoprotein (Mjoli and Kulpa, 1988) and a polynuclear Fe(III)-complex (Ingledew, 1986)

Electrons from the oxidation of ferrous iron cannot reduce NAD^+, FAD and many of the other components of the electron transport chain. The oxidative processes establishes a proton motive force across the cytoplasmic membrane which can be used to drive ATP synthesis or to produce reducing equivalents back up the respiratory chain to NAD^+. Under acidic conditions, acidophilic bacteria are capable of maintaining a near-neutral cytoplasmic pH (Ingledew, 1982; Cobley and Cox, 1983; Michels and Bakker 1985; Matin, 1990). In such cases, the pH difference across the cytoplasmic membrane represents a natural proton motive force that can play a role in ATP synthesis. Biochemical studies of electron flow in *T. ferrooxidans* suggest that acidophilic bacteria take advantage of the preexisting proton gradients on their environment for energy-generating purposes (Ingledew, 1982; Cox and Brand, 1984; Norris and Ingledew, 1992). However, to retain a neutral intracellular pH, protons entering the cell through the proton translocating ATPase (deriving the phosphorylation of ADP in the process) must be consumed. Therefore, it is here the oxidation of ferrous iron plays an important role. The oxidation of Fe^{2+} to Fe^{3+} ($2Fe^{2+} + \frac{1}{2}O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O$) is a proton-consuming reaction. There were reports suggesting that the reaction of $\frac{1}{2}O_2 + 2H_2 \rightarrow H_2O$ occurs on the inner face of the cytoplasmic membrane, whereas the reaction $Fe^{2+} \rightarrow Fe^{3+}$ occurs in the outer face of the membrane (Ehrlich *et al.*, 1991; Norris and Ingledew, 1992). Electrons from Fe^{2+} are accepted in the periplasmic space by rusticyanin and are subsequently transferred to cytochrome a. Cytochrome a donates electrons to $\frac{1}{2}O_2$ with the two protons required to form water which come from the cytoplasm. An influx of

protons via the ATPase replenishes the proton supply and, as long as Fe^{2+} remains available, the natural proton motive force across the membrane can continue to drive ATP synthesis (e.g. Blake and Shute, 1987; Hazra *et al.*, 1992; Cox and Brand, 1984; Hooper.and DiSpirito, 1985).

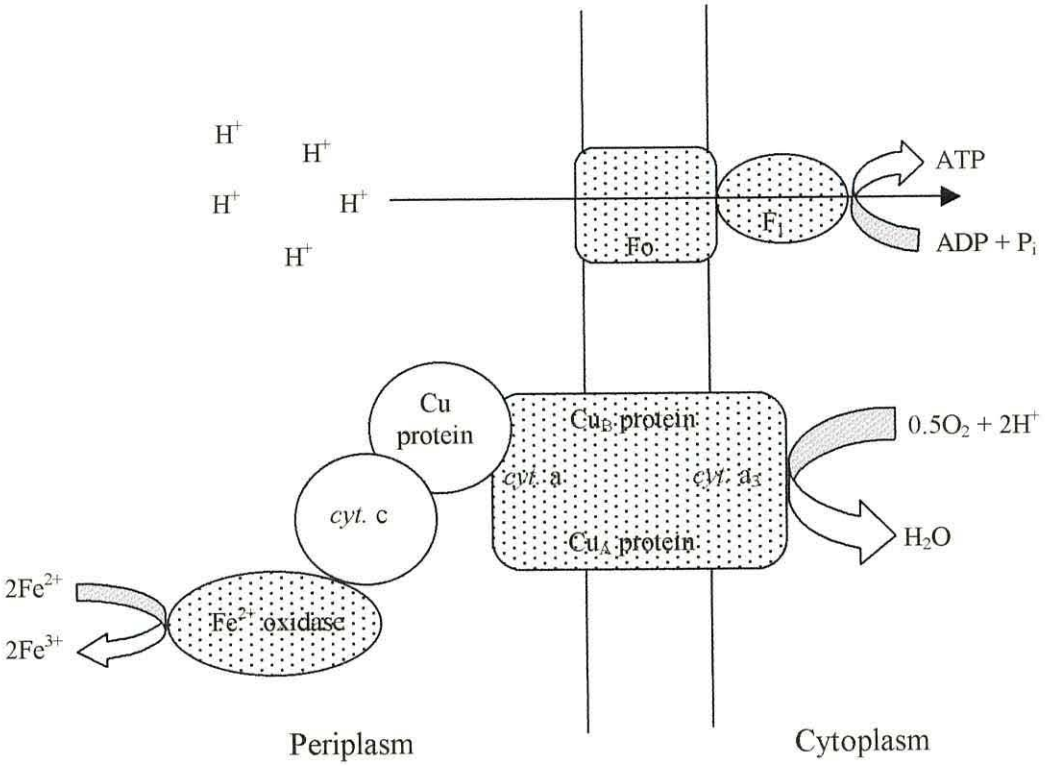


Fig1.4: Schematic representation of possible arrangement for the Fe^{2+} oxidation electron transport system of *Thiobacillus ferrooxidans*. Fe^{2+} oxidase, Fe(II)-cytochrome c552; *cyt* c, soluble cytochrome c552; Cu protein, rusticyanin: Cytochrome a (*cyt* a), cytochrome a3 (*cyt* a_3), Cu_A protein and Cu_B protein are likely components of a terminal oxidase. Fo and Fi are the membrane-integral and membrane-associated portions, respectively of ATP synthase (redrawn from Kuenen, 1999).

1.5.2 Ferric iron reduction by acidophilic bacteria

Microbial reduction of ferric iron has been mostly studied in neutrophilic environments, where it is linked to an anaerobic or a facultative aerobic mode of life. In either case, ferric iron may be used as terminal electron acceptor, coupled to the

oxidation of organic or inorganic compounds (Lovley, 1991). Three groups of iron reducing neutrophilic bacteria have been described (e.g. Jones, 1986; Johnson, 1995c): fermentative (e.g. *Bacillus* spp., *Escherichia* spp.), non-fermentative (*Pseudomonas* spp., *Geobacter metallireducens*, *Shewanella putrefaciens*) and sulphate reducing bacteria (e.g. *Desulfovibrio* spp.). Ferric iron reduction has also been reported in fungi (Dela Torre and Gomez-Alarcon, 1994), but their importance for iron cycling appears to be inferior to bacteria.

Ferric iron reduction by acidophiles was first demonstrated by Brock and Gustafson (1976), with cell suspensions of the obligately autotrophic acidophilic mesophiles *T. ferrooxidans* and *T. thiooxidans*, who showed that the reduction of ferric iron is coupled to the anaerobic oxidation of elemental sulphur and also pointed out that the free energy charge of the reaction ($S + 6Fe^{3+} + 4H_2O \rightarrow HSO_4 + 6Fe^{2+} + 7H^+$) is negative (-314 kJ/mol S at pH 2; in Pronk and Johnson, 1992). Ferric iron reduction has also been reported in some acidophilic mesophilic heterotrophs such as *Acidiphilium* spp. and the facultatively heterotroph *A. acidophilum* (Johnson and McGinness, 1991; Bridge, 1995), and recently in some iron-oxidising moderately thermophilic bacteria (strains ALV, THWX, YTF-1, TH1 and TH3; Bridge, 1995; Bridge *et al.*, 1998). However, little is known about the involvement of acidophilic microorganisms in the reductive part of the iron cycle, the enzymology or the kinetics of the reductive process (Pronk and Johnson, 1992; Bridge, 1995).

Microbial dissimilatory reduction of ferric iron greatly influences the biogeochemical cycles of carbon as well as other metals which are associated with ferric iron deposits and minerals (Lovley, 1991; 1993), and may have been the first globally significant mechanism for the oxidation of organic matter to carbon dioxide in an anoxic atmosphere, before sulphate, nitrate or oxygen respiration processes evolved (Walker,

1987). A number of models have been proposed to explain the mechanism of ferric iron reduction by bacteria (Jones, 1986) and these include (i) bacterial alteration of environmental parameters (such as pH, E_h) which induces spontaneous chemical reduction of ferric iron, (ii) the use of ferric iron as a general environmental electron sink associated with substrate level phosphorylation or regeneration of NAD, and (iii) ferric iron acting as an electron acceptor from an electron transport system.

A few components of the ferric iron-reducing system have been identified in acidophilic autotrophs. Sugio *et al.* (1985, 1987, 1988a,b and 1992a) have demonstrated the existence of a hydrogen sulphide:ferric ion oxidoreductase (SFORase) and a sulphite:ferric ion oxidoreductase in *T. ferrooxidans* strain AP19-3 grown under aerobic conditions on elemental sulphur. These enzymes use ferric iron as an electron acceptor during the oxidation of sulphide and sulphite ions, respectively. Further work showed that SFORase activity was present in a wide range of acidophilic iron-oxidising bacteria, including four strains of *Leptospirillum ferrooxidans*, eight strains of *T. ferrooxidans* and the moderate thermophiles *S. thermosulfidooxidans* BC1, *S. acidophilus* ALV and *Acidimicrobium ferrooxidans* TH3 (Sugio *et al.*, 1992b). A comparison between sulphite oxidases from various thiobacilli and Fe(III)-dependent sulphite-oxidases from *T. ferrooxidans* AP19-3 indicated that these were different enzymes, as the latter was not able to use cytochrome c as electron acceptor and the former could not use ferric iron as an electron acceptor.

Corbett and Ingledew (1987) noted that anaerobic Fe(III)-dependent sulphur oxidation by *T. ferrooxidans* NCIB 8455 was inhibited by HOQNO (n-heptyl 4-hydroxyquinoline N-oxide), a specific inhibitor of the cytochrome bc_1 complex. This suggested that participation of at least part of the respiratory chain on the Fe(III)-dependent sulphur oxidation process. Das *et al.* (1992) also reported an increase in the

cytochrome content of Fe(III)-reducing cells of *T. ferrooxidans* grown under anaerobic conditions compared with aerobically-grown cells, and the involvement of those cytochromes in ferric iron reduction.

1.6 Sulphur compound oxidation by acidophilic bacteria

Sulphur-oxidising bacteria are extremely heterogeneous, and are commonly known as colourless sulphur bacteria. The term 'colourless' discriminates these organisms from the phototrophic sulphur oxidising bacteria (Kelly, 1989). Oxidation of sulphur compounds by sulphur-oxidising bacteria results in accumulation of sulphate and consequent acidification of poorly-buffered growth media. The essential character of sulphur-oxidising bacteria is the ability to catalyse the oxidation of inorganic sulphur compounds and to couple this oxidation to conservation of energy as ATP. It has become clear that there is a large number of such compounds which can be oxidised by bacteria for this purpose, some of which are water soluble (tetrathionate, thiosulphate, sulfite), and some are extremely insoluble (elemental sulphur, pyrite) (Kelly, 1989; Hazeu *et al.*, 1988). The following balanced reactions summarise the aerobic oxidation of various sulphur compounds, summarised by Smith and Strohl (1991):

$$S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} \quad ; \triangle G_o -195.7 \quad (1.1)$$

$$SO_3^{2-} + 0.5O_2 \rightarrow SO_4^{2-} \quad ; \triangle G_o -61.7 \quad (1.2)$$

$$S_4O_6^{2-} + 3.5O_2 + 3H_2O \rightarrow 4SO_4^{2-} + 6H^+ \quad ; \triangle G_o -108.1 \quad (1.3)$$

$$S^o + H_2O + 1.5O_2 \rightarrow SO_4^{2-} + 2H^+ \quad ; \triangle G_o -140.4 \quad (1.4)$$

The most studied sulphur oxidisers are the mesophilic acidophilic bacteria of the genus *Thiobacillus*, and reports have often focused on the enzymology and mechanism of sulphur compound oxidation (e.g. Kelly *et al.*, 1997; Suzuki *et al.*, 1992; Kelly, 1985; Hallberg *et al.*, 1996). Many sulphur compounds can be used by these bacteria as

electron donor including elemental sulphur, thiosulphate, tetrathionate, sulphide and sulphite (e.g. Pronk *et al.*, 1990c, Leduc and Ferroni, 1994; Kelly, 1985). However, there is no single unifying pathway to account for the oxidation of sulphur compounds by these bacteria.

Oxidation of reduced inorganic sulphur compounds (RISCs) coupled to growth has been reported for moderately thermophilic bacteria of the genus *Sulfobacillus* (e.g Norris, 1990; Norris *et al.*, 1986). Sulphur oxidation has not been studied to any significant degree in these bacteria. However, metabolism of RISCs by the type strain of *S. thermosulfidooxidans* has been reported, which showed the capacity of this bacterium to oxidise various sulphur compounds commonly found in liquors during the bioleaching of sulphide minerals (Krasil'nikova *et al.*, 1998).

1.6.1 Sulphur compound disproportionation

A unique form of energy metabolism in some anaerobes has been described, in which sulphur compounds of intermediate oxidation state are metabolised in the absence of an extraneous electron acceptor. The term 'disproportionation' refers to the splitting of a sulphur compound into two new compounds, one of which is more oxidised and one of which is more reduced than the original substrate (Canfield and Thamdrup, 1994). In this process, both electron-donating and electron-accepting atoms are originally within the same substrate molecule and the oxidation level of products equals that of the reactant, i.e. inorganic sulphur fermentation (Smith and Strohl, 1991; Canfield and Thamdrup, 1994). In 1987, Bak and Cypionka published a description of the novel reaction sequence in which thiosulphate is disproprotionated by the sulphate reducer *Desulfovibrio sulfodismutans*. The proposal is summarised in the following reactions:

$$S_2O_3^{2-} \rightarrow SO_3^{2-} + S^o \qquad (1.5)$$

$$SO_3^{2-} + H_2O \rightarrow SO_4^{2-} + 2H^+ + 2e^- \quad (1.6)$$

$$S^\circ + 2H^+ + 2e^- \rightarrow H_2S \quad (1.7)$$

$$\text{Net: } S_2O_3^{2-} + H_2O \rightarrow SO_4^{2-} + H_2S; \quad \triangle G^\circ = -21.9 \text{ kJ} \quad (1.8)$$

In addition, sulphite can also be disproportionated to sulphate and sulphide following the reaction 1.5.(Bak and Pfennig, 1987):

$$4SO_3^{2-} + 2H^+ \rightarrow 3SO_4^{2-} + H_2S; \quad \triangle G^\circ = -235.6 \text{ kJ} \quad (1.9)$$

In these reactions electrons from either $S_2O_3^{2-}$ or SO_4^{2-} enter the electron transport chain and eventually reduce other molecules of $S_2O_3^{2-}$ or SO_4^{2-}, respectively, to H_2S.

Disproportionation of elemental sulphur has also been reported for sulphate reducers such as *Desulfobulbus propionicus* and *Desulfovibrio desulfuricans* (Lovley and Phillips, 1994; Fuseler and Cypionka, 1995). These bacteria disproportionate elemental sulphur completely to sulphate and sulphide with no detectable sulphur intermediates (Canfield and Thamdrup, 1994). The overall stoichiometry of this reaction is:

$$4S^\circ + 4H_2O \rightarrow 3H_2S + SO_4^{2-} + 2H^+; \quad \triangle G^\circ = +48 \text{ kJ} \quad (1.10)$$

The $\triangle G^\circ$ value indicates that disproportionation of elemental sulphur is an energetically unfavourable reaction. However, reports show that sufficient energy to support growth of sulphur disproportionating bacteria exists if the H_2S formed in reaction 1.6 is reoxidised to S°, in the presence of either Mn^{4+} or Fe^{3+} (Thamdrup *et al.*, 1993; Lovley and Phillips, 1994; Canfield and Thamdrup, 1994). Therefore, the stoichiometry of reaction 1.10 is altered to:

$$3S^\circ + 4H_2O + MnO_2 \rightarrow 2H_2S + SO_4^{2-} + 2H^+ + Mn^{2+} + 2OH^- \quad (1.11)$$

or

$$3S^\circ + 2Fe(OH)_3 \rightarrow 2FeS + 2H_2O + SO_4^{2-} + 2H^+ \quad (1.12)$$

Thus, unlike sulphite- or thiosulphate-disproportionating bacteria, sulphur-disproportionating bacteria require an external electron acceptor to drive the energetics of their reaction.

Unlike sulphur oxidation, the enzymology of sulphur disproportionation has not been elucidated, though, it was reported that proton motive force formation is central to the bioenergetics of these energy-yielding reactions (Smith and Strohl, 1991).

1.7 Bioleaching of sulphide minerals

The biooxidation of sulphide minerals is now an established industrial technology for the pretreatment of refractory arsenical gold ores, and for the bioleaching of copper and uranium (Hansford and Vargas 1999; Rawlings and Silver, 1995; Agate, 1996). Bioleaching processes depend on the abilities of acidophilic microorganisms to attack and dissolve mineral deposits (section 1.7.2). The reasons why the application of bioleaching in the mining industry has grown in recent years have been reviewed elsewhere (e.g. Ehrlich, 1999; Ehrlich and Brierley, 1990). In many places, rich surface ore deposits have been exhausted and mining companies have been forced to work lower grade surface ore deposits or to mine at greater depths. Research has been looking into methods to recover metals from ores containing lower metal values, and for ways of recovering the small quantities of metal left after physical processing of mineral concentrates. While the metal content of an ore is a major factor in the cost of metal recovery by conventional mining techniques, it is of less importance to the cost of metal recovery through bioleaching. Bacterial activity was reported to be effective in removing nearly all the metal from a 0.3% or from a 0.03% copper ore (Barrett *et al.*, 1993; Agate, 1996). In addition, it is often possible to carry out bioleaching of deep or low grade deposits *in situ*, thereby saving the cost of bringing vast tonnages of ore and waste rock to the surface (McNutty and Thompson, 1990; Brierley and Brierley, 1999).

Various types of acidophilic bacteria have been isolated from industrial leaching operations or from sites of natural leaching that are capable of attacking mineral

sulphides (Durand, 1997; Kelly, 1988; Harrison, 1984). The most important mesophiles are the chemolithotrophic bacteria *T. ferrooxidans*, *T. thiooxidans* (section 1.4.1.1.1) and *L. ferrooxidans* (section 1.4.1.1.2). In addition, there are a number of acidophiic heterotrophic bacteria (mostly assigned to the genus *Acidiphilium*) which grow in a close commensal relationship with the chemolithotrophic bacteria by scavenging organic carbon excreted by the chemolithotrophs (section 1.4.1.3). In recent years, more attention has been given to the thermophilic metal-mobilising bacteria which have the prospect of higher rates of mineral degradation and which may be more profitable commercially. Two distinct groups of iron oxidising acidophilic thermophiles are recognised, the moderately and the extremely thermophilic acidophiles (section 1.4.2). These include members of the genus *Sulfobacillus*, which grow on pyrite, chalcopyrite and similar ores. However, their requirement of some form of organic substrates or enhanced concentrations of carbon dioxide for maximum growth, have been perceived as a major drawback to their use in commercial bioleaching. The extremely thermophilic mineral degrading microorganisms are archaea (section 1.4.3). *Sulfolobus* spp. have been shown to metabolise sulphidic minerals such as pyrite and chalcopyrite, though one of the unusual characteristics of archaea is that they have cell walls lacking in peptidoglycan. As a result, these organisms are more fragile and more sensitive to the abrasive forces found in many leaching processes than bacteria, which may be one of the reasons why, in spite of their rapid leaching ability, no industrial-scale bioleaching process using *Sulfolobus* species is currently in operation. More recently, mixed cultures consisting of various acidophiles with different metabolic capacities such as iron and sulphur oxidation or carbon dioxide fixation have been showed to accelerate oxidative dissolution of pyrite (e.g. Norris and Kelly, 1982; Clark and Norris, 1996; Tuovinen *et al.*, 1991).

The rate of oxidation of sulphide minerals depends on their chemical reactivity (Baldi *et al.*, 1992; Wiersma and Rimstidt, 1984). This, in turn, depends on the composition of the metal sulphide (in terms of the various metal sulphides present), morphology (particle size and surface area) and crystal lattice imperfections on the mineral surface. Sulphide minerals often display non-stoichiometric composition (i.e. varying ratio of atoms in the crystal molecule) and also contain varying amounts of other metals, which make it difficult to establish an accurate stoichiometry for the reactions involved in bioleaching (Tuovinen *et al.*, 1991). Moreover, leaching of mineral ores is a complex process involving a variety of concurrent oxidative reactions (mediated by molecular oxygen, ferric iron and bacteria), and the conditions determining which chemical processes will prevail and which are not yet fully understood (Tuovinen *et al.*, 1991; Hansford and Vargas, 1999).

1.7.1 Chemical reactions in mineral sulphide oxidation

Pyrite is an extremely insoluble mineral but under aerobic conditions and in the presence of water, oxidation of metal sulphides occurs spontaneously as a purely chemical process. This process has been represented for pyrite by the following equations (e.g. Lundgren *et al.*, 1986; Evangelou, 1995):

$$FeS_2 + 3.5O_2 + H_2O \rightarrow FeSO_4 + H_2SO_4 \quad (1.13)$$

$$2FeSO_4 + H_2SO_4 + 0.5O_2 \rightarrow Fe_2(SO_4)_3 + H_2O \quad (1.14)$$

Following these reactions, the ferric iron formed is itself a powerful oxidant and consequently accelerates the dissolution of metal sulphides. In the course of this chemical reaction, Fe^{2+} ions and elemental sulphur (S°) are generated. This Fe^{3+}-mediated sulphide mineral oxidation (equation 1.15) is also known as the 'indirect leaching mechanism'. Generally, dissolved oxygen or Fe^{3+} ions are oxidising agents for

mineral sulphides in leaching operations and in the environment. The chemical oxidation of disulphides e.g. pyrite (FeS_2) and metal sulphides (MS) is summarised by the following equations (Schippers and Sand, 1999; Sand *et al.*, 1999):

$$FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 16H^+ + 2SO_4^{2-} \quad (1.15)$$

$$MS + 2Fe^{3+} \rightarrow M^{2+} + S^\circ + 2Fe^{2+} \quad (1.16)$$

$$S^\circ + 1.5O_2 + H_2O \rightarrow 2H^+ + SO_4^{2-} \quad (1.17)$$

The initial step in the mechanism of pyrite oxidation (reaction 1.13) has been suggested to involve the adsorption of oxygen molecules, as a monolayer, onto partially protonated pyrite surface. However, in most natural conditions, oxygen (as dissolved oxygen) does not directly attack the pyrite surface, but rather reacts with ferrous ions adsorbed on its surface and consequent to oxidation of ferrous to ferric iron by donating electrons to dissolved oxygen, reducing it to water (Moses and Herman. 1991). Subsequently, the absorbed ferric iron rapidly accepts electrons from pyrite, and is finally reduced to ferrous iron which again reacts with dissolved oxygen. However, faster rates of pyrite oxidation are observed in the presence of Fe^{3+} ions rather than when dissolved oxygen acts as an oxidant (Lawson, 1982; Schipper *et al.*, 1996). The faster rate of pyrite oxidation by Fe^{3+} is due to the fact that Fe^{3+} can bind chemically to the pyrite surface whereas O_2 cannot. The mechanisms of Fe^{3+} mediated leaching have been investigated by several researchers (e.g. Lawson, 1982; Luther, 1987) and consists of the following observations: (i) sulfoxy species ($S_2O_3^{2-}$) are intermediates produced during the process of pyrite oxidation either by Fe^{3+} or O_2; (ii) O_2 does not oxidise $S_2O_3^{2-}$ as readily as Fe^{3+} so that when O_2 is the sole pyrite oxidant sulfoxy compounds are more prevalent. In addition, ferric iron has also been reported to be the oxidant of pyrite in anaerobic environments (Moses *et al.*, 1987). The mechanism proposed above is also supported by Sand *et al.* (1995) who further suggested that thiosulphate produced reacted via

tetrathionate, sulphane monosulphonic acid and trithionate in a cycle to produce sulphate and elemental sulphur (reaction 1.11 – 1.13). This mechanism was reported to apply to pyrite (FeS_2), molybdenite (MoS_2) and wolframite (WS_2), and has been referred to as the 'thiosulphate mechanism', and has been shown to apply to both chemical and biological leaching (Schippers and Sand, 1999; Sand *et al.*, 1999). A simplified scheme is presented in Fig. 1.5

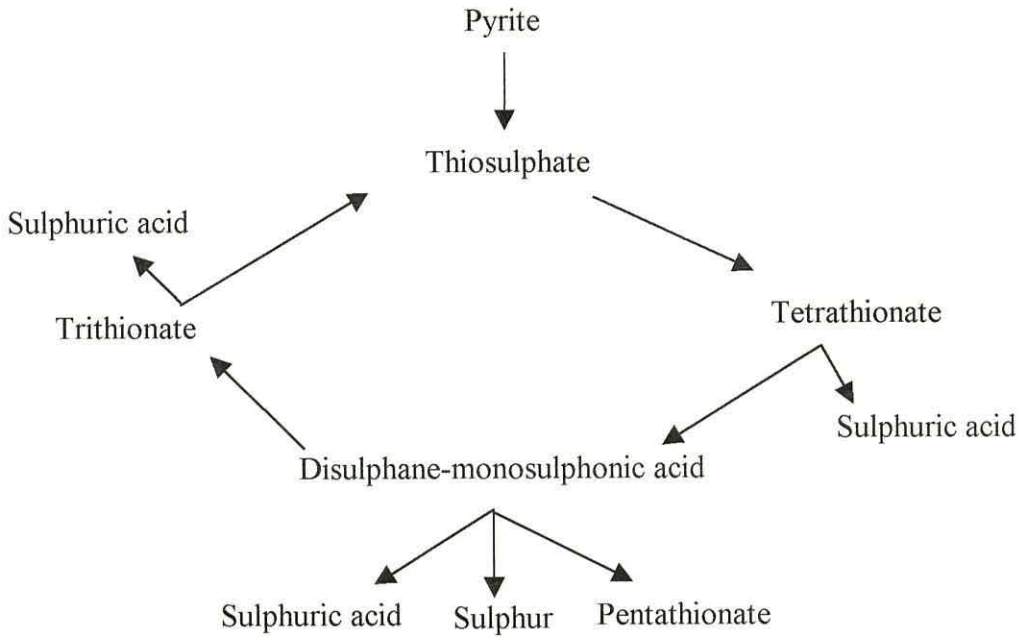


Fig. 1.5: Simplified scheme of the 'thiosulphate mechanism' for pyrite oxidation (adapted from Schippers *et al.*, 1996)

For all other sulphide minerals which are acid-soluble to some extent, the reaction with ferric iron yields elemental sulphur (Schipper and Sand, 1999). This proceeds by the formation of intermediary polysulphides (Steudel, 1996), which has prompted to the proposal of another indirect oxidation mechanism via polysulphides (Schipper and Sand, 1999; Sand *et al.*, 1999). The 'polysulphide mechanism' applies to metal sulphides such as sphalerite (ZnS), chalcopyrite ($CuFeS_2$), orpiment (As_2S_3), galena (PbS), hauerite

(MnS_2) and realgar (As_4S_4). The following equations summarises the two indirect oxidation mechanisms (Sand *et al.*, 1999):

"Thiosulphate mechanism":

$$FeS_2 + 6Fe^{3+} + 3H_2O \rightarrow S_2O_3^{2-} + 7Fe^{2+} + 6H^+ \quad (1.18)$$

$$S_2O_3^{2-} + 8Fe^{3+} + 5H_2O \rightarrow 2SO_4^{2-} + 8Fe^{2+} + 10H^+ \quad (1.19)$$

"Polysulphide mechanism":

$$MS + Fe^{3+} + H^+ \rightarrow M^{2+}\ 0.5H_2S_n + Fe^{2+} \quad (1.20)$$

$$0.5H_2S_n + Fe^{3+} \rightarrow 0.125S_8 + Fe^{2+} + H^+ \quad (1.21)$$

$$0.125S_8 + 1.5O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+ \quad (1.22)$$

The end product, elemental sulphur, produced via the polysulphide mechanism is biologically oxidised to sulphuric acid, which explains the ability of the non-iron oxidising *T. thiooxidans* to leach some metal sulphides such as ZnS. In the case of chalcopyrite there is an alternative proposed mechanism which involves only an acid initial attack of the sulphide (in Hansford and Vargas, 1999):

$$CuFeS_2 + 2H_2SO_4 \rightarrow CuSO_4 + FeSO_4 + H_2S \quad (1.23)$$

$$2H_2S + 2Fe(SO_4)_3 \rightarrow 4FeSO_4 + 2H_2SO_4 + 2S^\circ \quad (1.24)$$

The net result of these two reactions is the same as that of the direct ferric sulphate attack of the chalcopyrite:

$$CuFeS_2 + 2Fe(SO_4)_3 \rightarrow CuSO_4 + 5FeSO_4 + 2S^\circ \quad (1.25)$$

1.7.2 Role of acidophilic bacteria in sulphide mineral oxidation

The mechanisms of bacterial pyrite oxidation have mainly been derived from investigations of metal ore bioleaching. The mechanisms have been studied extensively (Konishi *et al.*, 1992; Mustin *et al.*, 1992; Wakao *et al.*, 1984) and classified as (a) direct metabolic reactions, mediated 'directly' by the bacteria, and (b) indirect metabolic

reactions, which are mediated via biologically-generated ferric iron acting as the chemical oxidant.

The direct mechanism of mineral leaching requires physical contact between bacteria and the mineral surface and results in the localised oxidation of the sulphide mineral. Several studies have shown that acidophilic microorganisms attach to sulphide minerals, such as pyrite (Bennett and Tributsch, 1978; Rodriguez-Leiva and Tributsch, 1988), chalcopyrite (McGoran *et al.*, 1969), zinc sulphide (Konishi *et al.*, 1992) and the pyrite fraction of coal (Dugan and Apel, 1978; Bagdigian and Myerson, 1986). Palencia *et al.* (1991) suggested that pyrite is directly attacked by iron-oxidising bacteria according to reaction (1.26):

$$4FeS_2 + 15O_2 + 2H_2O \rightarrow 2Fe_2(SO_4)_3 + 2H_2SO_4 \qquad (1.26)$$

Konishi *et al.* (1992) suggested that the microbial oxidation of pyritic sulphur to sulphate was due to the multiplication of *T. ferrooxidans* on the pyrite surface using pyritic sulphur as substrate. This was supported by electron microcopic observations of pyrite granules after bacterial leaching, where cylindrical-shape and dimensions appeared to be 'fingerprints' of bacterial cells (Rodriguez-Leiva and Tributsch, 1988), and resulted in increased porosity and specific surface area of the mineral grains (Mustin *et al.*, 1992).

Attachment of *T. ferrooxidans* cells to mineral surfaces was reported to be rapid and, mostly, irreversible (Blake *et al.*, 1995), though this appears to be strain-dependent (Ghauri, 1991; Espejo and Ruiz, 1987). Attachment of iron and/or sulphur oxidising bacteria onto surfaces is a selective process, though this is still not well understood. Bagdigian and Myerson (1986) reported selective adherence of *T. ferrooxidans* cells to the pyrite phase in dispersed coal. It has also been shown that *L. ferrooxidans* is chemotactically attracted to Ni(II), Fe(II) and Cu(II) (Acuña *et al.*, 1992) and *T. ferrooxidans* to thiosulphate (Chakraborti and Roy, 1992).

The involvement of extracellular polymeric substances (EPS) excreted by leaching bacteria in bioleaching has also been reported (Gehrke *et al.*, 1995; Tributsch, 1999). The primary attachment to pyrite is mediated by exopolymer-complexed iron (III) ions (Gehrke *et al.*, 1995) or other metal ions (Ferris *et al.*, 1989) as an electrochemical interaction with the negatively charged surface of the substrate. Considering the bacterial surface properties, attachment to sulphur is presumably dominated by hydrophobic (van der Waals) attraction forces, while sorption to pyrite is due to electrostatic interaction, probably combined with some involvement of hydrophobic forces (reviewed in Sand *et al.*, 1999; Hansford and Vargas, 1999). In contrast, Blake *et al.* (1995) and Ohmura *et al.* (1993) concluded that electrostatic interactions did not provide part of the driving force for adherence to the substrate pyrite. However, in acidophilic bacteria, the exact nature of the attachment mechanism remains unclear.

Several research groups have published results which indicate the existence of an organic layer surrounding the cell of chemolithotrophic bacteria, which forms a film between the cell and the mineral surfaces where the corrosion mechanisms takes place (Devasia *et al.*, 1993; Arredondo *et al.*, 1994; Gehrke *et al.*, 1995). The external polysaccharide layer (EPS) present in *T. ferrooxidans* and *L. ferrooxidans*, although chemically distinct in the two bacteria, was found to be produced 10-fold more in the presence of pyrite, compared to ferrous iron medium (Gehrke *et al.*, 1995). The presence of this EPS layer appeared to confer a modified surface charge or hydrophobic character to the surface of bacteria cells. This could be related to the binding of ions onto the EPS layer, in particular ferric iron (Blake *et al.*, 1994; Sand *et al.*, 1995).

It was recently suggested by Sand *et al.* (1995; 1999) that the 'direct', i.e. enzymatic attack mechanism of leaching does not exist. This was supported by the

studies of bacterial attachment to the surface of the mineral sulphide via the EPS, the latter catalyses or enhances indirect leaching by creating a favourable microenvironment where oxidation reactions occur. Ferric ions bound to the EPS layer mediate the chemical attack on the surface of the mineral sulphide, releasing ferrous iron hexahydrate and thiosulphate (Sand *et al.*, 1995). Ferrous iron is subsequently microbially oxidised to ferric iron, and thiosulphate undergoes chemical reactions in the 'thiosulphate mechanism' of indirect leaching following reactions (1.14 – 1.15), or is microbially oxidised. These reduced sulphur compounds may be included in periplasmic polythionates granula, as described for *T. ferrooxidans* (Steudel, 1989).

1.7.3 Factors influencing biological leaching

The bacterial oxidation of metal sulphides is effected by prokaryotes like *T. ferrooxidans*, *T. thiooxidans*, *L. ferrooxidans*, *Sulfobacillus*, *Acidianus* spp. and some others. The acidity, temperature, chemical composition of the medium and the characteristics of the leaching substrate influence the nature of the microbial populations that will develop both qualitatively and quantitatively, and may therefore affect the chemical reactions taking place (Tuovinen *et al.*, 1991; Lundgren and Silver, 1980). Nutritional requirements for microbial growth (carbon, oxygen, nitrogen, phosphate, sulphur and trace elements) are generally met by the composition of the leaching medium and from the environment, both in natural environments and synthetic media. An increase in concentrations of sulphide mineral substrates, i.e. pulp density, tends to result in increased metal solubilisation; however, high concentrations may cause a decrease in the leaching process by limiting the mass transfer of nutrients, especially oxygen and carbon dioxide, to the microorganisms (Lundgren and Silver, 1980; Baldi *et al.*, 1992).

Due to the exergonic nature of the mineral leaching process (e.g. Beck, 1967; Rawlings, 1997), elevated temperatures have often been found in leaching sites, even though these may vary considerably. Increasing temperature results in the augmentation of chemical reactions and, within limits, in faster microbial activity (Lundgren and Silver, 1980). The effects of temperature on mineral sulphide oxidation depends on the value of their activation energy, and hence their reactivity (Ahonen and Tuovinen, 1991; Ahonen and Tuovinen, 1992). At elevated temperatures the solubility of gases, such as oxygen and carbon dioxide is also reduced, which may result in a decrease in leaching efficiency. In the former case, this is due to a limitation in aerobic metabolism, as many microorganisms involved in the leaching of sulphide minerals do so, mostly, under aerobic conditions; in the latter case, a decrease in leaching efficiency may be due to a decrease in the availability of carbon source for chemolithotrophs and therefore in their growth and activity (Norris, 1990; Norris *et al.*, 1989).

The acidity of the solution influences the types of microbial populations which will thrive, the solubility of substrates for microbial oxidation, and the rate of chemical oxidation of ferrous iron. An optimum pH between 1.0 and 2.5 was reported for the oxidation of various sulphide minerals (Lundgren and Silver, 1980). At pH values above 3.0, the chemical oxidation of ferrous iron becomes increasingly rapid, reducing the level of substrate available for biological oxidation. Combined with this, the low solubility of the product (ferric iron) above pH ca 2.5 would lessen its role as a chemical leaching oxidant.

The influence of some potentially toxic metals on mineral oxidising bacteria is a consequence of their presence in the composition of mineral ores and their solubilisation during the leaching process. The relative sensitivity of acidophilic bacteria to toxic metals varies with the microorganism and is a function of previous exposure to the metal

in question (physiological or genetic adaptation), growth substrate, chemical composition of the growth medium and growth phase (Norris, 1989). Metal toxicity in acidophilic microorganisms was reviewed by Norris (1989) though, again, the majority of studies have been carried out with chemolithotrophic iron oxidisers.

Binding of metals to environmental constituents (e.g. organic compounds), precipitation, and ionic interaction were reported to decrease their toxic effects by decreasing their availability in solution (Gadd and Griffiths, 1978). Decreased toxicity of metals (uranium, thallium and rubidium) to *T. ferrooxidans* was reported in the presence of potassium (Norris, 1989). Mechanisms of metal resistance developed by microorganisms include production of hydrogen sulphide, production of organic compounds, uptake and accumulation and metal transformation (Gadd and Griffiths, 1978). In acidophilic microorganisms, resistance to mercury has been reported in various strains of *T. ferrooxidans*. This involves the enzymatic reduction and volatilisation of mercury, and appears to be constitutive and chromosomally-determined (Rawlings and Kusano, 1994; Norris, 1989). Resistance to uranium by washed cell suspensions of *T. ferrooxidans* is possibly related to its accumulation by the bacterium (Norris, 1989). In other cases, e.g. copper, zinc and cadmium, tolerance by *T. ferrooxidans* has been attributed to a limited binding or accumulation of the metals by the bacteria, and toxicity of silver to *T. ferrooxidans* was reported to be associated with a progressive accumulation of the metal by the cells (Norris, 1989).

The use of mixed cultures of acidophilic microorganisms in leaching operations may have some advantages over the use of pure cultures, and various reviews have been published on the subject (e.g. Norris and Kelly, 1982; Norris, 1990; Tuovinen *et al.*, 1991). Acidophilic heterotrophs and the chemolithotrophs *T. ferrooxidans* and *L. ferrooxidans* have been reported to form stable associations (e.g. Hallmann *et al.*,

1992), both in iron mineral media and in media containing sulphidic minerals. Mineral leaching by some of these mixed cultures has, however, resulted in contrasting results. The presence of *Acidiphilium* spp. in mixed culture with chemolithotrophic bacteria was shown to enhance the leaching activity of *L. ferrooxidans* (Hallmann *et al.*, 1992) and to enhance solubilisation of cobalt from a ore concentrate by *T. ferrooxidans* (Wichlacz and Thompson, 1988), while some other mixed cultures of *T. ferrooxidans* and *A. acidophilum* (Norris and Kelly, 1982) or *Acidiphilium* SJH did not (Johnson *et al.*, 1990). Furthermore, pure cultures of the heterotrophic iron-oxidising mesophile '*F. acidophilus*' (T-23) were reported to be not capable of pyrite leaching under autotrophic conditions, but oxidative dissolution of pyrite was found to be accelerated in co-culture with *T. thiooxidans* (Bacelar-Nicolau and Johnson, 1998).

The potential utilisation of acidophilic chemolithotrophs with complementary abilities, such as iron and sulphur oxidisers, to attack sulphide minerals has also been investigated (Sand *et al.*, 1992; Norris *et al.*, 1988; Helle and Onken, 1988). More extensive leaching of pyrite was reported with mixed cultures of *T. ferrooxidans* and some strains of *L. ferrooxidans* compared with pure cultures of either bacteria (Norris and Kelly, 1982; Norris *et al.*, 1988). A similar consortium did not enhance desulphurisation of coal when compared to a pure culture of *L. ferrooxidans*, though the formation of the sulphur layer was avoided in the mixed culture (Merretig *et al.*, 1989). Interactions between *T. ferrooxidans* and *T. thiooxidans* have been described (Norris and Kelly, 1982), but there is no general agreement on the influence of the latter organism in leaching. In some cases, the presence of *T. thiooxidans* was found to enhance the solubilisation of mineral sulphides or desulphurisation of coal (Norris and Kelly, 1982; Dugan and Apel, 1978). Mixed cultures of moderately thermophilic microorganisms with different abilities to fix carbon dioxide might also offer potential for bioleaching

operations elevated (40° - 60°) temperatures, where carbon dioxide may become limiting. This was suggested by the more extensive oxidation of ferrous iron by a mixed culture of *Ad. ferrooxidans* and *Sulfobacillus* spp. under atmospheric carbon dioxide concentrations, when compared with the respective pure cultures grown under the same conditions, and which was comparable to that observed for *Sulfobacillus* spp. under carbon dioxide-enhanced atmospheres (Clark and Norris, 1996).

1.8 Scope of the current project

The present study focused on five strains of acidophilic bacteria isolated from various acidic and geothermal sites. These microorganisms, in common with some chemolithotrophic acidophiles, were able to oxidise ferrous iron and sulphide minerals. It was therefore of interest to characterise these bacteria in detail and establish their taxonomic relationships to other known acidophilic microorganisms isolated from similar environments.

The major part of this thesis describes research into the metabolic versatility of these bacteria. The study was initiated with basic identification based on morphologies (cellular and colony), and was followed by investigations into physiological and nutritional characteristics of these acidophilic bacteria. It was also intended to study the iron and sulphur oxidation abilities of these bacteria and the importance of these elements to their metabolism. Apart from characterisation of these bacteria on the basis of their abilities to oxidise iron and sulphur, studies described in this thesis also sought to elucidate their potential application or possible role in the bioleaching of sulphide minerals. Characterisation studies also included molecular-based techniques i.e; (i) analyses of DNA base content (G + C mol %) and (ii) comparative analyses of 16S rRNA gene sequences.

(2)

MATERIALS AND METHODS

The following sections in this chapter describe all the materials and methods that were used routinely in the present study. Those used for specific experiments, and details of any modifications applied to the basic procedures, are described in respective chapters. All chemicals used were supplied by either Merck-BDH Laboratory Supplies, Dorset, U.K. or Sigma Chemicals Ltd., Dorset, U.K., unless stated otherwise, and were, where possible, of AnalaR grade.

2.1 Microorganisms

The mesophilic iron-oxidising acidophilic bacteria used throughout the studies included: i) iron-oxidising autotrophs: the type strain of *T. ferrooxidans* (ATCC 23270); *T. ferrooxidans* strain DSM 9465 and the type strain *L. ferrooxidans* (DSM 2705); ii) iron-oxidising heterotroph "*Ferromicrobium acidophilum*" proposed type strain T-23 (DSM 11138); iii) mesophilic iron-oxidising acidophilic bacteria, isolated from volcanic areas on the island of Montserrat, summarised in Table 2.1 (Atkinson *et al.*, 2000) and iv) moderately thermophilic, iron-oxidising bacteria isolated from a waste dump rock at Golden Sunlight Mine, Montana, U.S.A.. Details of isolation for the moderately thermophilic isolate, coded GSM, are given in Chapter 5. The type strains of *T. ferrooxidans*, *L. ferrooxidans* and "*F. acidophilum*" were revived from the acidophilic culture collection of the University of Wales, Bangor. *T. ferrooxidans* DSM 9465 was obtained from the Deutsche Sammlung von Microorganisen und Zelkulturen (DSMZ), Germany.

2.1.1 Montserrat isolates: origin and routes of isolation

Samples of water and sediments were collected at various sites on the island of Montserrat (W.I) as part of an expedition involving a group of U.K. based scientists, during March 1996. Most of the sites sampled were within the geothermal regions of the island; these areas were mapped and major characteristics of sites determined *in situ*. Recent cataclysmic volcanic activity on the island has since decimated these areas of Montserrat. Over 20 sites were sampled in all, and many were extremely acidic (pH < 3), though only four of these are referred to in this report (Table 2.1).

Samples were serially diluted and spread onto a variety of solid media specifically designed for isolating acidophilic bacteria (section 2.2.2), or else subjected to enrichment in liquid media (10 mM ferrous sulphate/0.02% (w/v) yeast extract, 10 mM ferrous sulphate, 1% (w/v) pyrite, 1% (w/v) elemental sulphur) adjusted at pH 2.0 and then streaked onto solid media (section 2.2.2). For isolation of mesophilic acidophiles, liquid enrichment cultures and solid media were incubated at 30°C for up to 3 weeks. Plates were examined and preliminary identification of isolates made on the basis of colony morphologies and cell characteristics (Johnson and Roberto, 1997). Isolates were purified by repeated single colony isolation and purity of cultures checked periodically by streaking liquid cultures onto yeast extract medium (to detect any heterotrophic contaminants) or iron sulphate overlay plates (to detect any autotrophic contaminants)

Table 2.1 : Origin of four iron-oxidising bacteria from Montserrat

Isolate	Site of Origin and Characteristics	Route of Isolation
S-10	Lower Gages Soufrière pH 1.9 ; E +472 mV, T 20°C ; Cond. 1955 μScm^{-1}	Ferrous iron/yeast extract liquid medium → iron/tetrathionate solid medium.
M-12	Galways Soufrière pH 3.4 ; E +235 mV, T 24°C ; Cond. 1450 μScm^{-1}	Ferrous iron liquid medium → ferrous iron solid medium
RIV-14	White River Mouth pH 3.2 ; E +500 mV, T 30°C ; Cond. 1741 μScm^{-1}	Ferrous iron/yeast extract liquid medium → iron/tetrathionate solid medium
L-15	Galways Soufrière pH 2.6 ; E -120 mV, T 50°C ; Cond. 1822 μScm^{-1}	Ferrous iron/yeast extract liquid medium → iron/tetrathionate solid medium

2.2 Media and culture conditions

Bacterial isolates were maintained in appropriate liquid media and routinely subcultured every two or three weeks. Cultures were usually inoculated at 1 to 2% (v/v) in 50 ml medium contained in 100 ml Erlenmeyer flasks or in 100 ml medium contained in 250 ml Erlenmeyer flasks. Autotrophic iron- and sulphur-oxidising bacteria were grown in ferrous iron (section 2.2.1.1) and sulphur liquid media (section 2.2.1.2), respectively. Mixotrophic and heterotrophic iron- and/or sulphur-oxidising acidophiles were maintained in ferrous iron, supplemented with 0.02% (w/v) yeast extract, or 5 mM glycerol plus 0.5 mM of potassium tetrathionate. Culture purity was routinely checked by streaking onto overlay plates (sections 2.2.2.1 and 2.2.2.2), to detect any contamination with iron- and sulphur-oxidising acidophiles, and also onto ferrous iron/yeast extract solid medium (section 2.2.2.3) to check for heterotroph contaminants.

2.2.1 Liquid Media

Liquid media used a basal salts solution described by Leathen *et al*; (1956), originally formulated for *Thiobacillus* species. It contained of the following salts (g/l):

$(NH_4)_2SO_4$	;	1.5
KCl	;	0.5
$MgSO_4 \cdot 7H_2O$	;	5.0
KH_2PO_4	;	0.5
$Ca(NO_3)_2 \cdot 4H_2O$	;	0.14

A trace elements solutions was routinely added at 1 ml/l culture. This contained (g/l): $ZnSO_4 \cdot 7H_2O$ (10.0), $CuSO_4 \cdot 5H_2O$ (1.0), $MnSO_4 \cdot 4H_2O$ (1.0), $CoSO_4 \cdot 7H_2O$ (1.0), $Cr._2(SO_4)_3 \cdot H_2O$ (0.5), H_3BO_3 (0.6), $NaMoO_4 \cdot 2H_2O$ (0.5), and $NaVO_2$ (0.1), $NiSO_4 \cdot 6H_2O$ (1.0), $NaSeO_4 \cdot 10H_2O$ (1.0), $NaWO_4 \cdot 2H_2O$ (0.1). The trace elements solution was acidified to pH 2.0 with H_2SO_4 and stored at 4°C.

2.2.1.1 Ferrous iron medium

A solution of ferrous sulphate ($FeSO_4 \cdot 7H_2O$), was sterilised separately and added to the basal salts / trace elements solution after sterilisation (120°C, 20 minutes) and cooling, to give the final required concentration of ferrous iron (generally 10 to 20 mM). The stock solution of ferrous sulphate was prepared as a 1 M solution (pH 1.8), filter sterilised through 0.22 µm nitrocellulose membranes (Millipore GVWP) and stored at 4°C.

2.2.1.2 Sulphur media

Two sulphur media were used routinely, containing either elemental sulphur or tetrathionate as energy source. The same basal salts/trace elements solution was used as

described in section 2.2.1. The medium was acidified with sulphuric acid to pH 3.0 (or as required) prior to heat sterilisation (121°C, 20 minutes).

Potassium tetrathionate was added from a 100 mM filtered-sterilised stock solution. Elemental sulphur (flowable sulphur) was prepared using the method of Germida (1985). The original sulphur suspension (THAT flowable sulphur, Stoller Enterprises Inc., Texas, U.S.A.) was washed in distilled water centrifuged (5000 r.p.m. for 5 minutes.) and process repeated three times. The purified sulphur, suspended in distilled water, was sterilised by autoclaving for 30 minutes at 110°C. Any flocculated S° formed during sterilisation were removed from solution, and the concentration of S° remaining in suspension was determined from its dry weight (105°C).

2.2.1.3 Heterotrophic media

Heterotrophic cultures utilised a modified basal salts solution containing (g/l):

$(NH_4)_2SO_4$	;	4.5
KCl	;	0.5
$MgSO_4 \cdot 7H_2O$	;	5.0
KH_2PO_4	;	0.5
$Ca(NO_3)_2 \cdot 4H_2O$	;	0.14

Trace elements solution (section 2.2.1) was added at 1 ml/l. One of the following organic substrates was included in this medium from a sterile stock solution: (i) 0.5 M sugar (e.g. fructose glucose, etc.), (ii) 0.5 M organic acid (e.g. acetic acid, lactic acid, etc.), or (iii) alcohol (e.g. glycerol, methanol, etc.). Stocks solutions were sterilised by either autoclaving, (121°C,for 20 minutes) for all sugars or filtering through 0.22 µm nitrocellulose membranes for organic acids and alcohols.

2.2.1.4 Sulphide minerals media

Two types of sulphide minerals were used: i) pyrite (FeS_2) ; containing 80% (w/w) FeS_2, obtained as rock samples from Cae Coch pyrite mine, North Wales, U.K., and ii) chalcopyrite ($CuFeS_2$) supplied by Mr. N. le Roux (Warren Springs laboratory, U.K.) consisting of 34% (w/w) chalcopyrite and 28% (w/w) pyrite/pyrrhotite(FeS_2). Both ores had been previously ground into fine powder of < 61μm particles (Ghauri, 1991).

All the pyrite was acid-washed before used. Pyrite was suspended in 0.1 M hydrochloric acid with continuous stirring for about 5 minutes, to remove any ferric iron precipitates on the surface of the pyrite particles. The pyrite was then harvested by centrifugation (10,000 r.p.m. for 5 minutes) and rinsed with distilled water 3 to 4 times, or until a ferric iron-free solution was obtained in the final rinse. After the final rinse, the pyrite was collected and dried at 100°C.

Media were prepared using the basal salts described in section 2.2.1, supplemented with 0.1% trace elements (section 2.2.1.1), and pH adjusted to 1.9 - 2.0 with sulphuric acid. Ground pyrite or chalcopyrite was added to the basal salts solution, at a concentration of 1 or 2% (w/v) and the suspensions were heat sterilised (121°C, 20 minutes).

2.2.2 Solid Media

In order to study microorganisms, it is highly desirable or necessary to be able to grow them in defined media in pure culture. Cultivation of acidophiles in an appropriate liquid media is seldom problematic, though there have been numerous reports of difficulties to grow them on solid media. However, the most successful solid media formulations have been developed by Johnson (1995a) for enumerating and

differentiating mixed populations of acidophilic bacteria. Most of these are variants of an 'overlay' technique and were used throughout the present study.

2.2.2.1 Ferrous iron overlay medium (Feo)

Ferrous iron – overlay plates were specifically formulated to grow iron-oxidising acidophilic bacteria. The medium described by Johnson and McGinness (1991) was reported to have a high plating efficiency for more than 50 iron-oxidising acidophilic isolates, including strains of *T. ferrooxidans*, *L. ferrooxidans* and moderately thermophilic bacteria. The medium was designed to overcome the inhibitory effects of organic compounds (predominantly agarose hydrolysis products) on the growth of iron-oxidising acidophiles. This was achieved by incorporating *Acidiphilium* SJH in the lower layer of the medium and covering the set gel with a sterile overlayer.

Feo medium was prepared with three solutions, sterilised individually and combined after sterilisation.

Solution A : Basal salts / TSB

The solution contained (g/l) : $(NH_4)_2SO_4$ (1.25), $MgSO_4 \cdot 7H_2O$ (0.5) and TSB (0.25). Trace elements solution (section 2.2.1.1) was added at a final concentration of 0.1% (v/v), pH adjusted to 2.5 with sulphuric acid and heat sterilised (121°C, 20 minutes).

Solution B : Gelling agent

A 2% (w/v) suspension of agarose type I (Sigma Ltd.) was prepared in distilled water, and heat sterilised at 121°C for 20 minutes.

Solution C : Ferrous sulphate

A 1 M solution of ferrous sulphate was prepared, acidified with sulphuric acid to pH 2.0 and filter sterilised through 0.22μm Millipore membranes. The solution was stored in sterile bottles at 4°C.

Acidiphilium SJH, inoculated in the under-layer, was grown in 0.025% (w/v) TSB liquid medium supplemented with 10 mM galactose and 25 mM ferrous sulphate, adjusted at pH 2.5. This procedure was carried out in order to adapt *Acidiphilium* SJH to grow on galactose (a major hydrolysis product of agarose) and to tolerate 25 mM ferrous iron (the concentration in the combined medium).

Sterile solutions A and B were held at 55°C in a water bath and combined with solution C in the ratio 29 : 10 : 1 (v/v/v). An active culture of *Acidiphilium* SJH was also added (~ 2.5%; v/v) to the medium. The molten medium was then mixed thoroughly, dispensed into sterile petri plates (~ 20 ml) and allowed to set. When solidified, it was covered with sterile medium as a thin overlayer, swirling each plate carefully to ensure the liquid mix covered the underlayer. The top layer contained the same concentration of agarose and ferrous iron but no *Acidiphilium* SJH. The medium was used after one to two days following preparation or stored in room temperature for up to one month.

2.2.2.2 Ferrous iron – tetrathionate overlay medium (FeSo)

The mixed iron/sulphur medium, FeSo, was a second overlay medium in which acidophilic heterotroph *Acidiphilium* SJH was included in a lower layer of a two-phase gel. The FeSo medium is a variant of Feo medium, but contains potassium tetrathionate in addition to ferrous iron and TSB. This mixed medium was formulated to promote growth of sulphur-oxidising acidophiles such as *T. thiooxidans* whilst at the same time facilitating growth of a wide variety of iron-oxidising acidophiles (e.g. *T. ferrooxidans*,

L. ferrooxidans and *Sulfobacillus* spp.) as well as some acidophilic heterotrophs. In addition, it also facilitates differentiation and preliminary identification of isolates on the basis of the colony morphologies including 'metabolic switching' during incubation (for *T. ferrooxidans*) reported by Johnson (1995a).

The FeSo medium was prepared in the same way as Feo medium, except that filtered-sterilised potassium tetrathionate was also added to solution A (basal salts / TSB) after heat sterilisation, to give a final concentration of 2.5 mM in the complete medium. The concentration of tetrathionate present in the solid medium has been found to be somewhat inhibitory to *Acidiphilium* SJH, and therefore it was necessary to adapt the heterotroph to the chemical conditions within the solid medium by pre-growing it in a liquid medium containing 25mM ferrous sulphate, 2.5 mM potassium tetrathionate, 10 mM galactose and 0.025% (w/v) TSB, at pH 2.5 before being inoculated (5% v/v) to the molten underlayer gel solution.

2.2.2.3 Ferrous iron – yeast extract medium (Fe/YE)

A non-overlay, ferrous iron – yeast extract medium, (Fe/YE) was used during the course of this study for culture of heterotrophic acidophilic bacteria (e.g. to check for culture purity). This medium were also prepared with three different solutions which were prepared and sterilised separately:

Solution A : Basal salts / YE

This solution was prepared with (g/l): $(NH_4)_2SO_4$ (0.45), KCl (0.05), $MgSO_4 \cdot 7H_2O$ (0.5), KH_2PO_4 (0.05), $Ca(NO_2)_3$ (0.01) and yeast extract (0.2). The pH was adjusted to 2.5 or 3.0 with sulphuric acid and the solution was heat sterilised (121°C, 20 minutes).

Solution B : Gelling agent

A suspension of 2% (w/v) agarose type I (Sigma Ltd.) was prepared in distilled water and heat – sterilised (121°C for 20 minutes).

Solution C : Ferrous sulphate

A 1 M stock of ferrous sulphate was prepared and adjusted at pH 2.0 with sulphuric acid. This solution was then filter-sterilised through 0.22μm Millipore membranes and stored at 4°C.

Solution A and B were cooled and held at approximately 50°C prior to mixing in the ratio 3 : 1. When cooled, a volume of solution C was added into solution A, to give a final concentration of between 1 to 2 mM ferrous iron in the complete medium. Solution B was then added and the mixed solution dispensed into sterile petri plates. The solidified Fe / YE plates were stored for up to 3 weeks at 4°C.

2.3 Determination of Microbial Biomass

2.3.1 Optical densities

Bacterial biomass was measured spectrophotometrically by determining culture optical densities (OD) at 600 nm. Samples were removed from cultures and ODs read against a blank of distilled water. To avoid the interference caused by adsorption by ferric iron (for iron-grown cultures), aliquots (1 ml) were removed, harvested by centrifugation at 10,000 r.p.m. for 10 minutes (Microcentaur MSE, Sanyo), the resulting pellets washed in basal salts solution (pH 2.0) and resuspended in the same volume of basal salts before OD 600nm was measured, again using a water blank.

2.3.2 Total cell counts

2.3.2.1 Acridine Orange Direct Counts (AODCs)

The AODC technique used in this studied was a modified version of the method described by Hobbie *et al.* (1977). Culture aliquots (0.1 ml) were filtered through 0.22μm black polycarbonate membranes (Nuclepore Ltd.), rinsing (cells on the membrane) first with acidified (pH 1.0; H_2SO_4) distilled water followed by a second rinse with alkaline (pH 11.0; NaOH) distilled water. Cells were then fixed on the membrane by using 4% (v/v) glutaraldehyde for 10 minutes before a final rinse using alkaline water. Acridine orange (0.5 ml of a 0.1% acridine orange in 4.5 ml distilled water) was added to the tower to stain the cells and left to stand for 6 minutes in darkened conditions. The excess staining solution was removed using suction and the membrane was washed with 5 ml of distilled water. The above procedure was carried out directly in a Millipore tower assembly fitted with a vacuum pump. Each black polycarbonate membrane was placed on the top of a cellulose membrane filter (0.45μm diameter pore; Millipore) to give a better distribution of vacuum pressure. Each membrane was pre-wetted *in situ* with 1 ml of distilled water, which was filtered through to give good filter-support contact. The membrane was removed from the tower assembly and air-dried before being mounted on a glass slide with immersion oil (Olympus) and covered with a coverslip.

All solutions used in this method were filtered through 0.22μm membranes prior to use. Counts were made using a fluorescence microscope (section 2.4.1.3) at a magnification x1000. Bacterial counts were estimated from counts of 20 randomly chosen fields of view and a total number of bacteria of at least 200 cells. An eye-piece micrometer disk was used to delineate the area of the field used for counting. Accordingly, the total number of cells per ml, n, was given by the following expression.

$$n = \frac{x.A.d}{v.a} \qquad (2.1)$$

where x is the average count per area. A is the effective filtration area of membrane, d is the dilution factor of the sample counted, a is the graticule area and v is the volume of sample used.

2.3.2.2 Thoma bacteria counting chamber

Direct counts of bacteria using a Thoma counting chamber did not require staining of cells. This chamber consists of a glass slide, a section of which is precisely ground to a depth of 20 μm below the surface and this is surrounded by a moat. An area of 1 mm^2 on the platform is marked with a Thoma-type grating of engraved lines into 400 small squares (≡ 2500μm^2) and the volume of each square is 5 x 10^{-5} μl. The chamber is closed with a coverslip specially made for this chamber. For counting of active and motile cells, the bacterial suspension was fixed by adding formaldehyde to 5% (v/v) final concentration and mixed thoroughly. Dense cultures were diluted to give between 40 to 200 x 10^6 cell / ml. Samples (~30μl) were then carefully pipetted on to the ruled area of the chamber's platform and the coverslip lowered evenly for a uniform distribution of the cell suspension over the area of contact. The preparation was then examined using a phase-contrast microscope (section 2.4.1.2) using a x40 objective lens. The average number of bacteria per square was calculated from counts made in enough squares to yield significant total numbers of cells. Counts were optimum in preparations which contained between 2 and 10 bacteria per square. The average count of cells per square was multiplied by 2 x 10^7 and any dilution factor used, to give the bacterial count per ml. in the original sample.

2.3.3 Plate counts on solid media

The plate count technique is a method for the enumeration of viable microorganisms. The number of viable cells present in an aqueous sample is assessed by the number of colonies formed on the solid medium, after incubation for a certain period of time in specific conditions. The method is based on the assumption that each colony originates from a single bacterial cell.

Samples of bacterial cells were diluted following a serial dilution procedure (10-fold) and 0.1 ml aliquots were plated onto selective solid media. The solid media selected for each case were chosen according to section 2.2.2. A maximum of five replicates were made of each dilution. Plates were analysed to give counts of between 10 and 200 colonies per plate. When counting the plates, those with less than 10 colonies or no growth were excluded. Statistical analysis (ANOVA) was made based on results obtained from a minimum of three replicates count. Bacteria were grown at 30°C (mesophiles) or 45°C (moderate thermophiles) for up to 1-2 weeks and colonies were counted under a stereo scan microscope (section 2.4.1.1).

2.4 Microscopy

2.4.1 Light Microscopy

2.4.1.1 Stereo scan microscopy

A stereo scan microscope (Leitz wild M32; Heebrugg, Switzerland) was used to examine and characterise bacterial colonies grown on various types of solid media, using magnifications of x50 to x400. An Olympus OM-10 camera was fitted to the microscope to photograph colonies.

2.4.1.2 Phase-contrast microscopy

Phase contrast microscopy allows the visualisation of colourless, small specimens which do not absorb enough light to be seen by bright-field microscopy. A Leitz Labolux (Switzerland) phase contrast microscope, fitted with a Zenike condenser and objective (magnification x800), was used to record morphological and behavioural characteristics of bacterial cells.

2.4.1.3 Fluorescence microscopy

The used of fluorescence microscopy required staining of the specimens with fluorescent dyes prior to viewing. The fluorochrome-treated specimens were illuminated with ultra-violet light to excite the dye and the emitted light formed the fluorescence image of the specimens. A fluorescence microscope, Jenalumar SH50 (Carl Zeiss, Germany) was used throughout these studies for bacterial counts using the AODC method (section 2.3.2.1), with a magnification x1000.

2.4.2 Scanning Electron Microscopy (SEM)

The scanning electron microscope used was a Hitachi S520 (Japan) operated at 10 kV and a magnification range of x100 to x40,000.

For sample preparations, bacterial isolates were first grown in appropriate media and harvested at the end of the exponential growth phase. A volume of culture aliquot (depending on the culture density) was filtered through a 0.22µm diameter white polycarbonate (PC) membrane (Nuclepore), washed with 0.1 M sulphuric acid to remove any ferric iron precipitates and rinsed with distilled water. Both solutions were filtered through 0.22µm Millipore membranes, to avoid contamination of any samples with other microbial cells. Fixation of the bacteria was carried out by immersing the membrane

filter in a glutaraldehyde solution (5% (v/v) in 0.1 M sodium cacodylate buffer) overnight. Following fixation, the specimen-membrane was dehydrated by replacing the glutaraldehyde with graded concentrations of ethanol (20, 40, 60, 80 and finally 100% v/v). This was done by immersing the membranes in the respective concentration of ethanol for 15 minutes each. The samples were finally left overnight in dried 100% ethanol at 4°C.

Samples were dried using a Polaron E3000 critical point dryer. Cells were flushed intermittently over two hours with liquid carbon dioxide at 20°C to replace the ethanol. When all the ethanol had been removed, the temperature and pressure were raised to 35°C and 1400 psi, respectively. In this way, carbon dioxide passes from the liquid to the gas state without the formation of a phase boundary, and the sample is dried with minimum structural distortion. The pressure was then released and carbon dioxide allowed to escape gradually.

Membranes were mounted onto aluminium stubs and coated with a layer of gold-palladium, 20 – 30 nm thick, to prevent charge build-up on the specimen and to hold the sample surface at a constant electric potential. A Polaron E5000 sputter SEM coating unit was used for this purpose, set at a current of 5 mA, and the membranes were coated for 15 minutes.

2.4.3 Transmission Electron Microscopy (TEM)

Bacteria were grown in appropriate media, harvested by centrifugation (10,000 r.p.m., 5 minutes, 4°C), washed in 10 mM sulphuric acid to remove ferric iron and finally rinsed in 0.2 M phosphate buffer, pH 7.2. Cells were fixed in 3 – 5% (v/v) gluteraldehyde (in 0.2M phosphate buffer, pH 7.2) for an hour, harvested by centrifugation, rinsed twice and finally resuspended in small volume of the same buffer (just enough to permit mixing

with molten agar in the following step). A 2% (w/v) agarose solution was prepared and kept molten at 45°C. Cells were encapsulated in agarose by adding a 1 – 1.5 volume of molten agarose to tubes containing cell suspensions using a warm Pasteur pipette, mixed rapidly and left to polymerise at room temperature. Agarose blocks were removed from the centrifuge tubes and cut into small cubes (approximately 1 mm^3). The cubes were then washed in buffer to remove smally pieces of agarose and free-floating cells.

Agarose cubes were then incubated in 1% (w/v) osmium tetroxide solution for an hour at room temperature, washed with distilled water and incubated in 2% (w/v) uranyl acetate overnight, and then again rinsed in distilled water. Samples were dehydrated in graded concentrations of ethanol (50%, 70%, 95%, 100%, 100%; v/v) for 10 – 15 minutes each, followed by two changes (of 15 minutes) in 100% (v/v) propylene oxide (intermediate solvent). Samples were then transferred to vials containing a mixture of Spurr resin/propylene oxide (1:1) and incubated on a rotator for 30 minutes. Finally the infiltrated agar cubes were embedded in Spurr resin by transferring a single cube into small Beem capsules full of fresh pure Spurr resin, and polymerised at 60°C overnight. Each specimen embedded in resin was trimmed with a rotating milling cutter to produce a flat-topped of pyramid with a surface area ~ 0.25 mm^3 for better imaging in the electron microscope. Thin sections were cut using an ultramicrotome (LKB-produkten, Sweden). The sections were placed on a Formvar-coated specimen grid and stained for 5 minutes with lead citrate. Stained grids were examined on a Philips EM6 transmission electron microscope at 60 kV.

2.5 Gram staining

When bacteria cells are stained with certain basic dyes, some may be decolorised by the use of organic solvents (e.g. ethanol), while others resist decolouration; these are referred to as Gram-negative and Gram-positive bacteria, respectively.

The ability to retain or lose the dye generally reflects differences in the structure of the cell wall, and is considered an important taxonomic characteristic. The mechanism of the reaction is still not completely understood. It is suggested that Gram-negative cells do not retain the dye due to higher permeability of their cell wall (possibly disrupted during the staining), while Gram-positive cells retain the dye due to lower permeability of the cell wall (presence of peptidoglycan and more extensive cross-linking than Gram-negatives).

The procedure followed was Hucker's modification of the method (in Lányi, 1987):

Solutions:

A. Crystal violet solution: A1: 2 g crystal violet in 20 ml ethanol (955, v/v)

A2: 0.8 g ammonium oxalate in 80 ml distilled water

Solution A1 and A2 were mixed and allowed to stand for 48 h before use. This solution is stable for 2 – 3 years.

B. Lugol's solution: 1 g crystalline iodine

2 g potassium iodine

300 ml distilled water

Potassium iodine and crystalline iodine were mixed together in a mortar, ground and dissolved in distilled water

C. Decolourising agent: ethanol (95%, v/v)

D. Counterstain - Safranin solution: 0.5% (w/v) safranin-O in distilled water

Procedure:

Iron-oxidising isolates (S-10, M-12, RIV-14, L-15 and GSM) were grown in ferrous iron/yeast extract liquid medium and stained during the exponential growth phase. A small volume of culture (~50 µl) was spread and heat-fixed on a glass slide, and then stained for 1 minute. with crystal violet solution. Bacterial smears were rinsed briefly under running water, treated with Lugol's solution for 1 minute. and, again, rinsed with distilled water. The smears were decolourised by running ethanol over the slide until no more dye was removed, and immediately rinsed with distilled water. The smears were finally counter-stained for 10 seconds with safranin solution and rinsed with distilled water. Following air-drying, they were examined under the microscope (magnification x1000). Gram-negative cells appeared red and Gram-positive cells appeared violet.

2.6 Analytical Analyses

2.6.1 Determination of Ferrous Iron

Three different methods were used to determine ferrous iron concentrations, depending on the required sensitivity of the analyses and experimental conditions (e.g. type of media).

2.6.1.1 Titrimetric method: potassium permanganate

Ferrous iron concentrations were determined with a solution of 1 mM potassium permanganate ($KMnO_4$) in sulphuric acid (H_2SO_4). An aliquot of 1 ml or 5 ml sample, was acidified with two or three drops of 25% (v/v) sulphuric acid prior to titration with permanganate. The reaction between permanganate and ferrous iron was determined by the point where a faint pink colour appeared.

The correlation between permanganate and ferrous iron concentration is given by the reaction stoichiometry,

$$Fe^{2+} \rightarrow Fe^{3+} + e^- \quad (2.2)$$

$$MnO_4^- + 8H^+ + 5e^- \longrightarrow Mn^{2+} + 4H_2O \quad (2.3)$$

Showing that 1 mole of permanganate reacts with 5 moles of ferrous iron. From this, concentrations of ferrous iron in sample aliquots could be determined. This method was used to determined > 2.5 mM ferrous iron.

2.6.1.2 Titrimetric method: ceric sulphate

Ceric sulphate ($Ce(SO_4)_2.4H_2O$) titration was used to measure ferrous iron concentration in tetrathionate-containing solutions, as this RISC was found to interfere with the potassium permanganate titration method. A 1 ml sample was acidified with two drops of 25% (v/v) sulphuric acid and 0.1 ml phenanthroline / ferrous sulphate indicator (1,10 phenanthroline-ferrous sulphate complex solution; BDH) were added. The mixture was titrated with ceric sulphate solution (1 M solution in 1 M H_2SO_4) until a change of colour from red to blue was obtained. The stoichiometry of the reaction between ferrous iron and ceric sulphate indicates that one mole of ceric sulphate oxidises one mole Fe^{2+}, therefore 1 ml of 1 mM ceric sulphate is equivalent to 1 ml of 1 mM ferrous iron in a sample aliquot:

$$2Fe^{2+} \rightarrow 2Fe^{3+} + 2e^- \quad (2.4)$$

$$2Ce(SO_4)_2 + 2H^+ + 2e^- \rightarrow Ce_2(SO_4)_3 + 4H_2SO_4 \quad (2.5)$$

As the phenanthroline / ferrous sulphate indicator contained some ferrous iron, a blank titration containing with 1 ml distilled water was carried out. The titre value of the blank was subtracted from that of the sample to give the actual concentration of ferrous iron present.

2.6.1.3 Colorimetric method: ferrozine

This assay is based on the chelation of ferrous iron by the (-N=C-C-N) group of the ferrozine molecule (3-(2-pyridyl)5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine ; Sigma Ltd.) to form a stable magenta-coloured complex ferrous iron-ferrozine, which exhibits a single sharp peak with a maximum absorbance at 562 nm (Stookey, 1970). The method used throughout these studies was a modification of the method described by Lovley and Phillips (1986) to determine concentrations of 0 to 1 mM ferrous iron. The complex formed was found to be stable for at least 1 hour in the dark.

Ferrozine reagent was prepared by dissolving 1 g of ferrozine in 1 litre of 50 mM Hepes buffer (pH 7.0). The reagent was stored at room temperature in the dark. A 50 µl aliquot of culture was added to 950 µl of ferrozine reagent, mixed thoroughly and stored in the dark, at 4°C, prior to absorbance being measured at 562 nm against a water blank. Standards were prepared with ferrous sulphate ($FeSO_4.7H_2O$) over the range of 0 to 1 mM.

2.6.2 Determination of total iron and copper: atomic absorption spectrophotometry

Samples (1 ml) were taken from cultures and centrifuged at 10,000 r.p.m., for 3 minutes to removed cells and particles of minerals. A 0.5 ml aliquot of the supernatant was combined with 0.5 ml of 6 M hydrochloric acid (HCl), and stored at room temperature. The concentration of total soluble iron and copper in solution was determined using Pye Unicam SP2900 double beam optic atomic absorption spectrophotometer (AAS) fitted with a Pye Unicam SP9-10 gas controller unit, at wavelength 248.3 nm (iron) or 324.8 nm (copper), using a fuel lean air/acetylene flame.

Calibration curves were made using standard iron and copper solutions, over the range 0 to 50 mg/L. Dilution of samples and standards were made with dilute acid (0.1 M HCl).

2.6.3 Determination of Reduced Inorganic Sulphur Compounds (RISC) by cyanolysis

Tetrathionate and thiosulphate were determined by cyanolysis at room temperature, and spectrophotometric estimation of the RISC compounds , described by Sorbö (1957) and modified by Kelly *et al.* (1969). Samples from RISC grown cultures were removed (1 ml) and centrifuged or filtered through 0.22 µm nitrocellulose membranes to remove bacterial cells and particles, producing clear samples.

Aliquots (0.24 ml) of each sample (diluted with distilled water when necessary) were added to 0.16 ml of potassium buffer, and 0.2 ml potassium cyanide and rapidly mixed by vortexing. The mixture was left for about 20 minutes to react at room temperature before the addition of 0.12 ml ferric nitrate, to form a dark red-coloured ferricyanide complex. To measure thiosulphate, copper (60 µl of 0.1 M copper sulphate; $CuSO_4$) was added (as catalyst) prior to addition of ferric nitrate. Each sample was made up to 1.0 ml with distilled water. Absorbance was read at 460 nm against a thionate-free blank, and compared with a standard curve over the range 0 to 1mM potassium tetrathionate ($K_2S_4O_6$). When assaying for thiosulphate, the final thiosulphate concentration is equivalent to the total thionate concentration obtained in the presence of copper minus twice the tetrathionate concentration found in the tetrathionate assay alone.

The reagents used in this assay were phosphate buffer solution, consisting of 0.2 M sodium dihydrogen orthophosphate (NaH_2PO_4) and 0.2 M sodium hydroxide (NaOH), mixed at 50 to 39 parts respectively. The solution of 1.5 M ferric nitrate ($Fe(NO_3)_3$) was

prepared in 4 N perchloric acid ($HClO_4$) and potassium cyanide (KCN) was prepared at 0.1 M, in distilled water.

2.6.4 Determination of Hexoses : anthrone method

Samples (1 ml) of bacterial cultures grown in liquid media containing fructose (or other hexoses) were spun down to remove bacterial cells or particles of pyrite. Concentrations of hexoses in supernatants were measured by adding 5.0 ml of chilled anthrone solution (below) to 1.0 ml of sample containing hexose, in a test tube (sample being diluted in distilled water where necessary). Solutions were mixed thoroughly by vortexing and kept on ice. The mixture was then incubated at 100°C for 10 minutes, rechilled on ice for approximately 5 minutes and absorbance was read at 620 nm.. The calibration curve was made using fructose at a range of between 0 to 1.0 mM, together with a hexose-free control.

The anthrone solution was made up in a mixture of concentrated sulphuric acid and water at ratio 5:2. In order to avoid polymerisation of anthrone, it was necessary to ensure that the acid was chilled (< 4°C) prior to addition of the anthrone at 0.2% w/v.

2.6.5 Protein measurement by the Bradford assay

A 1.0 ml culture sample or cell suspension was centrifuged (10,000 r.p.m. for 5 minutes) and the cell pellet resuspended in 1 ml of 0.5 M sodium hydroxide (NaOH). Bacterial cells were disrupted by heating cell suspensions in boiling water for 15 minutes. The amount of protein contained in the cell lysates was measured by mixing 100 µl of this extract and 1.0 ml of Bradford reagent and incubating for 2 minutes in the dark before measuring the absorbance at 595 nm. Dilution of samples in 0.5 M NaOH was done when necessary. The Bradford reagent was prepared by dissolving 100 mg of

Coomassie Brilliant Blue (G-250/L) in 1 litre of 5% (v/v) ethanol, containing 10% (v/v) of phosphoric acid.

The amount of protein was determined by comparing the optical density (OD 595 nm) value for each sample against a standard calibration curve. The standard curve was made in the range of 10 – 100 μg protein, using bovine serum albumin (BSA), diluted in 0.5 M NaOH.

2.7 Carbon dioxide fixation

Bacteria were grown in appropriate liquid media (sections 2.2.1.1 and 2.2.1.3). Flasks were sealed (with Suba seals) and cultures were supplemented with 1 % (v/v) of 0.5 M ($NaH[^{14}C]O_3$ (Amersham International). Samples were withdrawn (1 ml) from incubated cultures at regular intervals to monitor carbon dioxide incorporation by the bacteria. Samples were filtered through 0.22 μm membranes (Whatman Ltd.) under vacuum, washed with 0.1 M HCl to remove ferric iron deposits and rinsed with distilled water to remove any remaining HCO_3^-. The membranes were air-dried before being placed in scintillation vials and dissolved in 5 ml cocktail T-scintran. Finally, the radioactivity (^{14}C) of the samples was read in a Beckman scintillation counter. At the same time intervals, oxidation of ferrous iron was monitored by potassium permanganate titration (section 2.5.1.1). Cells numbers were counted using the Thoma chamber at the end of each experiment. The relationships between the two processes were determined by plotting CO_2 fixed (nmoles/ml) against the concentration of ferrous iron oxidised (μmol/ml), as described by Eccleston *et al.* (1985).

2.8 Chromosomal DNA extraction and purification of bacteria

The protocol used for the extraction of chromosomal DNA was that previously described by Wilson (1987). The following reagents were prepared:

a) TE buffer: 10 mM Tris-HCl + 1 mM EDTA, pH 8.0

b) SDS: 10 % sodium dodecyl sulphate in deionised water

c) RNAase: 10 mg RNAase / ml 0.1 M sodium acetate (pH 5.2) heated to 100°C for 15 minutes to inactivate DNAases and allowed to cool slowly to room temperature before adding 0.1 volume of Tris-HCl (pH 7.4)

d) Sodium acetate: 3 M sodium acetate in deionised water, adjusted to pH 5.2 with glacial acetic acid

d) Phenol/Chloroform: 25 phenol : 24 chloroform :1 isoamyl alcohol

e) Chloroform 24 chloroform : 1 isoamyl alcohol

Bacteria were grown in appropriate liquid media (section 2.2.1). Cells were harvested at late exponential phase by centrifugation (15,000 r.p.m. for 15 minutes at 4°C), pellets washed with 10 mM sulphuric acid to remove any ferric iron precipitates and then with TE buffer. The expected biomass from the cultures was approximately 10^{10} - 10^{11}.cells per ml of cell suspension.

Each of the cell pellets was resuspended in 567 µl TE buffer and 30 µl SDS solution plus 3 µl of proteinase K solution was added, and incubated for 1 hour (or until the solution was observed to clear) at 37°C without shaking. When preparing DNA from Gram-positive bacteria, 5 µl of a 100 mg/ml solution of lysozyme was added to the cell suspensions in TE buffer and incubated for 10 minutes at 37°C. SDS (30 µl) and proteinase K (5 µl) were then added, and followed by incubation at 37°C for one hour.

The DNA was extracted with 500 µl of phenol:chloroform solution by gentle inversion for about 5 minutes and the biphasic mixture was separated by centrifugation at 10,000 r.p.m. for 5 minutes. The upper aqueous layer containing DNA was carefully removed with a wide-bore pipette tip to avoid shearing of the DNA, followed by a second extraction into 500µl of the chloroform solution. Finally the DNA was precipitated with 70 µl sodium acetate and 500 µl isopropanol, by gently flicking the mixture until a stringy precipitate formed and the DNA was recovered as a pellet by centrifugation (10,000 r.p.m. for 15 minutes) followed by rinsing with 1 ml of 70% (v/v) chilled ethanol. DNA was then resuspended in 1 ml TE buffer overnight.

The concentration of DNA was measured spectrophotometrically at 260 nm. This involved measuring absorbance of the diluted (10-fold) DNA in deionised water against a water (dH_2O) blank. An absorbance value of 1.0 is equivalent to 50 µg DNA ml^{-1} dH_2O.

2.8.1 Agarose gel analyses of DNA

Agarose gel electrophoresis was performed to analyse > 1000 bp fragments of DNA. The following reagents were prepared:

a) 0.5 M EDTA: EDTA (46.53 g) dissolved in deionised water, adjusted to pH 8.0 with 10 M NaOH, and made up to 250 ml with deionised water.

b) 5X TBE: Tris (54 g) , boric acid (27.5 g) and 20 ml of 0.5 M EDTA stock. Made up to 1 litre with deionised water.

c) 6X DNA loading buffer: 0.25 % (w/v) bromophenol blue in 30% (v/v) glycerol.

A 0.7% (w/v) agarose gel was prepared using electrophoresis grade agarose in 0.5X TBE, melted and polymerised in a gel mold with a comb slotted vertically in the

melted gel to form the wells. The comb was then removed by gently but firmly pulling it straight up out of the solidified gel. Samples of DNA (10 µl) were added to 2 µl of the 6X DNA loading buffer prior to loading into respective wells on the agarose gel, which was then placed in an electrophoresis tank containing 0.5X TBE running buffer. The agarose gel was run at 60 volts for about an hour or until the blue dye had migrated to the desired position, represented by an approximately 300 bp product. The gel was then removed from the tray and stained by placing it into ethidium bromide bath for about 15 minutes. The pattern of DNA was observed and photographed on an ultraviolet (UV) light box.

2.8.2 DNA purification using caesium chloride gradient centrifugation

Bacteria were grown in appropriate liquid media (section 2.2.1). Cells were harvested at late exponential phase by centrifugation (15,000 r.p.m. for 15 minutes at 4°C) and genomic DNA was extracted as described in section 2.7. The concentration of the DNA was estimated spectrophotometrically at 260 nm (section 2.7).

In 10 ml test tubes, 0.9 g / ml caesium chloride was dissolved (by vortexing) in TE buffer (4 ml final volume), 20 µl ethidium bromide (from 25% stock) and a volume of DNA (to obtain between 350 to 400 µg DNA in TE/CsCl/ethidium bromide solution) added, and mixed gently by flicking to avoid shearing of DNA. The DNA/CsCl/ethidium bromide solution was then transferred into sealable centrifuge tubes and all tubes were balanced by weight to the nearest 0.01 g with caesium chloride solution (0.9 g / ml deionised water). DNA was separated by centrifugation in a Beckman Vti80 rotor, overnight at 55,000 r.p.m at 15°C. DNA was collected in eppendorf tubes and ethidium bromide was extracted with an equal volume of saturated butanol. Ethidium bromide-free DNA was then dialysed, firstly in 250 ml 0.1 X SSC

(15 mM NaCl + 1.5 mM trisodium citrate, in deionised water) to remove caesium chloride and secondly in 500 ml of 0.1 X SSC overnight. Finally, concentration of the purified DNA were measured using a spectrophotometer at 260 nm (section 2.7) prior to measurement of G + C contents (section 2.8.3).

2.8.3 Determination of DNA base composition

The determination of G + C (mol %) composition of chromosomal DNA was carried out using the melting profile method adapted by Dr. P. R. Norris (University of Warwick, U.K).

The DNA of the iron-oxidising bacteria was diluted in a volume of 0.1X SSC (section 2.8) to give a final concentration of 20 -30 µg DNA/ml SSC solution. Aliquots (1.5ml) were then scanned using a Hewlett Packard (HP) 8453A UV-visible spectrophotometer connected to a HP 89090A Peltier temperature controller. DNA melting temperature is the temperature at which a 50% increase in A260 nm is obtained, and was calculated using a DNA melt software package (HP 845x).

The base composition of the DNA was determined from its melting point, using the DNA melt software program of Maumur and Doty (1962):

$$G + C \text{ (mol \%)} = 2.44\ [(T_m - 81.5) - (16.6 \log M)] \qquad (2.6)$$

where T_m is the melting temperature of the DNA and M is the molar concentration of the cations in 0.1X SSC (= 0.0195 M).

2.9 Polymerase chain reaction (PCR) amplification of 16S rDNA fragments

The 16S rRNA gene of eubacteria was amplified by PCR using forward (5'-AGAGTTTGATCCTGGCTC-3') and reverse (5'-TACGGYTACCTTGTTACGACTT-

3') primers, complimenting to the positions 8 to 27 and 1510 to 1492 of *Escherichia coli* 16S rRNA, respectively (Lane, 1992).

Iron-oxidising acidophilic bacteria were grown in appropriate media to late exponential phase or early stationary phase, harvested by centrifugation (10,000 r.p.m., 5 minutes) and washed twice with 10 mM H_2SO_4 to remove any ferric iron precipitates. Cell pellets were then resuspended in 20 µl of PCR lysis solution (0.05 M NaOH + 0.25% SDS) and heated to 95°C for 10 minutes in a thermocycler (Progene Techne, Cambridge). The cell lysate was immediately diluted (10-fold) by adding 180 µl of autoclaved deionised water (MilliQ water).

Amplification of the 16S rRNA gene fragment was carried out using 1 µl of the diluted cell lysate in a 50 µl of PCR reaction. The reaction mix composed of; 0.5 µl Taq DNA polymerase (from *Thermus aquaticus*; Promega), 5 µl 10X reaction buffer (normally supplied with the enzyme polymerase), 5 µl of 25 mM MgCl buffer (also supplied with the polymerase), 5 µl of dNTPs (mixture of 2 mM each dATP, dCTP. dGTP and dTTP), 1 µl of each reverse and forward primers (100 ng/µl stock respectively) and finally made up to 50 µl with autoclaved MilliQ water. The PCR was started by denaturing the double stranded DNA by heating at 95°C for 3 minutes, followed by 30 cycles (95°C for 30 s of denaturation phase, 50°C for 30 sec of annealing phase and 72°C for 2 minutes to allow extension of the target DNA segment), and a final 10 minutes incubation at 72°C.

Following PCR, agarose gel analyses was carried out to confirm that the product was that of the expected fragment (16S rRNA genes). This was done by analysing 5 µl of the PCR reaction on an 0.7 % (w/v) agarose gel (section 2.6.1), on which a bright band of approximately 1500 bp was visible if the PCR was successful.

2.9.1 Cloning of the 16S rRNA gene of the acidophilic iron-oxidising bacteria

The amplified 16S rRNA genes (section 2.9) were cloned into the pCR2.1 vector (Invitrogen, Inc, Carlsbad, U.S.A) using the TOPO TA cloning kit, according to the manufacturer's instructions. The cloning reaction was carried out in a 5 µl reaction mix containing 1 µl of PCR product (section 2.9), 1 µl pCR@-TOPO vector and sterile MilliQ water, for 5 minutes at room temperature (~25°C) and immediately placed on ice. At this point, the cloned 16S rRNA genes were obtained. The process then continued with the transformation of the clones into competent cells. This was carried out by adding 2 µl of the cloning reaction to a vial of TOP10 One Shot competent cells (supplied with the cloning kit) and mixed by gently flicking the vial. This was followed by 30 minutes. incubation on ice, heat shock at 42°C for 30 sec and followed by chilling on ice for 2 minutes. A 250 µl of SOC medium (supplied with the cloning kit) was finally added to the transformation reaction prior to incubating at 37°C for 30 minutes. A 100 µl of the transformation mix was then spread onto a Luria Bertani (LB) plate containing 50 µg/ml ampicillin, which had been spread with 40 µl of X-gal (40 mg/ml stock in DMF (dimethylformamide) and dried in the 37°C prior to inoculation. All plates were incubated at 37°C overnight to allow development of white and blue colonies. Screening of positive clones was then carried out by PCR (section 2.9.2) and finally confirmed by RFLP (section 2.9.3) analyses.

2.9.2 PCR screening of cloned 16S rRNA genes

This procedure was carried out to screen positive transformants obtained during the transformation of cloned 16S rRNA into the competent cells (section 2.9.1). The PCR reaction was set up in a 20 µl reaction mix consisting of components described in section 2.9 for the PCR amplification of the original 16S rRNA genes, with the exception

that this reaction used the specific primers for the cloning vector i.e. M13 forward (5'-GTAAAACGACGGCCAG-3') and reverse (5'-CAGGAAACAGCTATGAC-3') primers. Four to five, well-separated white colonies (section 2.9.1) were selected, small amounts of bacterial biomass removed and mixed (with sterile toothpick) with 20 µl of PCR reaction mixture. The PCR was performed in a thermocycler, starting with denaturing the double stranded DNA by heating at 95°C for 10 minutes. before 30 cycles (95°C for 30 sec. of denaturation, 55°C for 30 sec of annealing and 72°C for 2 minutes of elongation of the target DNA fragment), and finally 10 minutes incubation at 72°C.

The resulting PCR product was then analysed on an agarose gel (0.7% w/v), using 5 µl of the newly made PCR product following the protocol described in section 2.8.1. The positive clones were visualised as bright bands at approximately 1600 base pair (bp) position.

2.9.3 Restriction fragment length polymorphism (RFLP) analyses of cloned 16S rRNA gene fragments

The purpose of the RFLP was to confirm that the PCR (section 2.9.2) gave the correct results and not the false negative results that can be obtained from mispriming or a contaminated template. The positive clones can be determined by comparing the RFLP pattern of the cloned inserts with the authentic 16S rRNA gene previously obtained from the original PCR reaction (section 2.9). RFLP was set up in a 10 µl digestion reaction mix, consisting of 5 µl of PCR reaction product (section 2.9.2), restriction enzymes (*Eco*R1 and *Msp*1; Promega; 0.5 µl of each), 1 µl 10X buffer B (supplied with the *Eco*R1 restriction enzyme), made up to 10 µl with autoclaved MilliQ water. The restriction digest reaction was carried out at 37°C for at least 1 hour.

The resulting product was then analysed on an agarose gel. For the agarose gel analyses of the RFLP digest, a similar procedure was followed as section 2.6.1 except that 3% (w/v) of high resolution agarose (type 3:1; Amresco) in 1X TBE buffer was used.

2.9.4 Miniprep of plasmid DNA

The purification of plasmid DNA was carried out using the QIAGEN kit, according to the manufacturer's instruction. The following reagents were used in the purification procedures:

Buffer P1 (resuspension buffer):	50 mM Tris-HCl, pH 8.0 ; 10 mM EDTA ; 100 µg/ml RNAase
Buffer P2 (lysis buffer):	200 mM NaOH ; 1% SDS
Buffer N3 (neutralisation buffer):	3 M potassium acetate, pH 5.5
Buffer PB (QIAfilter wash buffer):	750 mM NaCl ; 50 mM MOPS, pH 7.0 ; 15% isopropanol
Buffer PE (equilibration buffer):	750 mM NaCl ; 50 mM MOPS; pH 7.0 ; 15% isopropanol ; 0.15% TritonX-100
Buffer EB (elution buffer):	1.25 mM NaCl ; 50 mM Tris-HCl, pH 8.5

The positive transformant (section 2.9.2 and 2.9.3) was grown in an LB liquid medium supplemented with ampicilin (50 µg/ml) overnight at 37°C. Cells were then harvested by centrifugation (10,000 r.p.m. for 5 minutes) and the pellet was resuspended in 250 µl of the RNAase-containing Buffer P1, mixed by vortexing to ensure no cell aggregates were visible, followed by cell lysis in 250 µl of Buffer P2, mixed by gentle inversion to avoid shearing of DNA, for no more then 5 minutes. Buffer N3 (350 µl) was then added, mixed immediately by inverting the tubes to precipitate the salts and cell

debris, which were removed by centrifugation (10,000 r.p.m. for 10 minutes). The supernatant containing DNA was pipetted into an assembled spin column filtered with a collection tube, centrifuged (10,000 r.p.m. for 1 minute) and the flow-through liquid was discarded. The DNA-containing spin column was then washed by centrifugation, firstly with 0.5 ml Buffer PB, and secondly with 0.75 ml Buffer PE, and the flow-through liquid discarded in both cases. The DNA was eluted by adding 50 µl of Buffer EB to the spin column, and the flow-through liquid collected in a sterile fresh tube.

The concentration of DNA was then estimated by measuring the optical density of diluted DNA (10-fold) at 260 nm, as described in section 2.6.

2.9.4.1 Sequencing of cloned 16S rRNA genes

Plasmid DNA was precipitated and sent to MWG-Biotech, Germany for sequencing. The DNA (approximately 30 ng) was precipitated in 0.1 volume of 3 M sodium acetate and 2.5 volume of absolute ethanol, recovered by centrifugation (10,000 r.p.m. for 15 minutes) and washed with 300 µl of 70% (v/v) ethanol. Finally, the DNA pellet was recovered and left to dry at room temperature.

The purity of the cloned DNA was confirmed by comparing the RFLP pattern of the plasmid DNA with the original PCR product (section 2.9). A single digestion (*Eco*R1) reaction was set up in a 10 µl restriction digest reaction, containing approximately 200 ng DNA in the total reaction mix. The RFLP analysis was carried out following the procedure described in section 2.9.3.

2.9.4.2 Sequence analyses and phylogenetic tree assembly

The sequence data obtained from MWG-Biotech were compared with 16S rRNA sequences deposited in the Genbank database using the BLAST search program (Altschul *et al.*, 1997). The 16S rRNA gene sequences of various bacteria (including

those closely related to the unknown sequences, as indicated from the BLAST search) obtained from the Genbank database were aligned with those of the new sequence using ClustalW program (Thompson *et al.*, 1994). These alignments were then used to construct a distance matrix (Juke and Cantor, 1969), followed by phylogenetic tree construction by neighbour joining (Saitou and Nei, 1987). Bootstrapping and DNA parsimony analyses were also used for comparison. These algorithms were provided in PHYLIP version 3.5c (Felsenstein, 1993). Phylogenetic trees were viewed using Treeview software (Page, 1996).

2.10 Determination of pH

Solution pH was determined using a "phase rapid renew" electrode connected to a pH meter (series 3, DR359 Tx). The electrode was calibrated using a pH 2.0 buffer solution. When not in use, the electrode was stored in distilled water acidified with sulphuric acid to ensure that no iron precipitate formed on the electrode.

2.11 Determination of redox potential (E_h)

Redox potential (E_h) of solution was determined using a platinum redox electrode connected to an accumet, combined pH / E_h meter. The electrode was calibrated using a redox buffer solution (E_h + 770) and stored in distilled water when not in use.

(3)

Characterisation of the Gram-Negative Iron- and Sulphur - Oxidising Isolates from Montserrat (W.I)

3.1 Introduction

Bacteria have traditionally been divided into two major groups, i.e. Gram-negative and Gram-positive bacteria, on the basis of their reaction to the Gram stain. In this chapter, studies of two Gram-negative acidophilic isolates from Montserrat are described.

Over the past fifty years, there has been much interest in Gram-negative bacteria of the genus *Thiobacillus*, all of which have the ability to derive energy from the oxidation of inorganic sulphur compounds. This genus is extremely heterogeneous and studies have been carried out to establish taxonomic relationships among species of *Thiobacillus* as well as their relationships to other Gram-negative acidophilic bacteria (such as *Leptospirillum ferrooxidans* and *Acidiphilium* spp.), based on 16S rRNA gene sequences. *Thiobacillus ferrooxidans* was the first iron-oxidising acidophilic bacterium to be isolated from acidic mine drainage water and has since been isolated from a variety of acidic environments, and described as being almost unique among the acidophilic thiobacilli in its ability to respire on both iron and sulphur as the sole source of energy (the halophile *Thiobacillus prosperus* also has this ability). Therefore, *T. ferrooxidans* has been one of the most studied iron- and sulphur-oxidising acidophiles, most notably due to the interest in economic applications of acidophilic bacteria in extraction of metals from sulphide minerals.

In recent years, knowledge of the diversity of acidophiles has increased and characterisation studies have progressed to allocate novel isolates to appropriate

taxonomic classes. Some have been identified and named while many others remain unclassified, which indicates that the biodiversity of the iron-oxidising acidophilic bacteria may be considerably greater than has hitherto been recognised. The latter includes *Thiobacillus* strain T3-2, which has been reported to grow autotrophically on sulphur and pyrite, though yeast extract, glucose or thiosulphate supplement is required for its growth on ferrous iron (de Siloniz *et al.*, 1993). In addition, *Thiobacillus* strain Funis has also been characterised which resembles *T. ferrooxidans* but differs from it in content of cytochromes and fatty acids and in its 16S rRNA gene sequence (Blake *et al.*, 1992).

In this chapter two Gram-negative iron-oxidising isolates (S-10 and M-12) isolated from volcanic sites on the island of Montserrat (section 2.1.1) are discribed. Various methods were used to characterise these isolates, including studies on sulphur- and iron-oxidising abilities, as well as molecular-based techniques i.e. comparative analysis of 16S rRNA gene sequences and chromosomal DNA base compositions.

3.2 Morphological characteristics of isolates grown in liquid and on solid media

3.2.1 Methodology

A total of 16 mesophilic Gram-negative, iron-oxidising Montserrat isolates were revived from the 'UWB acidophile culture collection' (Johnson, unpublished data) which had been maintained at 4°C in pyrite medium for over a year. However, only two isolates, coded S-10 and M-12, were chosen to be characterised in further detail, based on their tendency to grow as "*F. acidophilum*"-like colonies on overlay media. Repeated single colony isolation from overlay solid into liquid media was performed to ensure culture purity before further studies were carried out.

Both isolates were inoculated (1% v/v) into 100 ml shake flasks containing 50 ml of 20 mM ferrous sulphate medium (section 2.2.1.1), pH 2.0, supplemented, or not, with 0.02% (w/v) yeast extract. Cultures were grown in a shaking incubator (150 r.p.m.) at various temperatures (30°, 37° and 45°C) for up to 1 week, and oxidation of ferrous iron was determined by titration using potassium permanganate (section 2.6.1.1). Cultures were observed with a phase-contrast microscope (section 2.4.1.2) to record the cellular morphology of the isolates. Specimens for scanning electron microscopic observation (section 2.4.2) were also prepared for further characterisation of the bacteria.

Growth of the two isolates on solid media was also monitored by streaking the respective liquid-grown cultures onto selective solid media (sections 2.2.2.1 and 2.2.2.2), specially formulated for the growth of iron- and sulphur- oxidising acidophilic bacteria. Plates were incubated at 30°, 37° and 45°C. Colony formation was observed after 2 weeks of incubation using a stereo-scan microscope (section 2.4.1.1) and plates were reincubated for a further one or two weeks until the bacteria reached the stationary growth phase. Morphological characteristics of the colonies were recorded throughout the complete cycle of bacterial growth.

3.2.2 Results

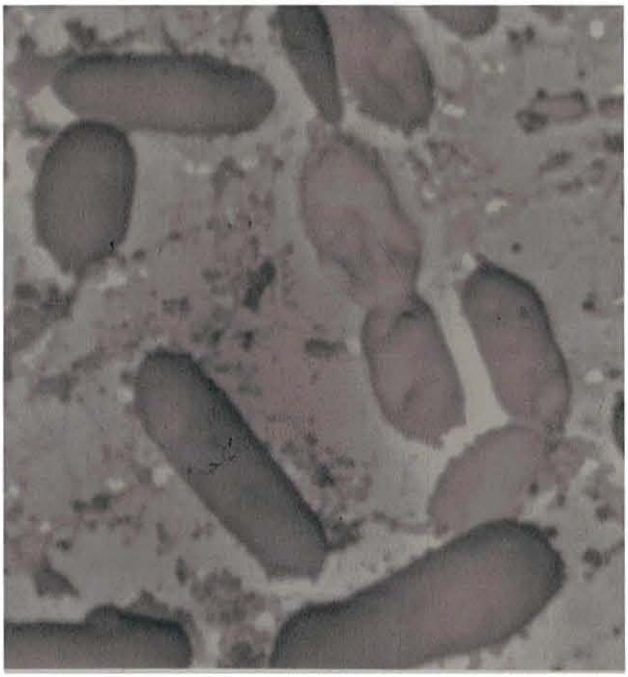
Cellular characteristics of these isolates were based on their shape, motility and abilities to form endospores. Observations from phase-contrast and scanning electron microscopy showed that S-10 and M-12 were very similar. Both were rod-shaped bacteria, and normally occurred as single or paired cells (Fig. 3.1). However they differed from each other by their motility, as the cells of isolate S-10 were always more motile than that of M-12 when grown under the same conditions. Spores were not detected at any growth phase in liquid media. The size of individual cells was 1.32 μm

long x 0.45 μm wide and 1.25 μm long x 0.48 μm wide for the isolates S-10 and M-12 respectively, grown in ferrous iron medium as determined from measurements of 20 cells in scanning electron micrographs. Neither of these isolates was able to grow at 45°C, either in liquid or on solid media. Both isolates stained Gram-negatively.

Growth was observed on plates incubated at 30 and 37°C but not at 45°C. Colonies displayed the same morphologies at these temperatures (Fig 3.2), confirming culture purity. Both isolates were able to grow on both ferrous iron and ferrous iron/tetrathionate overlay plates (Fig 3.2). Growth of both S-10 and M-12 on ferrous iron overlay medium differed slightly from those on ferrous iron/tetrathionate overlay during the early stages of growth, but developed into identical iron-encrusted colonies. On ferrous iron overlay plates, they appeared initially (between day 7 to 10) as small (< 1 mm), translucent, domed colonies with slightly irregular edges. Ferric iron precipitates started to appear after *ca.* 10 to 14 days and became more evident with prolonged incubation (20 days). Colonies were subsequently found to develop central 'crators' and their shape became plateau-like rather than domed with extended incubation (> 25 days).

Colony morphologies of both isolates grown on ferrous iron/tetrathionate plates were found to be more gelatinous in the early growth phases, and less ferric iron precipitates were formed compared to colonies grown on ferrous iron overlay medium. These developed initially as small, colourless translucent colonies which subsequently became more opaque, with small white/creamy central cores. Ferric iron precipitates were observed to cover the centre of each colony and with prolonged incubation, colonies were heavily ferric stained.

(a)



(b)

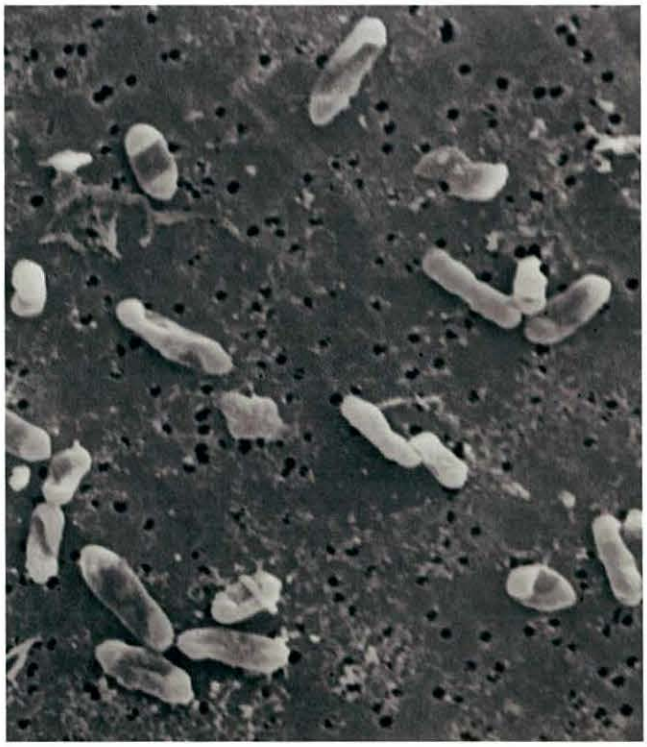
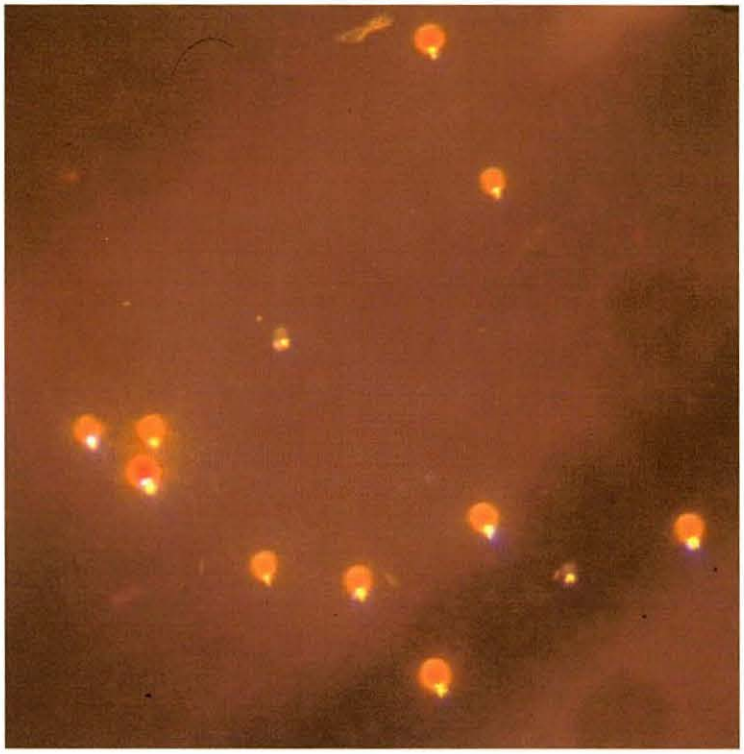


Fig. 3.1 : Scanning electron micrographs of the Gram-negative isolates (a) S-10 and (b) M-12 during growth on 20 mM ferrous iron, pH 1.9

(a)



(b)

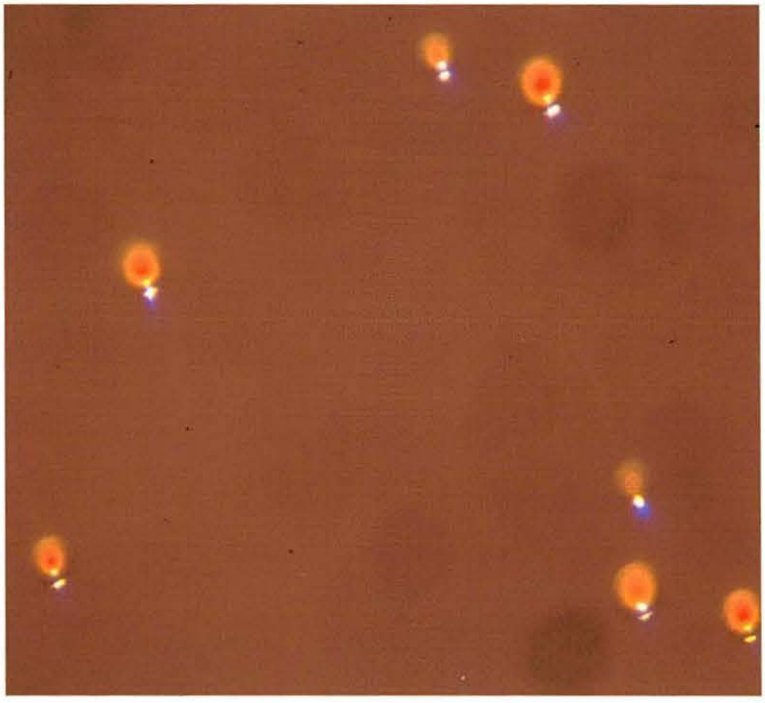


Fig. 3.2: Colonies of iron-oxidising isolates (a) S-10 and (b) M-12 grown on ferrous iron overlay solid medium, after 21 days incubated at 30°C.

3.3 Ferrous iron oxidation by Gram-negative Montserrat isolates

Ferrous iron oxidation by isolates S-10 and M-12 was evident from their first isolation, though the effects of growth conditions on iron oxidation and the relationship between growth and iron oxidation was initially unclear. The following experiment was carried out to study ferrous iron oxidation by both isolates, as affected by various media compositions.

3.3.1 Correlation between growth and ferrous iron oxidation

3.3.1.1 Methodology

Isolates S-10 and M-12 were grown in 100 ml shake flasks containing 50 ml basal salts medium, supplemented with 20 mM ferrous iron, or medium amended with 0.02% (w/v) yeast extract, at 30 °C.

Cultures were then inoculated (2% v/v) into triplicate 250 ml Erlenmeyer flasks containing 100 ml basal salts, poised at pH 1.9, to which were added with 25 mM ferrous sulphate, 25 mM ferrous sulphate / 0.02% (w/v) yeast extract or 25 mM ferrous sulphate / 2.5 mM potassium tetrathionate. Bacteria were incubated in an orbital shaker (150 r.p.m) at 30°C, and samples were removed at regular intervals to analyse ferrous iron concentration titrimetrically, using potassium permanganate (section 2.6.1.1). Culture doubling times, based on rates of ferrous iron oxidation, were determined using semi-logarithmic plots of the concentration of ferrous iron oxidised against time. Cells were occasionally observed under the phase contrast microscope and culture purity was checked at the end of the experiment by streaking onto ferrous iron overlay (section 2.2.2.1) and yeast extract non-overlay solid media (section 2.2.2.3).

3.3.1.2 Results

Culture purity was maintained throughout the experimental period. Ferrous iron oxidation by isolates S-10 and M-12 in ferrous iron medium supplemented (or not) with either yeast extract or tetrathionate was monitored, and mean generation times determined, as summarised in Table 3.1. Growth of both isolates was found to be coupled with iron oxidation (Fig 3.3).

Both isolates were capable of complete iron oxidation in all media tested. Culture doubling times were found to be significantly less in ferrous iron/yeast extract (7 – 7.5 h) medium then in yeast extract-free media (8.5 – 9.4 h). Additional of tetrathionate to ferrous sulphate medium resulted in a significant increase in culture doubling time of isolate S-10 but was not found to affect the culture doubling time of isolate M-12 ($P>0.05$).

Table 3.1: Cultures doubling times (t_d), based on ferrous iron oxidation, by Gram-negative Montserrat isolates grown in various media. Results are mean values ± standard deviation (n = 3)

Medium	Mean generation time,t_d (h)	
	S-10	M-12
25 mM Fe^{2+}	8.44 ± 0.13 *a*	9.35 ± 0.25 *a*
25 mM Fe^{2+} / 0.02% YE	7.28 ± 0.26 *b*	7.42 ± 0.10 *b*
25 mM Fe^{2+} / 2mM tetrathionate	9.15 ± 0.25 *c*	8.9 ± 0.90 *ac*

a - *c* : values not followed by the same letters are significantly different ($P < 0.001$)

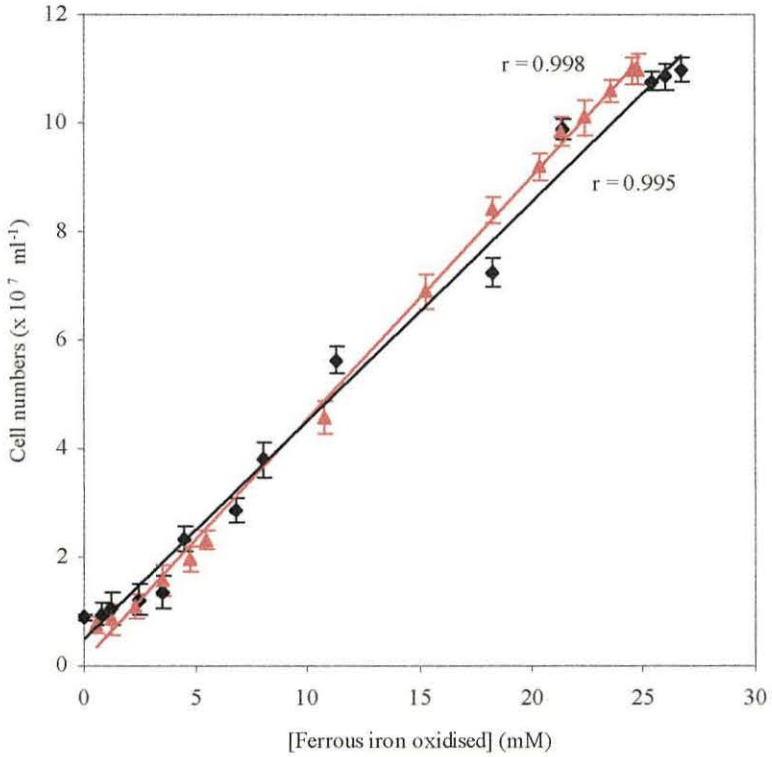


Fig. 3.3: Relationship between growth and iron oxidation by isolates S-10 (▲) and M-12 (◆) grown in 30 mM ferrous sulphate medium, pH 2.0 at 30°C.

3.3.2 Specific rates of iron oxidation

3.3.2.1 Methodology

Pure cultures of S-10 and M-12 were inoculated (1% v/v) into 2 litre flasks containing 1 litre of 30 mM of ferrous iron or 30 mM ferrous sulphate/0.05% (w/v) yeast extract media, adjusted to their pH optima (section 3.6.1), i.e. pH 1.8 for M-12 and pH 1.5 for S-10. Cultures were incubated shaken (120 r.p.m.) at 30°C and ferrous iron oxidation was monitored. Bacteria were harvested at the late-exponential growth phase by centrifugation (10,000 r.p.m. at 4°C for 15 minutes), rinsed, and finally resuspended sterile basal salts pH 1.8 or pH 1.5 (section 2.2.1). One millitre aliquots of the cell suspensions were removed for protein analysis using the Bradford assay (section 2.5.5).

Cells numbers were estimated using the bacteria Thoma counting chamber at *ca.* 10^8 to 10^9 cell/ml suspension.

Bacterial suspensions (5% v/v) were inoculated into triplicate universal bottles containing 10 ml of basal salts poised at pH 1.8 (M-12) or pH 1.5 (S-10). These were equilibrated at 30°C in a water bath, continuously aerated with compressed air and ferrous sulphate added at 4 mM final concentration. Samples were taken immediately following ferrous iron addition, and regularly at 5 minute intervals for up to 30 or 45 minutes. Iron oxidation was determined by measuring the concentration of ferrous iron using the ferrozine assay (section 2.6.1.3).

3.3.2.2 Results

A linear relationship between ferrous iron oxidation and time by cell suspensions of both isolates S-10 and M-12 was observed (Fig. 3.4a and 3.4b). Specific rates of iron oxidation by these bacteria, grown under different conditions are shown in Table 3.2, together with data for previously-studied strains of chemolithotrophic iron-oxidising mesophiles *T. ferrooxidans* and *L. ferrooxidans*.

Specific iron oxidation rates by S-10 and M-12 grown in ferrous iron medium were found to be much smaller than those determined elsewhere for *T. ferrooxidans* and *L. ferrooxidans*. In general, the effects of yeast extract on the specific rates of iron oxidation rates were similar for both isolates. Specific rates of iron oxidation for both isolates grown in yeast extract-free medium were found to be significantly ($P < 0.001$) greater than for cells grown in yeast extract-containing medium.

Table 3.2: Specific rates of iron oxidation by the Gram-negative Montserrat isolates in comparison with values documented for other mesophilic iron-oxidising acidophilic bacteria. Values shown for S-10 and M-12 are mean of 3 replicate experiments ± standard deviations.

Bacteria	Growth conditions	Specific rates (μmoles/min/mg protein)
S-10	Fe^{2+} Fe^{2+} / yeast extract	157.1 ± 8.64 *a* 106.7 ± 2.93 *b*
M-12	Fe^{2+} Fe^{2+} / yeast extract	170.2 ± 9.2 *a* 134.6 ± 3.00 *c*
T. ferrooxidans^{T} [(a)] (ATCC 23270) n = 4	Fe^{2+}	559.6 ± 35.6 *d*
L. ferrooxidans[(a)] (strain CF12) n = 4	Fe^{2+}	422.0 ± 19.9 *e*

[(a)]: Bacelar-Nicolau, (1996); specific activity was determined with cells grown aerobically in 100 mM ferrous iron, pH 1.7 at 30°C

a, - *e* : values not followed by the same letters are significantly different ($P < 0.001$)

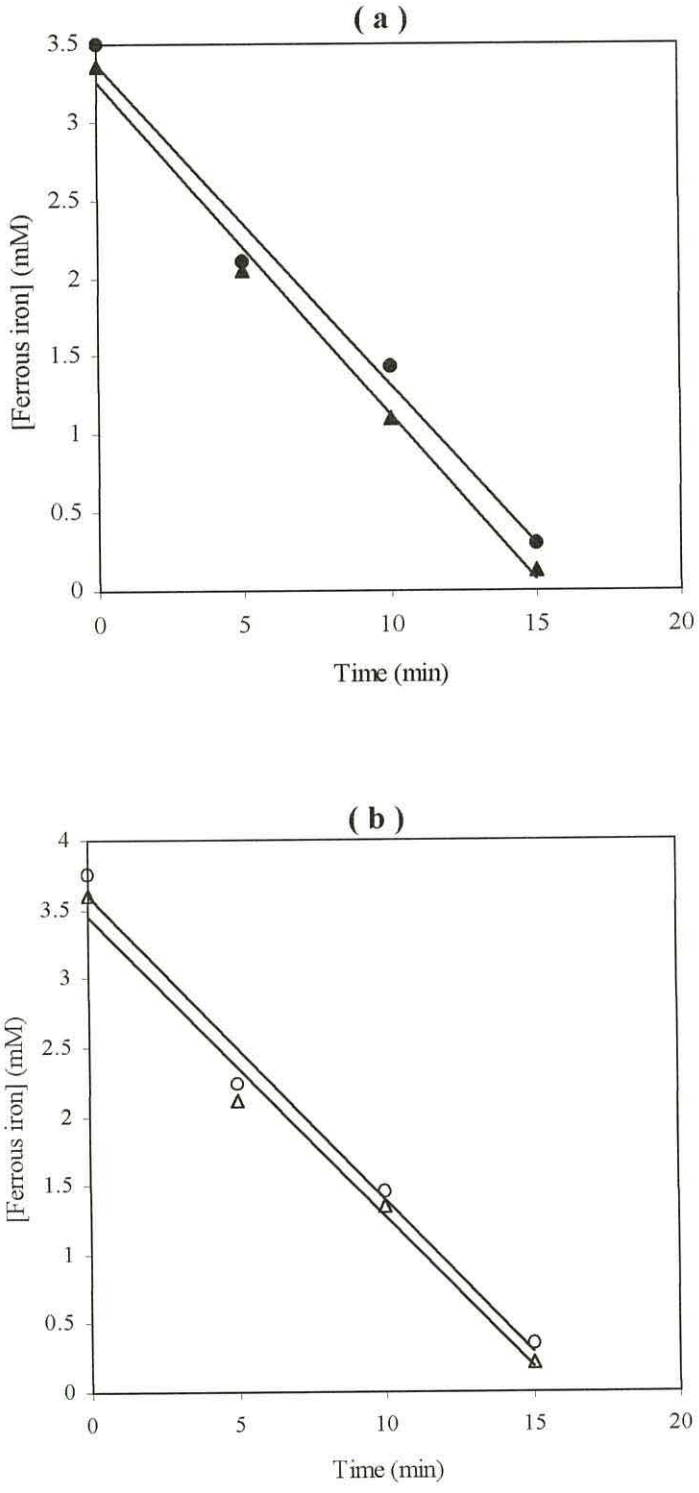


Fig. 3.4: Ferrous iron oxidation by S-10 (●○) and M-12 (▲△) grown aerobically in 30 mM ferrous iron medium (a) supplemented (solid symbols) or (b) not (open symbols) with 0.05% (w/v) yeast extract. Experiments were carried out at 30°C, in a 10 ml reaction mixture containing (a) 0.015 and 0.014 mg protein (b) 0.011 and 0.009 mg protein for S-10 and M-12, respectively.

3.4 Effect of yeast extract on growth yields of Gram-negative Montserrat isolates

3.4.1 Methodology

The Gram-negative Montserrat isolates S-10 and M-12 were grown in 10 mM ferrous sulphate medium supplemented with 0.02% (w/v) yeast extract, and culture purity was checked by streaking the liquid-grown cultures onto yeast extract solid media (section 2.2.2.3) to check for any heterotroph contaminant.

The effects of yeast extract on bacterial growth yields were assessed by inoculating (in triplicate) the pure cultures in 100 ml shake flasks containing 50 ml of 20 mM ferrous sulphate medium adjusted at pH 1.9, and the same medium supplemented with either 0.02% or 0.05% (w/v) yeast extract. Cultures of the type strain of *T. ferrooxidans* (ATCC 23270), *T. ferrooxidans* (strain DSM 9465) and the heterotrophic iron oxidiser, "*Ferromicrobium acidophilum*" (T23) were also prepared for comparison. All cultures were incubated at 30°C, with shaking (150 r.p.m), for up to 1 week.

Growth yields were estimated by cell counts (AODCs; section 2.3.2.1), and viable cell counts (section 2.3.3) of cultures using ferrous sulphate overlay solid medium (section 2.2.2.1).

3.4.2 Results

Culture purity was confirmed from the start and end of the experiment. The effects of yeast extract on growth yields of the Gram-negative Montserrat isolates compared to those of iron-oxidising chemolithotrophs *Thiobacillus ferrooxidans* (ATCC 23270 and DSM 9465) and the heterotroph "*F. acidophilum*" (T23), are summarised in Fig. 3.5.

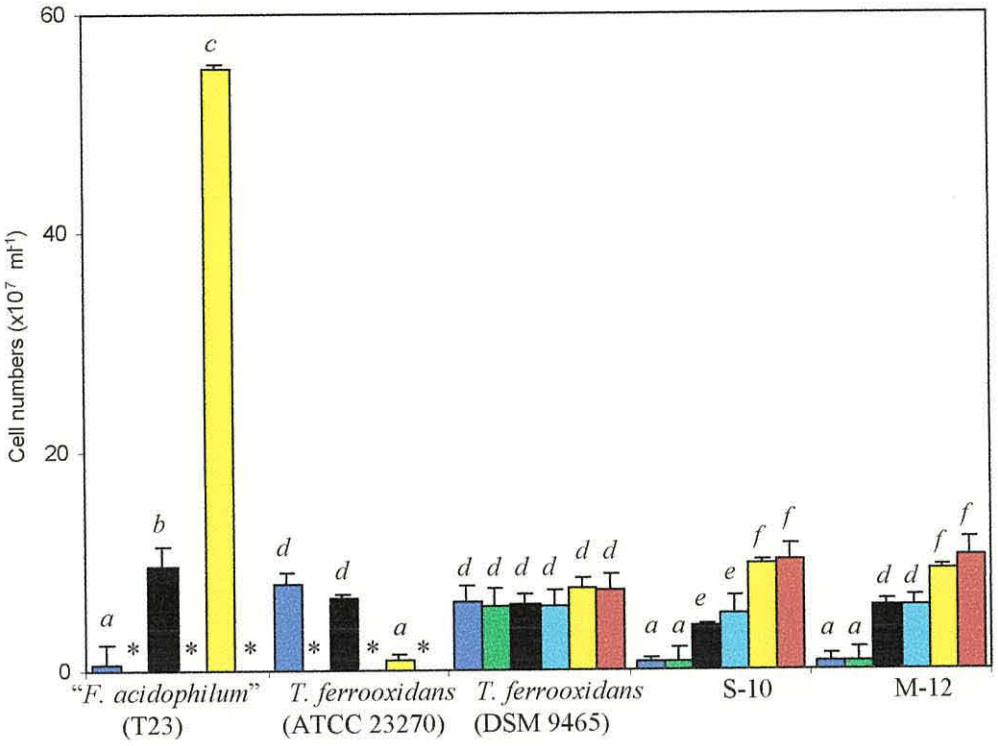


Fig. 3.5: Effect of yeast extract on growth yield of Gram-negative Montserrat isolates S-10 and M-12 compared to those of chemolithotrophic *T. ferrooxidans* (ATCC 23270), *T. ferrooxidans* (DSM 9465) and the iron-oxidising heterotrophic "*F. acidophilum*". Values are shown for total (Fe ■; Fe/0.02% YE ■; Fe/0.05% YE ■) and viable (Fe ■; Fe/0.02%YE ■; Fe/0.05% YE ■) cell counts. (*a – f*): values not followed by the same letters are significantly different ($P < 0.01$)
*: viable cells were not determined

As with the heterotrophic iron-oxidising "*F. acidophilum*", significant increases in cell numbers were displayed by the Gram-negative Montserrat isolates (S-10 and M-12) in cultures containing yeast extract, though numbers of "*F. acidophilum*" in yeast extract-containing medium were found to be significantly ($P < 0.01$) greater than those of the Montserrat isolates. In contrast, growth of the type strain *T. ferrooxidans* (ATCC 23270) was suppressed in the presence of 0.05% yeast extract, as indicated by significant ($P < 0.01$) decrease in cell numbers by 10-fold, i.e.; from 7.8×10^7 cell/ml (Fe medium) or 6.5×10^7 cell/ml (Fe/0.02% YE medium) to 8.2×10^6 cell/ml (Fe/0.05% YE). Growth yields of *T. ferrooxidans* (DSM 9465) are increases in 0.05% yeast extract-containing

medium, though cell numbers were not significantly different with either in the absence or in the presence of 0.02% yeast extract. Due to the effects of yeast extract displayed by S-10 and M-12, heterotrophic growth was tested by growing these isolates in ferrous sulphate/glycerol liquid medium though a similar cell numbers were shown by cultures grown in ferrous sulphate and ferrous sulphate/glycerol liquid media (8.2×10^6 cell/ml and 7.8×10^6 cell/ml).

3.5 Oxidation of reduced inorganic sulphur by the Gram-negative Montserrat isolates

All species of the genus *Thiobacillus* are able to oxidise various inorganic sulphur compounds (e.g. sulphide, thiosulphate, tetrathionate, polythionates etc.). Research on the biochemistry of sulphur metabolism has suggested a fundamentally common oxidation mechanism in different *Thiobacillus* spp.. However, some aspects appear unique to particular thiobacilli, such as the proposed role for a ferric iron-reducing system in sulphur oxidation by *T. ferrooxidans* (Sugio *et al.*, 1985). The experiments described below were designed to test the ability of the two Gram-negative iron-oxidising acidophilic isolates, S-10 and M-12 to oxidise sulphur in batch cultures, under both aerobic and anaerobic conditions.

3.5.1 Aerobic oxidation of elemental sulphur and tetrathionate

3.5.1.1 Methodology

The ability of isolates S-10 and M-12 to oxidise inorganic sulphur compounds in the form of elemental sulphur and tetrathionate was examined. Both isolates were grown in 10 mM ferrous sulphate medium, pH 1.9, at 30°C. Pure active cultures were then inoculated (2 % v/v) into duplicate flasks containing 50 ml basal salts (section 2.2.1.2),

supplemented with either 1.6 g/l (≡ 50 mM) elemental (flowable) sulphur or 2 mM potassium tetrathionate. The effect of yeast extract was studied by inoculating the cultures into the same media amended with 0.02% (w/v) yeast extract. Cultures were initially adjusted to pH 3.0, and were incubated at 30°C, shaken (120 r.p.m). Uninoculated cultures were also set up and run simultaneously as controls to monitor any abiotic sulphur oxidation.

Samples were taken regularly and sulphur oxidation was monitored by measuring changes in culture pH. Concentrations of tetrathionate were monitored by cyanolysis (section 2.6.3). Cells were counted using the Thoma counting chamber (section 2.3.2.2) and cell protein was analysed using the Bradford assay (section 2.6.5) for cultures grown in tetrathionate medium, at the end of the experiment.

3.5.1.2 Results

Isolates S-10 and M-12 were able to oxidise both elemental sulphur (Fig. 3.6) and tetrathionate (Fig. 3.7). A similar pattern of sulphur oxidation was also observed with both isolates grown either in the presence or the absence of yeast extract.

Elemental sulphur was not analysed directly. However, as sulphur oxidation is an acid producing process ($S^{\circ} + H_2O + 1.5O_2 \rightarrow SO_4^{2-} + 2H^+$), the decline in pH was used as an indication of sulphur oxidation. Rapid oxidation of sulphur was observed with both isolates though an extended lag period was observed with isolate S-10 grown either in the presence or the absence of yeast extract. Oxidation of elemental sulphur by S-10 was found to be slightly suppressed in the latter stages in yeast extract-containing medium. This was indicated by the final pH recorded for S-10 grown in the presence of yeast extract, which was slightly higher (pH 1.68) than that in yeast-extract free medium (pH 1.42). In contrast, elemental sulphur oxidation by isolate M-12 was not affected by

yeast extract. A similar pattern of sulphur oxidation was displayed by this isolate grown in either yeast extract-containing and yeast extract-free medium.

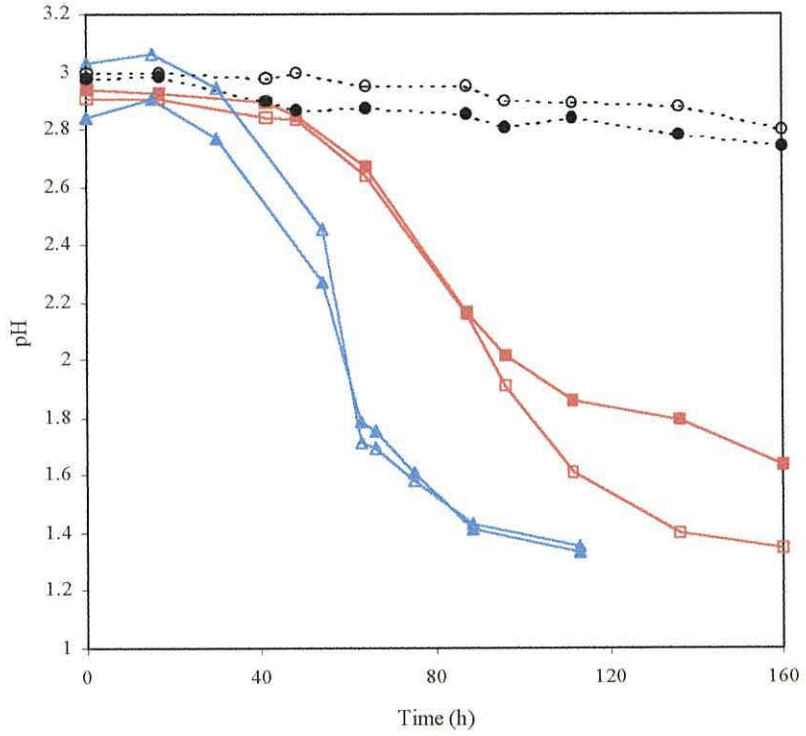


Fig. 3.6: Elemental sulphur oxidation by the Gram-negative isolates S-10 (■□) and M-12 (▲△) in medium containing 1.6 mg/l S° (opened symbols) and the same medium supplemented with 0.02% (w/v) yeast extract (solid symbols), initially adjusted to pH 3.0. Sulphur oxidation was monitored by measuring the pH of the cultures over time. Data for control uninoculated experiments are also shown (●○; broken lines)

Complete oxidation of tetrathionate by both S-10 and M-12 was observed, though more rapid oxidation was displayed in yeast extract-containing medium. Transient accumulation of thiosulphate was observed during tetrathionate oxidation in yeast extract-free medium. Growth yields of isolates S-10 and M-12 were found to be greater in yeast extract-containing medium, as indicated by cell numbers at stationary phase in yeast extract-containing medium (5.8×10^8 and 4.7×10^8 cell/ml, respectively) compared to those in yeast extract-free medium (7.6×10^7 and 8.9×10^7 cell/ml, respectively). This was also supported by protein concentrations found in S-10 and M-12 cultures at the end

of the experiment which were significantly higher ($P < 0.05$) in yeast extract-containing (0.57 and 0.49 mg/ml) than that in yeast extract-free (0.28 and 0.32 mg/ml) cultures.

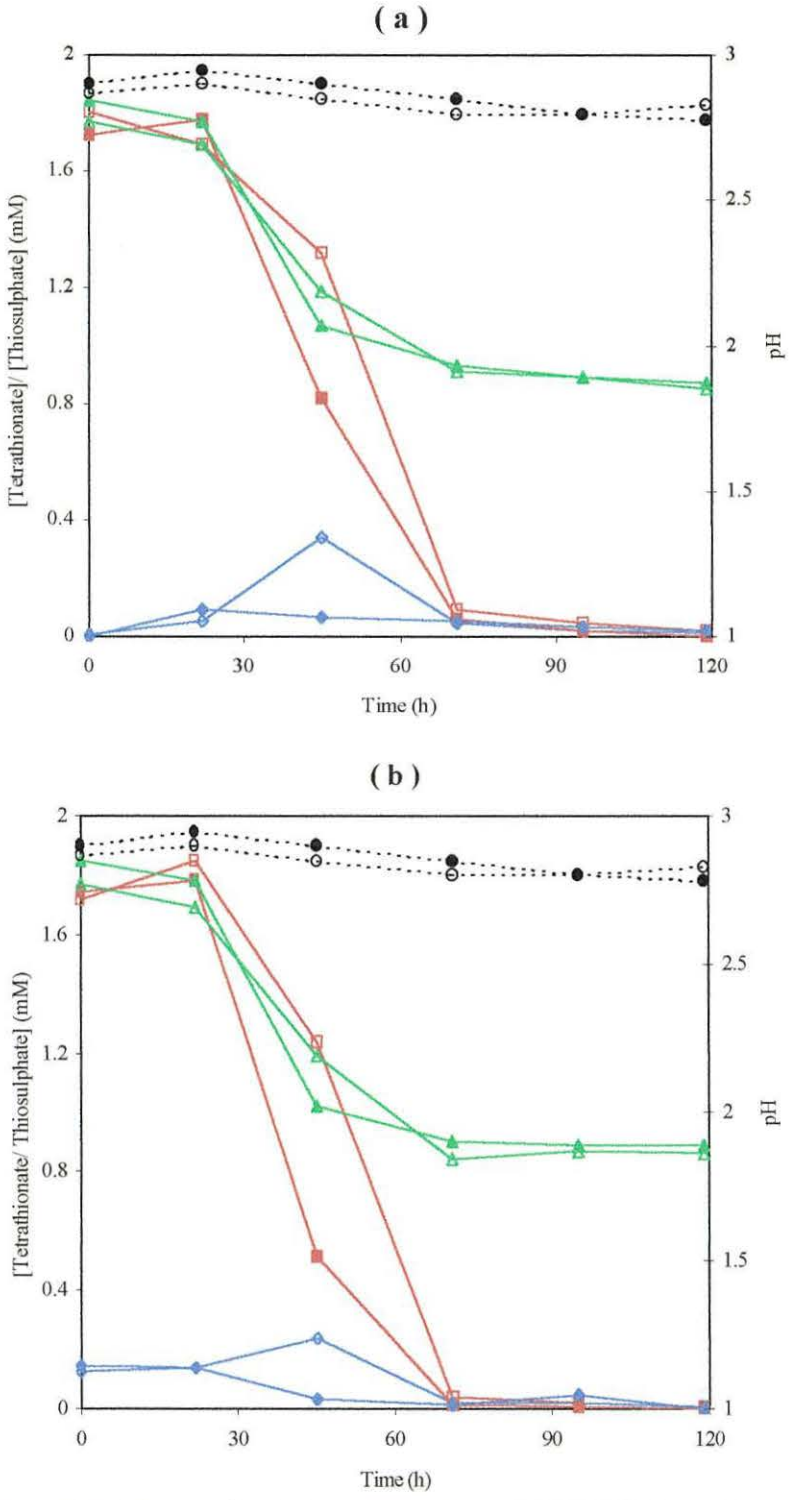


Fig. 3.7: Oxidation of tetrathionate by isolates (a) S-10 and (b) M-12 in medium supplemented (solid symbols) or not (open symbols) with 0.02% (w/v) yeast extract. Growth was monitored by measuring the concentration of tetrathionate (■□) and changes of pH (▲△). Thiosulphate (◆◇) produced during tetrathionate oxidation was also determined. Control experiments; tetrathionate (●○; broken lines); no thiosulphate was detected in experimental controls.

3.5.2 Anaerobic oxidation of sulphur and tetrathionate

3.5.2.1 Methodology

Isolates S-10 and M-12 were grown aerobically in 100 ml shake flasks containing 25 ml sulphur medium (section 2.2.1.2) containing approximately 0.32 g/l (≡ 10 mM) of elemental (flowable) sulphur. Growth was observed under the phase-contrast microscope (section 2.4.1.2).

Assessment of anaerobic oxidation of sulphur was carried out by inoculating sulphur-grown cultures into serum bottles containing 50 ml of oxygen-free sulphur medium, pH 1.5, in duplicate. In order to establish anoxic conditions, the basal salts solution was pre-gassed with nitrogen. A portable oxygen-monitoring unit was used to check oxygen concentrations in the medium during N_2-sparging. Oxygen-free basal salts were dispensed into serum bottles, closed with Suba-seals and sealed with metal caps prior to autoclaving.

Serum bottles were then supplemented with 0.32 g/l elemental sulphur (or 3 mM tetrathionate), 25 mM ferric iron, and 10 mM sodium bicarbonate as the carbon source. Ferric iron and sodium bicarbonate were added aseptically from 1 M stock solutions, adjusted to pH 1.7 and 3.0 respectively. Active sulphur-grown cultures were inoculated (2 % v/v) into the media, and cultures were incubated at 30°C. Samples (1.0 ml) were removed regularly using sterile needles and syringes. Reduction of ferric iron was monitored by measuring the concentrations of ferrous iron using the ferrozine assay (section 2.6.1.3), and bacterial cells were counted using the Thoma counting chamber (section 2.3.2.2). Tetrathionate concentrations were determined by cyanolysis (section 2.6.3).

3.5.2.2 Results

Isolates S-10 and M-12 were able grow anaerobically, with either elemental sulphur or tetrahionate as electron donor and ferric iron as an electron acceptor. As a consequence, sulphur compounds were oxidised and ferric iron was reduced.

Elemental sulphur concentrations were not analysed in this experiment. However, growth of both isolates was found to be coupled with ferric iron reduction, indicating that these isolates were able to use elemental sulphur as electron donor to grow under anaerobic condition (Fig. 3.8). Isolate M-12 was found to grow better than isolate S-10 in anaerobic sulphur cultures. This was indicated by greater cell numbers found with M-12 than with S-10 throughout the experimental period. Final cell numbers detected were 6.8×10^8 and 2.5×10^9 cell/ml for isolates S-10 and M-12, respectively.

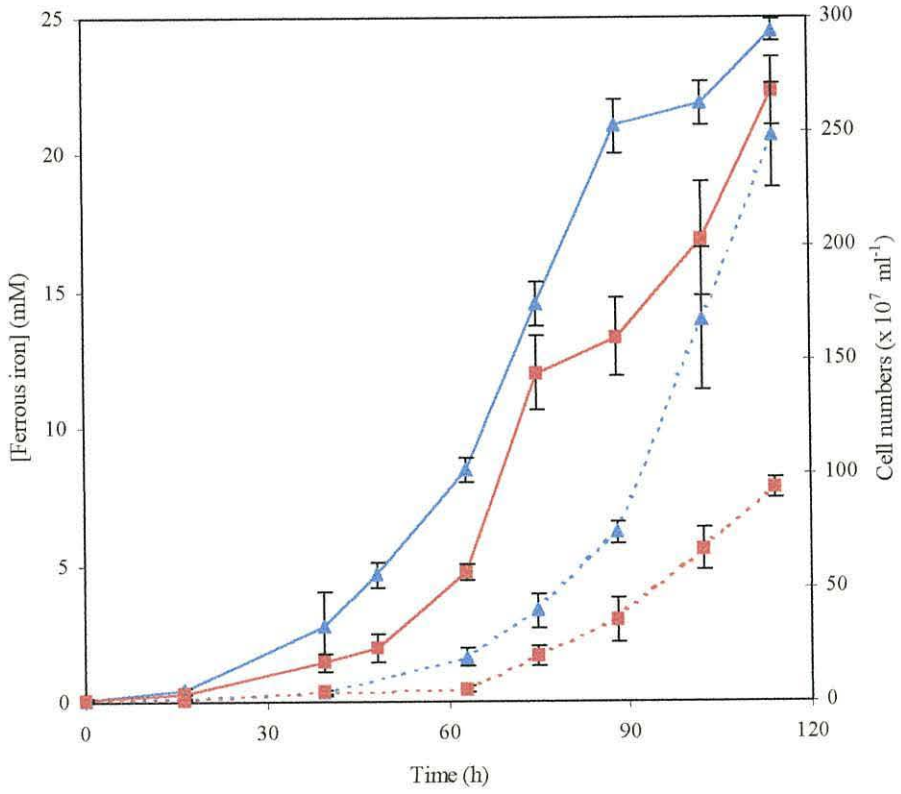


Fig. 3.8: Anaerobic growth of isolates S-10 (■) and M-12 (▲) in elemental sulphur / ferric iron medium. Growth was monitored by measuring concentrations of ferrous iron (solid lines) and cell numbers (broken lines).

A similar pattern of tetrathionate oxidation under anaerobic growth conditions was found with isolates S-10 and M-12 (Fig 3.9). Complete utilisation of tetrathionate and ferric iron was detected with both isolates within *ca.* 6 days of incubation. Growth was found to be coupled with the tetrathionate oxidation and ferric iron reduction, as indicated by increases in cell numbers throughout the experiment. In addition, a distinct smell of hydrogen sulphide was noted when cultures were opened at the end of the experiment.

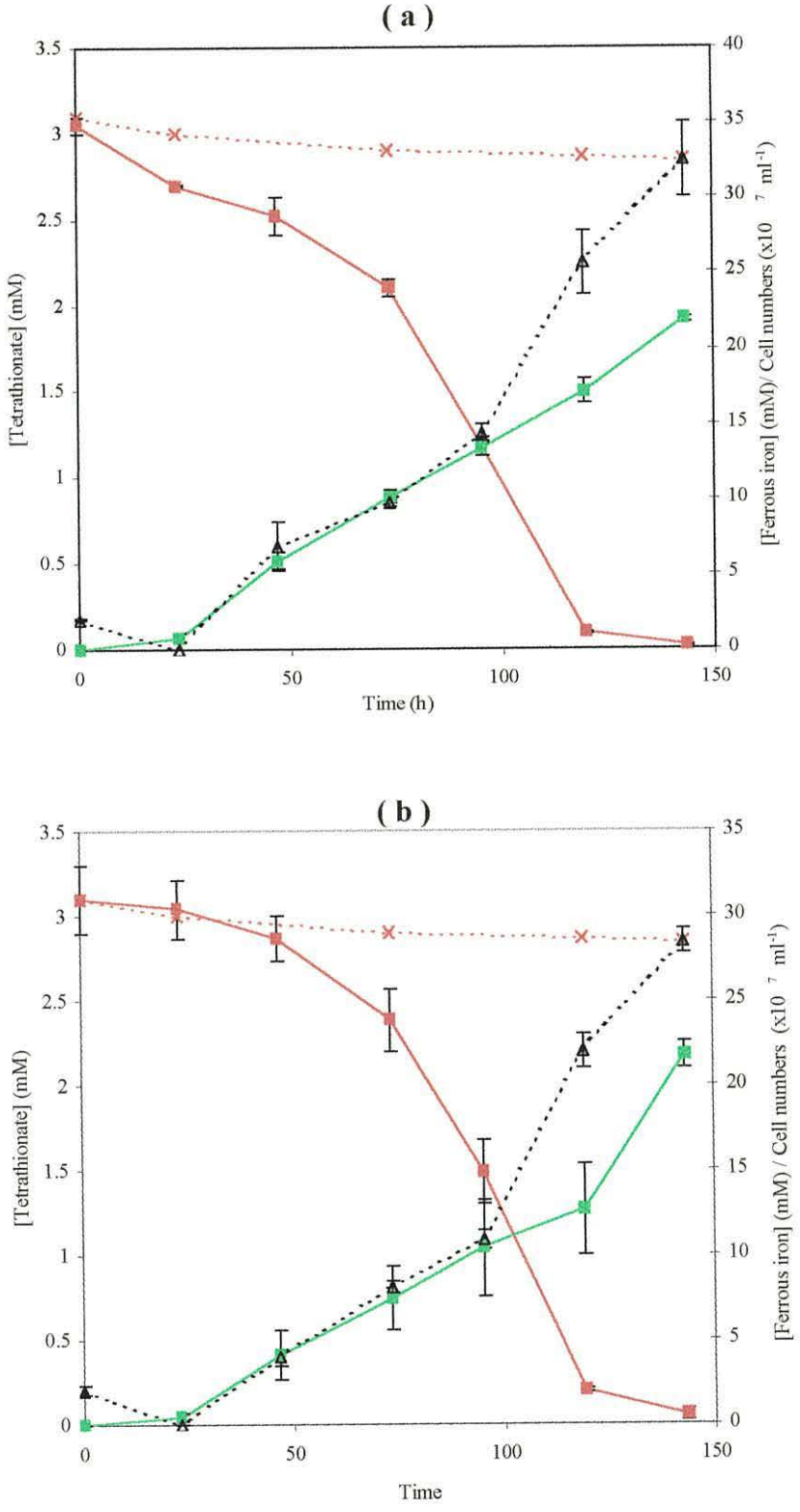


Fig. 3.9: Anaerobic growth of isolates (a) S-10 and (b) M-12 in anaerobic tetrathionate/ferric iron cultures. Symbols; tetrathionate (■), ferrous iron (■) and cell numbers (△; broken lines). Data for tetrathionate in control uninoculated cultures are also shown (×; broken lines)

3.6 Growth of the Gram-negative Montserrat isolates in a bioreactor

Temperature and pH are two fundamental parameters that affect bacterial growth. Therefore, optimisation of those parameters would be advantageous in order to control bacterial growth in commercial applications. Optimisation of these parameters was as fundamental part of the current characterisation studies. Experiments were carried out under controlled conditions using a LH-500 bioreactor (section 3.6.1 and 3.6.2).

3.6.1 Effect of temperature on iron oxidation

3.6.1.1 Methodology

The experiment was set up using a 2 l volume laboratory bioreactor, fitted with pH, temperature, aeration and agitation control units (LH Ltd., series 500). Working volumes were 1 or 1.5 l, and cultures were run in batch mode. Agitation was set at 150 r.p.m, pH was automatically controlled, using 1 M H_2SO_4 or 1 M NaOH, and aeration was set at 0.5 l/min.

Sterile basal salts was adjusted at pH 1.9 and supplemented with 25 mM ferrous sulphate. The temperature was controlled at 30°C and bacterial cultures were inoculated (*ca* 10% v/v) into the equilibrated bioreactor, from shake flask-grown cultures in 25 mM ferrous sulphate medium. In subsequent experiments, 20% (v/v) of the residual volume in the bioreactor was used as the inoculum and fresh medium was added to make up the total volume of 1 – 1.5 l. Temperature was set at 25, 30, 35 and 37°C, and each test was carried out in duplicate. Samples were withdrawn from the bioreactor vessel at regular intervals to monitor iron oxidation, by measuring ferrous iron concentrations titrimetrically using potassium permanganate (section 2.6.1.1). Culture doubling times were calculated from the semi-logarithmic plots of ferrous iron oxidised against time.

3.6.1.2 Results

Optimum growth temperatures of S-10 and M-12 were determined from culture doubling times (t_d) based on ferrous iron oxidation which had been shown to be coupled to growth (section 3.2.1). Specific growth rates (μ) were calculated using the equation μ (h^{-1}) = $\ln 2/t_d$. The effects of temperature on the specific growth rates of isolates S-10 and M-12 are displayed in Fig, 3.10, and show that the optimum growth temperatures were at 30° and 33°C, respectively. At the temperature optima, bacteria were capable of very rapid oxidation of ferrous iron, with equivalent culture doubling times of about 3.5h for both isolates. The maximum temperatures which allowed growth of isolates S-10 and M-12 were at 35° and 37°C, respectively; temperature minima were not determined.

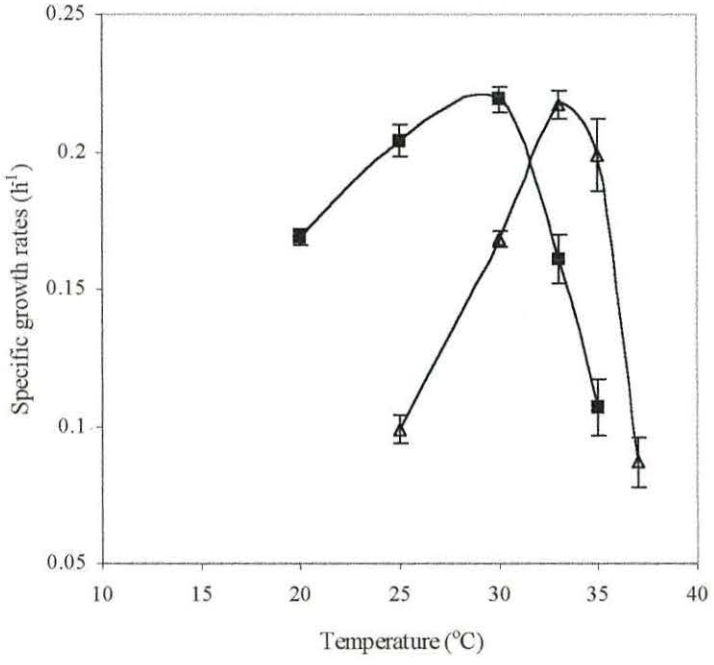


Fig. 3.10: Specific growth rates of the Gram-negative Montserrat isolates S-10 (■) and M-12 (△) based on rates of ferrous iron oxidation as a factor of temperature (pH 1.9 cultures).

3.6.2 Effect of pH on iron oxidation

3.6.2.1 Methodology

The same bioreactor set up (section 3.5.1.1) was used in this experiment, except that the temperature was fixed at the respective optimum value for each isolates while pH was varied.

The bacteria were grown in shake flasks containing 20 mM ferrous sulphate medium, pH 1.9. Active cultures were then inoculated (10% v/v) into the bioreactor which contained 1 - 1.5 l of 25 mM ferrous sulphate medium, equilibrated at 30° or 33°C for S-10 and M-12 respectively. The subsequent experiments were carried out using 20% (v/v) of the residual cultures as the inoculum, and fresh medium supplemented with ferrous sulphate was added to the bioreactor vessel. Samples were taken out at regular intervals and ferrous iron concentrations measured using potassium permanganate titration (section 2.6.1.1). The pH of growth medium was set at 1.0, 1.5, 1.6, 1.7, 1.8, and 2.0 (each test in duplicate). Culture doubling times were calculated based on the oxidation of ferrous iron, from semi-logarithmic plots of ferrous iron oxidised against time.

3.6.2.2 Results

These isolates showed differences in their pH optima, with S-10 (pH optimum 1.5) being more acidophilic than M-12 (pH optimum 1.8) (Fig.3.11). Furthermore, isolate S-10 was able to grow at pH 1.0 while isolate M-12 did not. The maximum growth rates recorded (at temperature and pH optima) were equivalent to culture doubling times of 2.8 h (S-10) and 3.0 h (M-12).

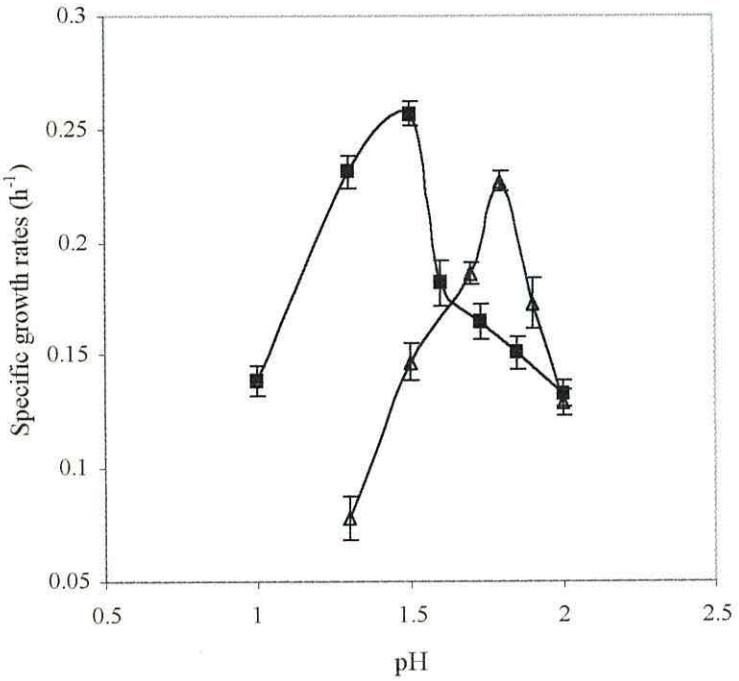


Fig. 3.11: Specific growth rates of the Gram-negative isolates S-10 (■) and M-12 (△) based on rates of ferrous iron oxidation as a factor of pH at 30° or 33°C, respectively.

3.7 Tolerance to some heavy metals

3.7.1 Methodology

The tolerance of the two Gram-negative Montserrat isolates to some heavy metals commonly found in leaching environments was tested, and compared with that of the type strain of *T. ferrooxidans* (ATCC 23270). Bacteria were grown in 10 mM ferrous sulphate medium pH 1.9. Active cultures were inoculated (2%) into a series of 100 ml shake flasks, each containing 50 ml of ferrous iron medium (section 2.2.1.1), supplemented with various concentrations of ferrous and ferric iron, copper, zinc or molybdenum. All metals were added as their sulphate salts, with the exception of molybdenum, which was added as sodium molybdate. Metal concentrations used were 5, 10, 25, 50, 100, 200 and 300 mM for copper and zinc, 0.05, 0.1, 0.2, 0.5 and 1.0 mM for

molybdate, 100, 200, 300 and 500 mM for ferrous iron and 10, 25, 50 and 100 mM for ferric iron. Cultures were incubated, unshaken at 30°C for up to 10 days, and ferrous iron oxidation was monitored, titrimetrically using potassium permanganate (section 2.6.1.1). In the case of ferric iron, 25 mM ferrous iron was added and 10 mM ferrous iron was added to media containing copper, zinc and molybdenum. Effects of ferrous and ferric iron on culture doubling times of the isolates were determined from semi-logarithmic plots of iron oxidation versus time.

3.7.2 Results

Tolerance of the bacteria to the heavy metals tested was determined from noting the effects of the metals on ferrous iron oxidation. Minimum concentrations of the metals (MIC) which completely inhibited bacterial oxidation of ferrous iron, are shown in Table 3.3.

As with T. *ferrooxidans*, isolates S-10 and M-12 were highly tolerant of elevated concentrations of the heavy metals tested, with the exception of copper and molybdenum. Molybdenum is among the most toxic anionic metals to acidophiles, with growth often inhibited at concentrations below 0.05 mM (Barrett *et al.*, 1993). Iron oxidation by S-10 and M-12 was completely halted by 0.1mM molybdate, at which concentration complete iron oxidation was observed with cultures of *T. ferrooxidans* (ATCC 23270). Zinc was the least toxic metal to all bacteria, with complete iron oxidation occurring in the presence of the highest concentration tested (300 mM). Copper was more toxic to the Montserrat isolates than to the type strain *T. ferrooxidans* with iron oxidation being completely inhibited by 100 mM copper. At 50 mM copper, S-10 and M-12 oxidised only 15 to 20% of the available ferrous iron. The Montserrat isolates showed similar tolerance to ferrous and ferric iron as the type strain of *T. ferrooxidans*. Increasing

concentrations of ferrous and ferric iron were found to increase culture doubling times (Fig 3.12).

Table 3.3: Tolerance of Gram-negative Montserrat isolates to some heavy metals in comparison to the type strain of *T. ferrooxidans* (ATCC 23270).

	Minimum inhibitory concentration of metals (mM)		
Metals	*T. ferrooxidans*	S-10	M-12
Fe^{2+}	>500	>500	>500
Fe^{3+}	>100	>100	>100
Cu^{2+}	>300	100	100
Zn^{2+}	>300	>300	>300
MoO_4^{2-}	0.2	0.1	0.1

'>' indicates that the concentration required to inhibit iron-oxidation by these bacteria was greater than the values shown (the maximum concentration tested).

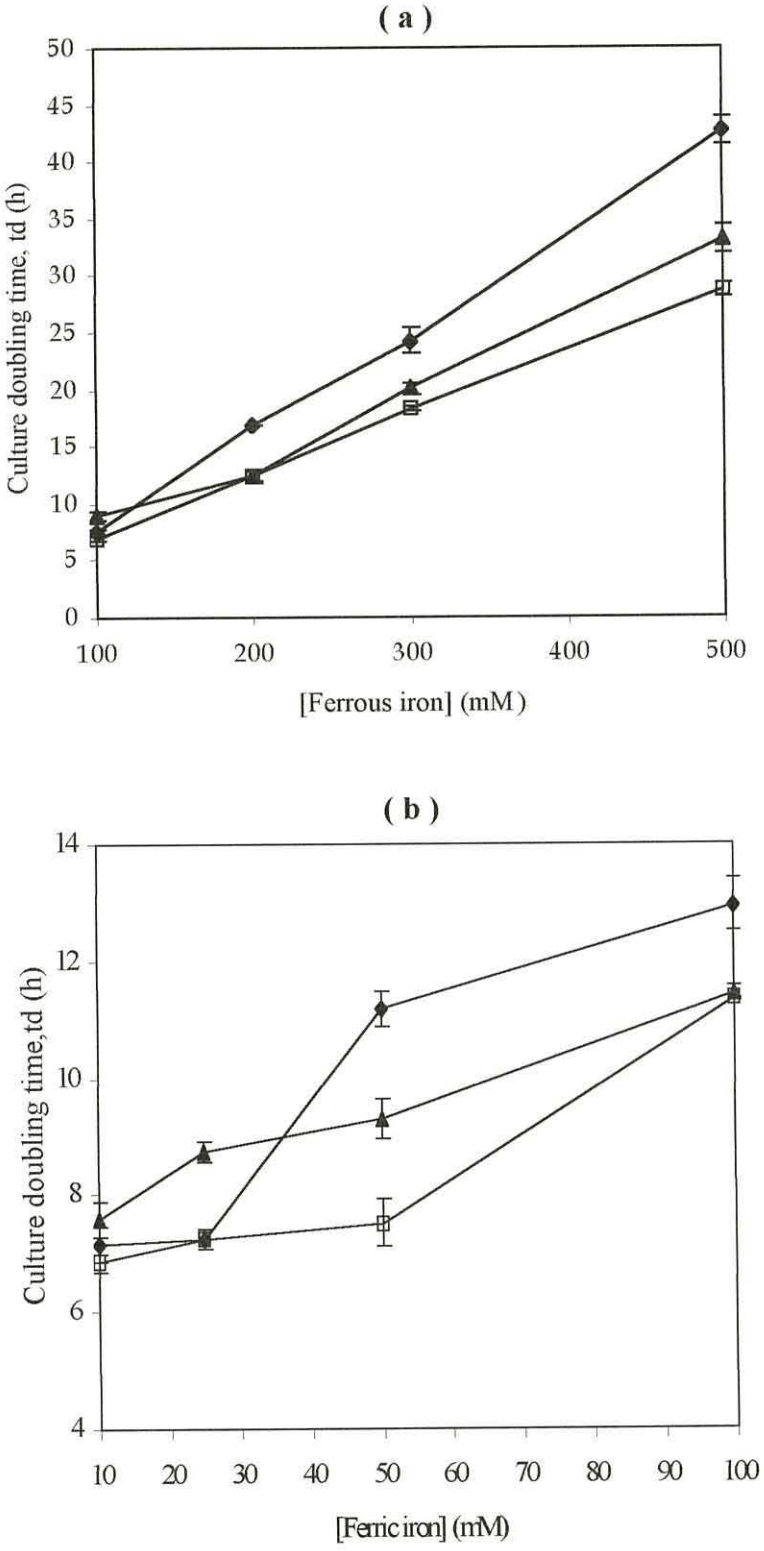


Fig. 3.12: Effect of (a) ferrous iron and (b) ferric iron on the culture doubling times of isolates S-10 (◆), M-12 (▲) and *T. ferrooxidans*^{T} (□).

3.8 Carbon dioxide fixation

3.8.1 Methodology

Carbon is a key component of living microorganisms and can be derived from either prefixed organic compounds or inorganic carbon dioxide (CO_2). In an attempt to assess carbon metabolism by the two Gram-negative iron-oxidising Montserrat isolates, S-10 and M-12 were grown in the radiolabelled carbon ($^{14}CO_2$)-containing ferrous iron medium, supplemented or not with 0.02% (w/v) yeast extract.

The bacteria were grown in 10 mM ferrous sulphate medium; pH 1.9, and active cultures inoculated (2% v/v) into duplicate 100 ml Erlenmeyer flasks containing 50 ml of 25 mM ferrous sulphate medium, with some media amended with 0.02% (w/v) yeast extract. The flasks were sealed (Suba seals) and cultures were then supplemented with 1 % (v/v) of 0.5 M ($NaH[^{14}C]O_3$ (Amersham International) with a specific activity of 11,640 dpm/μmol. Cultures were incubated at 30°C with shaking (150 r.p.m.) and carbon dioxide incorporation by the bacteria was monitored, as described in section 2.7. Iron oxidation was monitored by potassium permanganate titration (section 2.6.1.1). Cell numbers were counted using the Thoma chamber at the end of incubation. The relationship between the two processes were determined by plotting the CO_2 fixed (nmoles/ml) against the concentration of ferrous iron oxidised (μmoles/ml).

3.8.2 Results

Both Gram-negative Montserrat isolates, S-10 and M-12, were able to assimilate carbon from atmospheric carbon dioxide, and this was coupled to the oxidation of ferrous iron (Fig 3.13). However, carbon dioxide fixation by these bacteria, was slightly inhibited when 0.02% (w/v) yeast extract was present in the growth medium. This

indicated that these bacteria were capable of making use of yeast extract as an alternative carbon source.

Due to the observed effect of yeast extract on cell yields of S-10 and M-12 (section 3.4) it was more pertinent to note the effect of yeast extract on CO_2 fixation on a unit cell basis. At the stationary phase, cell numbers of S-10 and M-12 were 4.9×10^6 and 5.9×10^6 cell/ml in the absence of yeast extract, and 3.3×10^7 and 4.2×10^7 cell/ml in the presence of yeast extract. Using these numbers, the amount of carbon fixed (from CO_2) per cell was calculated, as summarised in Table 3.4. The inhibition of carbon dioxide fixation by these isolates was more apparent on this basis, with CO_2 fixation being reduced by about 4 and 5.5–fold for S-10 and M-12, respectively, in the presence of 0.02% yeast extract.

Table 3.4: Carbon dioxide fixation by Gram-negative Montserrat isolates in ferrous sulphate medium, supplemented, or not, with 0.02% (w/v) yeast extract.

Bacteria / Medium	CO_2- C fixed (g C/cell)	
	Fe^{2+}/ yeast extract	Fe^{2+}
S-10	1.82×10^{-13}	7.36×10^{-13}
M-12	1.40×10^{-13}	7.80×10^{-13}

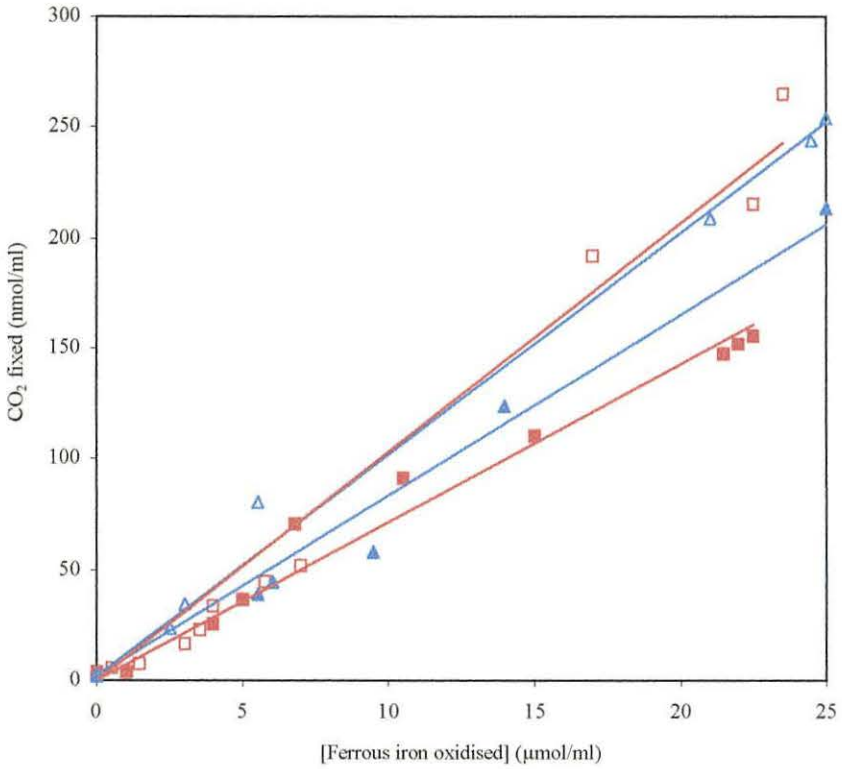


Fig. 3.13: Incorporation of carbon dioxide by isolates S-10 (■□) and M-12 (▲△) grown in medium containing 25 mM ferrous sulphate (opened symbols) and the same medium amended with 0.02% (w/v) yeast extract (solid symbols) in the presence of $NaH[^{14}C]O_3$.

3.9 Molecular characterisation of the Gram-Negative isolates

3.9.1 Analysis of 16S rRNA gene sequence.

3.9.1.1 Methodology

Cultures of isolates S-10 and M-12 were grown in a liquid medium, pH 1.8, containing 30 mM ferrous iron, and incubated at 30°C. Cells were harvested at the late exponential phase by centrifugation (10,000 r.p.m., 10 min. 4°C) washed with 10 mM sulphuric acid and rinsed twice in TE buffer (10 mM Tris-HCl + 1 mM EDTA; pH 8.0). Cells were lysed in 20 µl of PCR lysis solution (0.05 M NaOH + 0.25% SDS) by heating at 95°C for 10 minutes. One microlitre aliquotes of the resulting cell lysates were used in

subsequent 50 µl PCR reaction mixes. The 16S rRNA gene sequences were amplified using forward and reverse primers (section 2.9) and then cloned into the pCR2.1 TOPO TA cloning vector (section 2.9.1). The authentic clones were screened and confirmed using the PCR technique (section 2.9.2) and RFLP analysis (section 2.9.3), respectively.

Positive transformants were grown overnight in ampicillin-containing LB liquid medium and plasmid DNA was extracted and purified (section 2.9.4), followed by sequencing (section 2.9.4.1). Sequence data was analysed and a phylogenetic tree was constructed, as described in section 2.9.4.2.

3.9.1.2 Results

A near full-length of the 16S rRNA gene sequence was obtained from both Gram-negative isolates S-10 and M-12 (1460 bp). When compared with those of Genbank databases (BLAST program), the sequences revealed a close relationship with other members of mesophilic acidophiles of the genus *Thiobacillus*, with the closest being with *T. ferrooxidans* (DSM 9465) which showed 99% sequence identity. Binary level comparison between the 16S rRNA sequences from S-10 and M-12 also exhibited 99% sequence identity. Both Gram-negative Montserrat isolates showed 98% sequence identity (BLAST) with the type strain *T. ferrooxidans* (ATCC 23270). Using the aligned sequence of both Montserrat isolates and other selected bacteria from the Genbank database, a phylogenetic tree was constructed (Fig 3.14) which indicated that both Gram-negative Montserrat isolates clustered together with mesophilic chemolithotrophic acidophiles of the genus *Thiobacillus*.

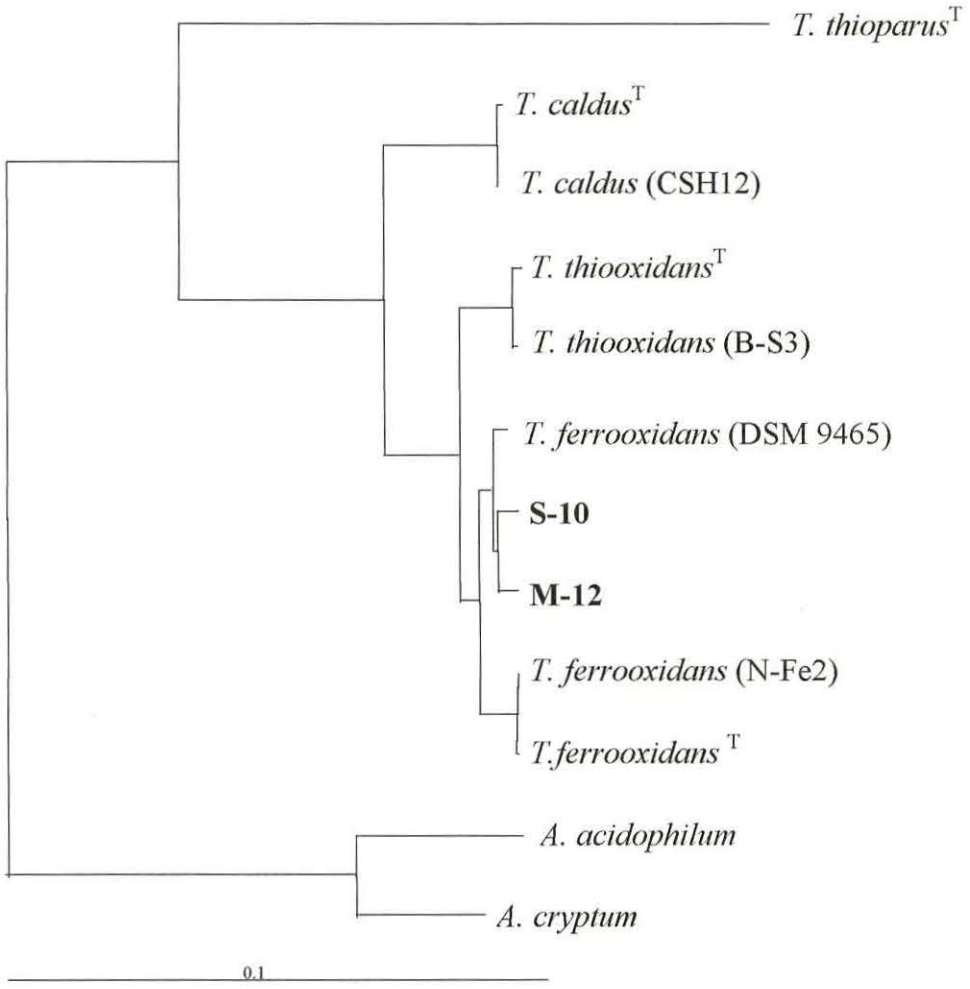


Fig. 3.14: Phylogenetic relationship of *Thiobacillus* strains S-10 and M-12, and other selected acidophilic *Thiobacillus* spp. and *Acidiphilium* spp. based on the comparative analysis of the 16S rRNA sequences contained in the Genbank. Branch lengths are proportional to the calculated evolutionary distances. The tree was rooted using *Korarchaeote* as an outgroup. The bar indicates 0.1 nucleotide substitutions per site.

3.9.2 Analysis of DNA base (G + C) composition

3.9.2.1 Methodology

Pure cultures of isolates S-10 and M-12 were grown in a 500 ml of 30 mM ferrous sulphate, pH 1.8, at 30°C. Cells were harvested during late exponential growth phase by centrifugation (10,000 r.p.m. 10 min, 4°C), washed in 10 mM sulphuric acid and rinsed twice in TE buffer (10 mM Tris-HCL + 1mM EDTA; pH 8.0).

The DNA was then extracted from the resulting cell pellets, using the protocol described in section 2.8, and subsequently purified using caesium chloride gradient centrifugation technique (section 2.8.2). The G + C contents of the chromosomal DNA were determined by melting point analysis in 0.1X SSC as described by Marmur and Doty (1962; section 2.8.3).

3.9.2.2 Results

The DNA base composition obtained for S-10 and M-12 were compared with the type strain of *T. ferrooxidans*, as summarised in Table 3.5. The G + C content of S-10 and M-12 were found to be significantly greater than that of the type strain *T. ferrooxidans*.

Table 3.5: DNA base composition of Gram-negative, *Thiobacillus*-like Montserrat isolates

Bacteria	Mol% (G + C)
S-10	65.4
M-12	65.6
T. ferrooxidans (ATCC 23270)*	58 -59

* Kelly and Wood, (2000)

3.10 Discussion

Over the last 50 years, many iron- and sulphur-oxidising mesophilic acidophilic eubacteria have been isolated. A number of *Thiobacillus*-like isolates were identified from various sites in Montserrat (Atkinson *et al.*, 2000), including isolates S-10 and M-12, though the preliminary identification (based on growth on solid media) was unclear, as colonies of both S-10 and M-12 resembled more closely those of the iron-oxidising heterotroph "*F. acidophilum*" than *T. ferrooxidans*. In this study these uncertainties were resolved through a detailed examination of bacterial characteristics, and physiological and phylogenetic comparison with the type strain of *T. ferrooxidans* (ATCC 23270). It was found that isolates S-10 and M-12:

- are mesophilic, obligately acidophilic Gram-negative bacteria;
- are more acidophilic than the type strain of *T. ferrooxidans*;
- are able to grow autotrophically and mixotrophically;
- have greater growth yields in ferrous iron medium supplemented with yeast extract;
- are able to oxidise iron, sulphur and sulphidic minerals (their ability to degrade sulphide minerals is discribed in Chapter 6);
- are able to reduce ferric iron under anaerobic condition i.e. are facultative anaerobes;
- have 16S rRNA gene sequences most closely related to the genus *Thiobacillus*.

Microscopic examination showed that S-10 and M-12 are non spore-forming bacteria mostly occurring as single straight rods, though occasionally as paired rods. Individual cells of both isolates (at the exponential phase grown on ferrous sulphate medium) had dimensions of an approximately 1 – 1.5 μm long by 0.2 - 0.5 μm wide.

However, S-10 and M-12 differed from each other as S-10 was extremely motile, whereas the motility of M-12 was less pronounced when grown under the same conditions. Both isolates were capable of growth on both ferrous iron and ferrous iron/tetrathionate overlay solid media with slight differences evident in the early phase of growth on ferrous iron/tetrathionate solid medium. However, similar colony morphologies were observed with protracted incubation. In general both cellular and colony morphologies of these isolates were similar to those reported for *T. ferrooxidans* (Ghauri, 1991).

Isolates S-10 and M-12 are obligately mesophilic acidophiles as evident from their optimum growth temperatures (30° and 33°C, respectively); neither grew at 45°C. However, S-10 was found to be more acidophilic than M-12, displaying optimum growth at pH 1.5 compared to pH 1.8 for M-12. On the basis of their optimum pH, both isolates were apparently more acidophilic than the type strain *T. ferrooxidans* (pH optima 2.0; Norris and Johnson, 1998). Furthermore, isolate S-10 was also capable of active growth on ferrous iron at pH 1.0, with an equivalent culture doubling time of ~ 5 h, though pH no ferrous iron oxidation was observed with M-12 at this pH.

The metabolic flexibility of both isolates had also been evident by their ability to grow aerobically or anaerobically under conditions where appropriate electron donors and acceptors were present. In general, the ability of S-10 and M-12 to grow on ferrous iron and sulphur compounds resembled strains of *T. ferrooxidans* (e.g. strain N-Fe4, N-Fe2 and DSM 9465; Durand *et al.*, 1997) but the two Gram-negative Montserrat isolates differed from *T. ferrooxidans* in their response to yeast extract. However, there was no evidence of heterotrophic growth of these isolates on defined organic substrates

as indicated by a similar cell numbers when grown in ferrous sulphate and ferrous sulphate/glycerol media (8.2×10^6 cell/ml and 7.8×10^6 cell/ml, respectively).

Faster rates of iron oxidation were observed with S-10 and M-12 in yeast extract-containing than in yeast extract-free medium, as indicated by a significant decrease ($P < 0.01$) in culture doubling times after 5 to 6 transfers through the same medium (~ 7 h in ferrous sulphate/yeast extract medium and ~8 to 9 h in ferrous sulphate medium). However, it was later found that cultures doubling times based on rates of iron oxidation of both isolates in ferrous sulphate/yeast extract medium increased to ~ 10 to 12 h, after *ca.* 15 to 20 transfers through ferrous sulphate medium, indicating the influence of bacterial adaptation under different growth conditions on iron oxidation activity.

The study of iron oxidation by the Gram-negative isolates S-10 and M-12 demonstrated the effects of yeast extract on the specific rates of iron oxidation. The specific rates of iron oxidation determined were less than those recorded for the mesophilic iron-oxidising *T. ferrooxidans* and *L. ferrooxidans*, grown under oxidising conditions on 100 mM ferrous sulphate (Bacelar-Nicolau, 1996). Specific rates of iron oxidation by S-10 and M-12 were significantly lower for cells grown in ferrous sulphate/0.05% (w/v) yeast extract medium than in ferrous sulphate medium. The effects of yeast extract on the specific rates of iron oxidation by the two Gram-negative Montserrat isolates resembled to those displayed by Gram-positive Montserrat isolates (Chapter 4), though the effects were less pronounced in the former. Lower specific rates of iron oxidation displayed by S-10 and M-12 grown in yeast extract-containing medium were possibly due to greater diversity of proteins and less 'iron-oxidase' produced by the bacteria under such conditions compared to those grown in autotrophic (organic-free) medium. This also indicated the ability of these isolates to utilise yeast extract available

in the medium to maximise biomass production, though the affinity for organic carbon was far less than those of obligately heterotrophic mesophiles (members from the genus *Acidiphilium*: e.g. *A. cryptum*; Harrison, 1980) and iron-oxidising heterotrophs (e.g. "*F. acidophilum*", strain T23; Bacelar-Nicolau, 1996).

Elemental sulphur and tetrathionate were also suitable substrates for the growth of both S-10 and M-12. Rapid oxidation of both sulphur species were observed with both isolates. The fact that these bacteria grew readily on iron and reduced inorganic sulphur compounds and derived energy from the oxidation of these inorganic compounds is again in common with *T. ferrooxidans*; (eg. Ingledew, 1982; Blake *et al.*, 1992; Norris, 1990; Leduc and Ferroni, 1994). Higher growth yields detected in the sulphur- than iron-grown Montserrat isolates reflected the fact that sulphur compounds are energetically more favourable, as shown by the calculations based on the redox potential and the number of electrons transferred during oxidation, which indicate that much more energy is available from tetrathionate (-588.2 kJ/mol) than iron (-91 kJ/mol) (Pronk *et al.*, 1990a,b; Kuenen, 1999). However, there were no apparent effects of organic materials (enhancement or suppression) on rates of oxidation of tetrathionate as these were similar in yeast extract-containing and yeast extract-free media.

Tetrathionate occurs as an intermediate during the oxidation of thiosulphate by *T. ferrooxidans*, *T. thiooxidans* and *A. acidophilum* (Kelly and Harrison, 1989). In this study tetrathionate was used as the substrate due to its greater stability in acidic solutions than thiosulphate. A number of studies have focused on the precise mechanism of reduced sulphur compounds oxidation by thiobacilli, though there is no single unifying pathway to account for the oxidation of RISCs by these bacteria. However several

different operative pathways have been put forward for thiosulphate oxidation, as summarised by Kelly (1989).

In this study, transient accumulation of thiosulphate was detected during the oxidation of tetrathionate by the Montserrat isolates. Based on the hypothetical cyclic pathway of thiosulphate and tetrathionate oxidation proposed for *Thiobacillus* spp. (Sinha and Walden, 1966), thiosulphate could have been formed from the hydrolysis of trithionate ($S_3O_6^{2-}$) produced as an intermediate in tetrathionate oxidation. Trithionate can be formed either chemically, by a reaction of tetrathionate with sulphite, or biologically, by the oxidation of S_3-sulphane-monosulphonic acid ($S_3O_3^{2-}$) (Pronk *et al*, 1990c; Kelly *et al.*, 1997). Rapid oxidation of thiosulphate and tetrathionate by the Montserrat isolates caused acidification of the growth medium (indicated by decrease in pH) as observed with other acidophilic *Thiobacillus* spp. (Meulenberg *et al.*, 1993; Kelly *et al.*, 1997). Production of thiosulphate via the hydrolysis of trithionate has also been reported for *T. thiooxidans* (Okuzumi, 1966; Pronk *et al.*, 1990c).

It was demonstrated that both Montserrat isolates were facultative anaerobes. Both S-10 and M-12 grew in media containing ferric iron and reduced sulphur (either elemental sulphur or tetrathionate). Growth was found to parallel the reduction of ferric iron and was accompanied by a decrease in the culture pH, in agreement with the proposed equations:

$S^\circ + 6Fe^{3+} + 4H_2O \rightarrow HSO_4^- + 6Fe^{2+} + 7H^+$ (Brock and Gustafson, 1976) (3.1)

$S_4O_6^{2-} + 14Fe^{3+} + 10H_2O \rightarrow 4SO_4^{2-} + 14Fe^{2+} + 20H^+$ (3.2)

Bacterial growth in anaerobic cultures was found to be directly related to the increase of the ferrous iron concentrations produced via ferric iron reduction, indicating that growth was not due to leakage of oxygen into the system. These observations were similar to

that of reported with *T. ferrooxidans* (ATCC 21834) grown anaerobically on ferric iron, with elemental sulphur (Dawes, 1986; Pronk 1991) or formic acid as electron donor (Pronk *et al* 1992; 1991a,b), which confirmed that the reaction involved in the anaerobic oxidation of elemental sulphur with ferric iron can provide the bacteria with the metabolic energy required for growth. Anaerobic ferric iron reduction displayed by the Montserrat isolates and other thiobacilli could be important in terms of their contribution to the dissimilatory reduction of iron, which has been and continues to be an important biogeochemical process in certain environments such those of acid polluted water and mining sites (Cummings *et al.*, 2000; Johnson and Bridge, 1997).

Based on the stoichiometry (reaction 3.2), 1 mM tetrathionate is required to reduce 14 mM ferric iron. However, in the experiments described, concentrations of tetrathionate (3 mM) were in access to that required to reduce 25 mM ferric iron (1.22 mM excess tetrathionate in theory). However, complete oxidation of tetrathionate was observed by both S-10 and M-12; indicating that there is another possible mechanism for tetrathionate oxidation by the bacteria under anaerobic condition. Since there was no remaining electron acceptor (ferric iron) in the medium, and both isolates do not reduce sulphate, there is the suggestion that tetrathionate was utilised via a disproportionation reaction ($S_4O_6^{2-} + 2H_2O \rightarrow 2SO_4^{2-} + 2H_2S$), as discussed for the Gram-positive Montserrat isolate RIV-14 (Chapter 4). In support of this hypothesis was the smell of hydrogen sulphide detected at the end of the experimental period. However, more thorough experimental work is required to confirm the ability of these isolates to disproportionate tetrathionate.

A hallmark characteristic of autotrophs, regardless of their physiological group, is the use of CO_2 as the carbon source for cellular constituents. In this study, CO_2 fixation

by the Gram-negative Montserrat isolates was similar to that reported for obligately chemolithotrophic acidophiles *T. ferrooxidans* and *L. ferrooxidans* (Gale and Beck, 1967; Johnson *et al.*, 1992). In this process, CO_2 is reduced and assimilated in the bacterial cellular as organic carbon. As with *T. ferrooxidans*, chemolithotrophic growth of both Gram-negative Montserrat isolates was displayed by their abilities to obtain energy and grow exclusively from the oxidation of iron and reduced inorganic sulphur using inorganic conditions. However, the response of S-10 and M-12 to the addition of yeast extract to ferrous sulphate liquid medium in terms of partial suppression of carbon dioxide assimilation and increased biomass production (the latter also being found in sulphur and tetrathionate media) suggested that the Montserrat isolates are facultative mixotrophs i.e. capable of simultaneously fixing inorganic carbon and assimilating pre-fixed carbon. An apparently mixotrophic *T. ferrooxidans* (strain FDI) was previously described by Barros *et al.*, (1984), though the stoichiometry between glucose utilised (2.5 g/l) and enhanced biomass production (20 mg/l) suggested a possible alternative fate for glucose under the experimental protocol used. Furthermore, a *Thiobacillus*-like isolate (T3.2) has been described which could grow in ferrous sulphate medium only if supplemented with yeast extract, glucose or thiosulphate (de Siloniz *et al.*, 1993). The fact that *T. ferrooxidans* ATCC 13598 is not strictly autotrophic was demonstrated by its ability to take up and incorporate radiolabelled amino acids when growing on ferrous iron or sulphur (Oliver and VanSlyke, 1988). In addition, *T. ferrooxidans* ATCC 21834 can grow anaerobically on formic acid (as electron donor, and ferric iron as electron acceptor) (Pronk *et al.*, 1992).

The ability to oxidise iron and sulphur suggested that S-10 and M-12 would grow readily on mineral sulphides. The oxidation of pyrite and chalcopyrite by the Gram-

negative Montserrat isolates is described in Chapter 6. One of the problems with bacterial leaching is the possibility that heavy metals which accumulate in leachate liquors can reach toxic concentrations. Several heavy metals are essential for bacterial growth but the requirement is normally at very low concentrations (μmoles/l). In the leaching environments concentrations of heavy metals (e.g. copper, zinc,) available may be high enough to inhibit growth of some leaching bacteria and thus would directly affect the leaching process. However, both Gram-negative Montserrat isolates were found to be highly tolerant of the heavy metals tested (e.g. Cu^{2+}: 100 mM, Zn^{2+}: >300 mM), though the sensitivity to copper was greater for the Gram-negative Montserrat isolates than for *T. ferrooxidans*. Metal tolerance of *T. ferrooxidans* has been enhanced using genetic manipulation (Roberto and Bruhn, 1993), though tolerant populations may be adapted, by subculturing, to elevated concentrations of heavy metals (Said, 1990).

The majority of morphological and physiological characteristics of S-10 and M-12 (Gram-negative, rod shaped and non spore-forming mesophilic acidophiles, capable of obtaining energy from iron and sulphur oxidation) suggested that they are strains of *T. ferrooxidans*. This was confirmed with the phylogenetic analysis based on 16S rRNA sequences, which showed their closest relatioship was with *T. ferrooxidans* (DSM 9465). Both isolates S-10 and M-12 have 99% level of binary sequence similarity (BLAST) with *T. ferrooxidans* (DSM 9465) and 98% with the type strain of *T. ferrooxidans* (ATCC 23270). The close phylogenetic relationship between the two Gram-negative Montserrat isolates and *T. ferrooxidans* DSM 9465 prompted speculation that this classified strain of *T. ferrooxidans* might also show a positive response (in terms of growth yield) to the addition of yeast extract to culture media. For this reason, the strain was obtained from the Deutsche Sammlung von Microorganismen und Zellkulturen

(DSMZ), and a parellel experiment carried out as with S-10 and M-12 (section 3.4). However, the results with *T. ferrooxidans* DSM 9465 were slightly ambiguous in that there was no significant increase in cell yield when yeast extract was added at 0.02%, though there was when the yeast extract concentration was 0.05%. The results with the Montserrat Gram-negative and *T. ferrooxidans*-like strains S-10 and M-12, and also for DSM 9465 suggest that adaptation to yeast extract (e.g. by repeated subculturing in yeast extract-containing medium) might be necessary in order to obtain clear evidence of mixotrophic growth. The situation regarding autotrophy and mixotrophy with bacterial strains designate as "*T. ferrooxidans*" is clearly somewhat unclear, and may well be related to strain variation. Based on the high similarity (physiologically and phylogenetically) between the Gram-negative Montserrat isolates and *T. ferrooxidans*, the former are currently considered to be strains of *T. ferrooxidans*. However, both isolates S-10 and M-12 showed a significantly higher G + C value (65%) than that of the type strain *T. ferrooxidans* (58 - 59%). Therefore it is necessary to carry out further work (DNA homology testing etc.) in order to confirm taxonomic position of these isolates. This also includes comparison of the G + C content of the Gram-negative Montserrat isolates and *T. ferrooxidans* DSM 9465 (the closest phylogenetic neighbour).

(4)

Characterisation of the Novel Mesophilic Gram-Positive Iron-Oxidising Acidophiles Isolated from Montserrat (W.I)

4.1 Introduction

Ferrous iron- and sulphide mineral-oxidising Gram-positive acidophilic bacteria that have been characterised previously are moderate thermophiles or ('thermotolerant') that grow optimally at temperature between 45 to 60°C (Norris, 1990). Those most readily isolated from hot springs and run-off from coal spoil heaps and acidic waters at mine sites are species of *Sulfobacillus:* *Sulfobacillus thermosulfidooxidans* (Golovacheva and Karavaiko, 1979) and *Sulfobacillus acidophilus* (Norris *et. al.*, 1996). These two species of sulfobacilli are distinguished by the lower mol % G + C content of the former and also by its failure to grow well autotrophically on elemental sulphur (Norris *et al.*, 1996).

Acidimicrobium, the most recently described genus of moderately thermophilic mineral-oxidising bacteria, so far consists of a single species, named *Acidimicrobium ferrooxidans*; this prokaryotes has been isolated from similar environments to *Sulfobacillus* spp. (Clark and Norris, 1996). As with *Sulfobacillus* spp., *Ad. ferrooxidans* is a Gram-positive bacterium, though it does not produce endospores. However, it is readily distinguished from *Sulfobacillus* spp. by its smaller size and also by its higher G + C content (67 – 69 mol%). In addition, *Ad. ferrooxidans* has a greater capacity to fix carbon dioxide than *Sulfobacillus* spp. and has been suggested to be a significant constituent of mixed cultures of moderate thermophiles that degrade sulphide minerals more rapidly than pure cultures in the absence of enhanced carbon dioxide concentrations (Clark and Norris, 1996; Norris and Johnson, 1998).

This chapter describes the first mesophilic Gram-positive, iron- and sulphur-oxidising acidophiles to be fully characterised. The bacteria were isolated from geothermal sites on the volcanic island of Montserrat (section 2.1.1). Various methods were used to characterise these isolates, including physiological and biomolecular approaches, the latter based on the comparative analysis of the 16S rRNA gene sequences with other known Gram-positive iron-oxidising acidophiles as well as on the basis of chromosomal DNA base (G + C) composition.

4.2 Morphological characteristic of the isolates on solid and liquid media

4.2.1 Methodology

A total of eight isolates of putative mesophilic heterotrophic iron-oxidising acidophiles were revived from the preserved Montserrat cultures in the 'UWB acidophile culture collection' (Johnson, unpublished data) maintained at 4°C in pyrite medium for over a year. However, in this study, only two of the isolates coded RIV-14 and L-15 were examined in detail. The purity of isolates RIV-14 and L-15 was first confirmed by streaking onto ferrous iron/tetrathionate overlay (section 2.2.2.2) and yeast extract plates (section 2.2.2.3). Repeated single colony isolation into ferrous iron/yeast extract liquid medium was performed to ensure cultures purity, before further studies were carried out (section 2.1.1).

Pure active cultures of isolates RIV-14 and L-15 were inoculated (1% v/v) into duplicate 100ml shake flasks containing 50 ml of sterile basal salts solution (section 2.2.1.1), pH 2.0 supplemented with 20 mM ferrous iron, or the same medium amended with 0.02% (w/v) yeast extract. Cultures were incubated, shaken (150 r.p.m.), at various temperatures (30°, 37° and 45°C) for up to 1 week, and oxidation of ferrous iron by the bacteria was determined. Cultures were observed under the phase-contrast microscopy to record the cellular morphology of the isolates. Specimens for scanning electron (section

2.4.2) and transmission electron microscopic (section 2.4.3) observation were also prepared for further characterisation of cellular morphologies observations were also prepared for further characterisation of cellular morphologies. Gram staining of cells was carried out as described in section 2.5.

Growth of the isolates on plates was also monitored by streaking the respective liquid-grown cultures onto the solid media, specially formulated for growth of iron- and sulphur-oxidising acidophilic bacteria (section 2.2.2), incubated at 30°, 37° and 45°C. Colony formation was observed after 2 weeks of incubation using a stereo scan microscope (section 2.4.1.1) and plates were reincubated for a further one to two weeks. Morphological characteristics of the colonies were recorded throughout this period.

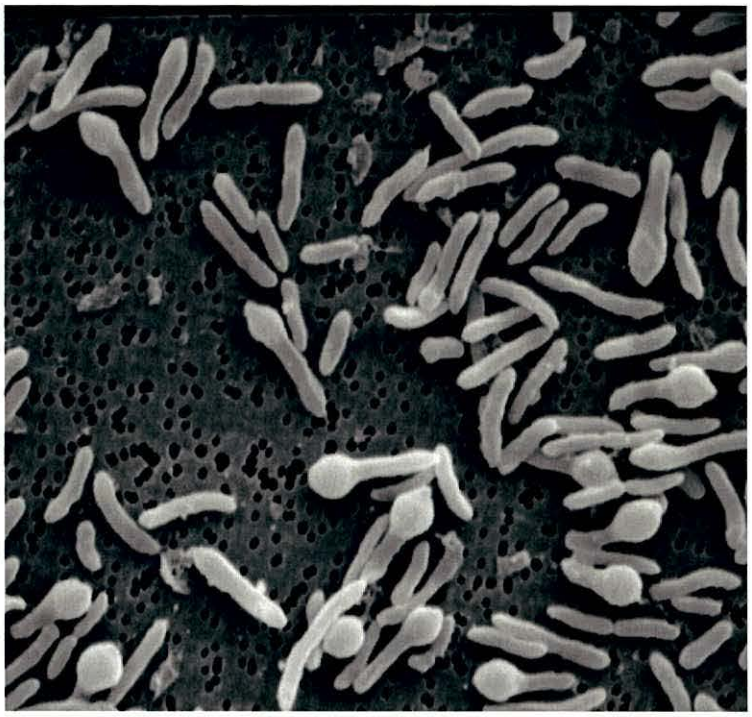
4.2.2 Results

Cellular and colony morphologies of isolates RIV-14 and L-15 were closely observed in this preliminary characterisation study. Phase-contrast observation revealed that these isolates normally existed as single and paired rod-shaped cells which tended to form chains consisting of 3 to 10 individual rods during late exponential growth phase (Fig 4.1a). The average size of individual cells was 2.25 μm long / 0.55 μm wide and 2.9 μm long / 0.72 μm wide, for isolates RIV-14 and L-15 respectively, grown in ferrous iron/yeast extract medium, as determined from measurements of 20 cells in scanning electron micrographs. Endospores were also observed in both cultures, were more commonly found in cells grown in autotrophic (ferrous iron) medium, or at the stationary growth phase in ferrous iron / yeast extract medium. Both RIV-14 and L-15 stained Gram-positive. Sections of sporulating cells of both isolates revealed a development of mature spore structure, with clearly visible spore coat layers (Fig 4.1b)

Both isolates RIV-14 and L-15 grew on ferrous iron/tetrathionate solid medium but neither was observed to grow on ferrous iron overlay solid medium (Fig. 4.2). Limited growth was displayed by these bacteria on non-overlay yeast extract medium. Colonies on ferrous iron/tetrathionate overlay plates were initially (*ca.* 4 to 6 days) small off-white, gelatinous, raised colonies with smooth edges and were slightly stained (with ferric iron) in the centre of individual colonies. These subsequently developed into larger, round colonies with flatter morphologies, and ferric iron staining became more pronounced with prolonged incubation at 30°C (*ca.* 15 to 20 days).

Isolates RIV-14 and L-15 grew on plates and liquid medium incubated at 30° and 37°C but no growth was observed in media incubated at 45°C. This indicated that these isolates were mesophilic, as with the Gram-negative Montserrat isolates, S-10 and M-12 (section 3.2.2).

(a)



(b)

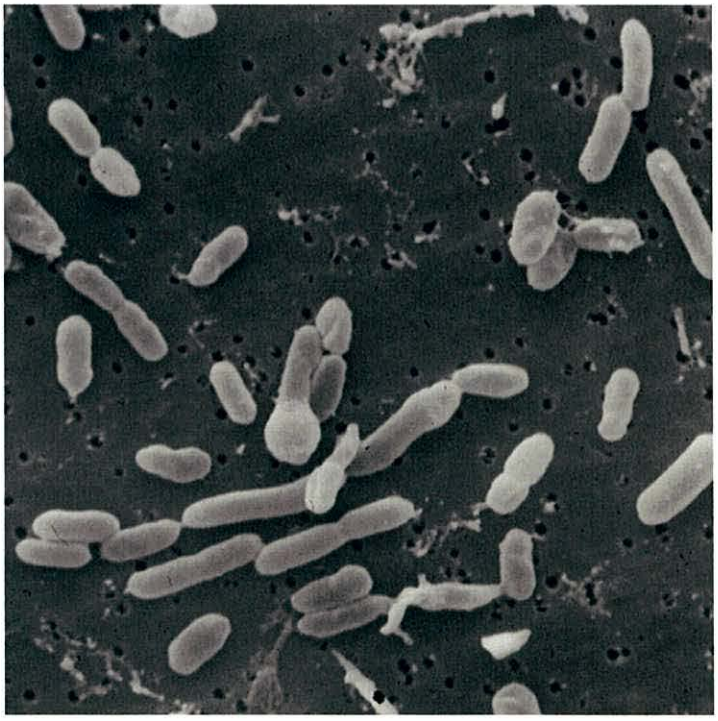


Fig. 4.1: Scanning electron micrographs showing cells of (a) RIV-14 and (b) L-15, during the exponential growth phase in medium containing 10 mM ferrous sulphate and 0.02% (w/v) yeast extract; pH 1.8

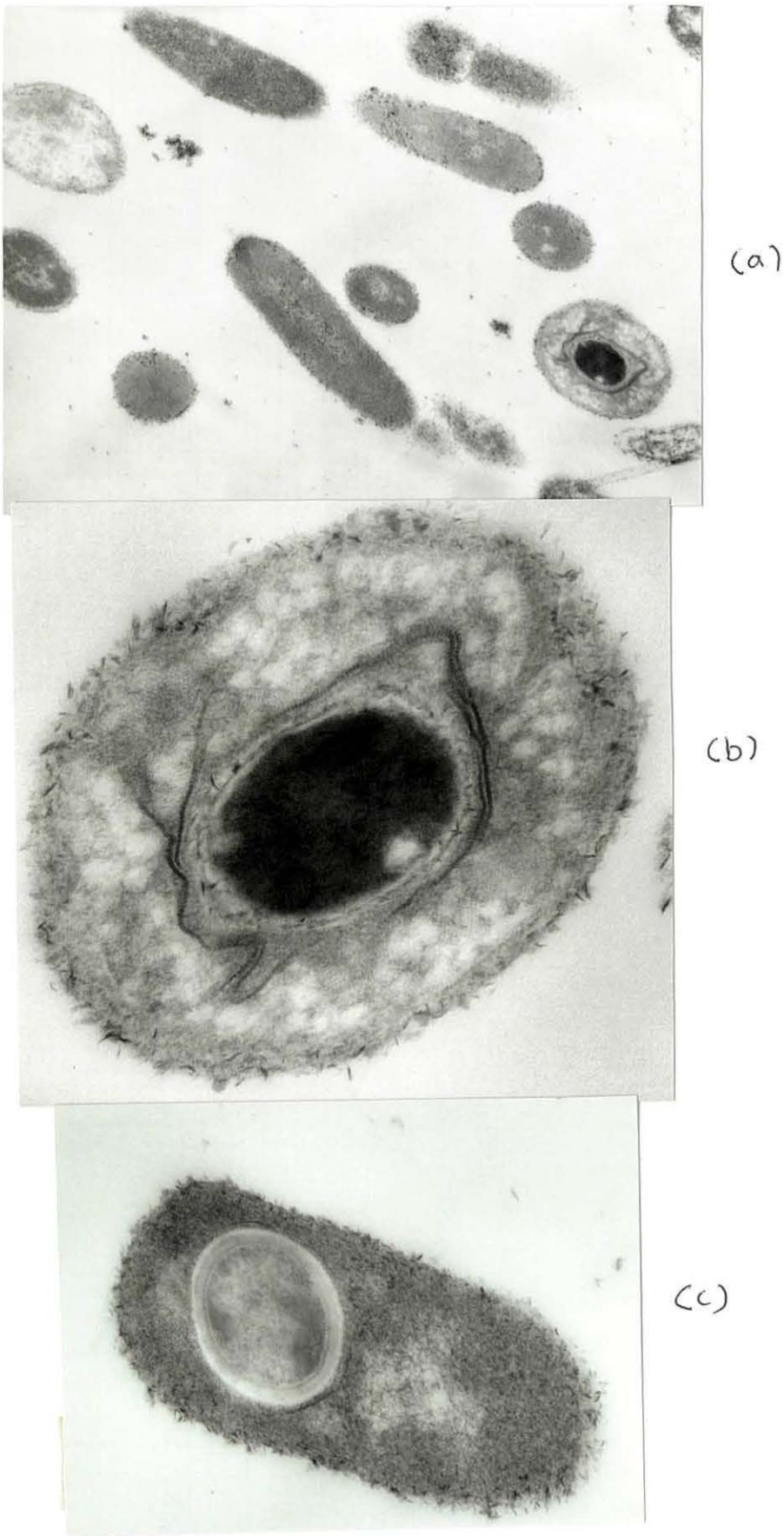
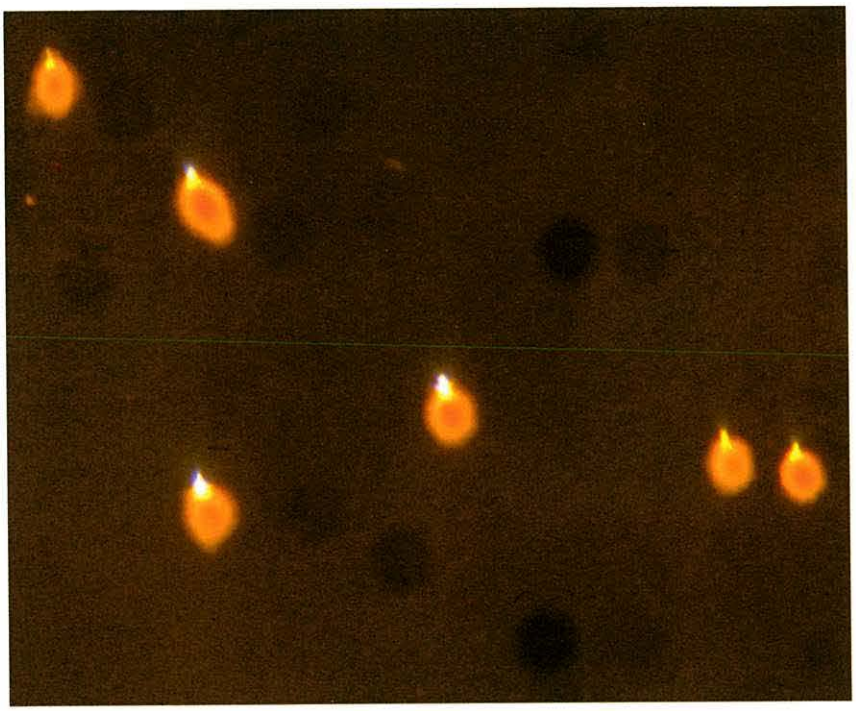


Fig 4.1b: Transmission electron micrographs showing; (a) longitudinal and transverse sections of normal and sporulating cells of RIV-14; (b) transverse section showing a sporulating cell of RIV-14 and (c) longitudinal section of a sporulating cell for isolate L-15

(a)



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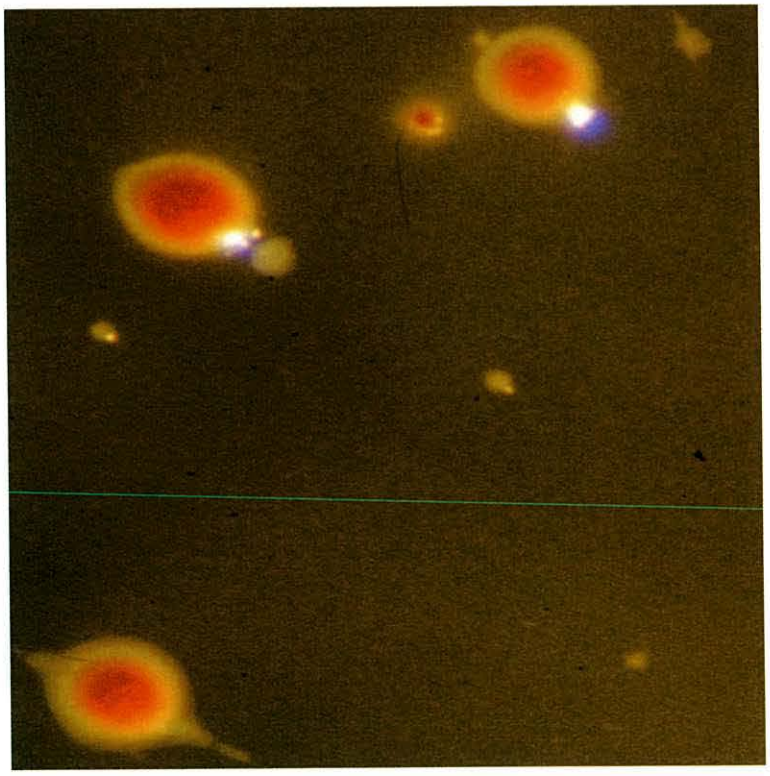


Fig. 4.2: Colony morphology of (a) RIV-14 and (b) L-15 (large colonies), grown on FeSo overlay solid medium, after 20 days incubated at 30°C

4.3 Aerobic and anaerobic growth of the Gram-positive Montserrat isolates on iron

Iron is the fourth most abundant metal in the earth's crust, and exists in reduced (Fe^{2+}) or oxidised (Fe^{3+}) forms. Under aerobic conditions, both oxygen and ferric iron (Fe^{3+}) can be used as electron acceptors by acidophilic heterotrophs though oxygen is used preferentially (Johnson and Pronk, 1992). However, under limited aeration conditions ferric iron, which is often abundant and present in the soluble form in extremely acidic environments (pH < 3), is a thermodynamically attractive alternative electron sink to oxygen (electrode potential of +780 mV). The ability of acidophilic isolates RIV-14 and L-15 to metabolise iron was investigated, under both aerobic and anaerobic conditions.

4.3.1 Ferrous iron oxidation in liquid media

4.3.1.1 Methodology

Pure culture of isolates RIV-14 and L-15 were grown in 100 ml basal salts liquid medium, pH 1.9, supplemented with 10 mM ferrous iron and 0.02% (w/v) yeast extract, at 30°C. At late exponential growth phase, the bacteria were harvested by centrifugation (10,000 r.p.m. for 15 minutes at 4°C), rinsed twice sterile pH 2.0 basal salts and the pellets finally resuspended in 5 ml of basal salts (section 2.2.1). This procedure was carried in order to remove any remaining organic materials present in the growth medium.

Cell suspensions of RIV-14 and L-15 were then inoculated (2% v/v) (in 4 replicates) into 250 ml Erlenmeyer flasks containing 100 ml of sterile 25mM ferrous iron medium; pH 1.9 (section 2.2.1.1), and the medium amended with either 0.02% (w/v) yeast extract or 2.5 mM potassium tetrathionate. The cultures were incubated at 30°C,

shaken at 150 r.p.m.. Growth of the cultures was monitored by measuring oxidation of ferrous iron. Samples were removed at regular intervals to measure ferrous iron concentrations by titration with potassium permanganate (section 2.6.1.1) or ceric sulphate (section 2.6.1.2). Culture doubling times, based on the ferrous iron oxidation by the bacteria, were determined using semi-logarithmic plots of concentration of ferrous iron oxidised against time. Cells were observed occasionally under the phase-contrast microscope (section 2.4.1.2) and culture purity was checked at the end of the experimental period by streaking on the ferrous iron/tetrathionate overlay solid medium (section 2.2.2).

4.3.1.2 Results

Culture purity was confirmed throughout the experimental period. Similar patterns of iron oxidation were observed with both isolates RIV-14 and L-15 (Fig.4.3). Iron-oxidising activity of these bacteria was found to be stimulated by the addition of either yeast extract or tetrathionate to the ferrous iron medium, as determined by culture doubling times (Table 4.1). Both isolates displayed limited ferrous iron oxidation in yeast extract-free medium, where 25 mM ferrous iron was present as the sole inorganic substrate. However addition of tetrathionate to the ferrous iron medium resulted in the complete oxidation of iron by both isolates, confirming that they were capable of autotrophic growth. Growth of both isolates was found to be coupled with iron oxidation (Fig. 4.4).

Although either potassium tetrathionate or yeast extract was required for the bacteria to grow and effectively oxidise iron, ferrous iron oxidation was stimulated to a greater extent by the inclusion of yeast extract than by tetrathionate. Rapid oxidation of ferrous iron in the yeast extract-containing medium, as indicated by culture doubling

times, produced a significant ($P<0.01$; Table 4.1) decrease of the growth rate compared to that in the inorganic growth medium. This indicated that the organic substrate (yeast extract) was a more favourable supplement for growth of the isolates, compared to the reduced inorganic sulphur compound (tetrathionate).

Table 4.1 : Cultures doubling times (td) of iron oxidation by Gram-positive Montserrat isolates grown in various media. Results are mean values ± standard deviation (n = 4)

Bacteria / Medium	Mean generation time, t_d (h)		
	Fe^{2+}	Fe^{2+} / YE	Fe^{2+}/ Tetrathionate
RIV-14	n.d	7.08 ± 0.56 *a*	18.9 ± 1.7 *b*
L-15	n.d	7.33 ± 0.25 *a*	19.02 ± 2.66 *b*

n.d. : doubling time was not determined due to the incomplete oxidation of ferrous iron by the bacteria.
a, *b*,: values not followed by the same letters are significantly different ($P < 0.01$)

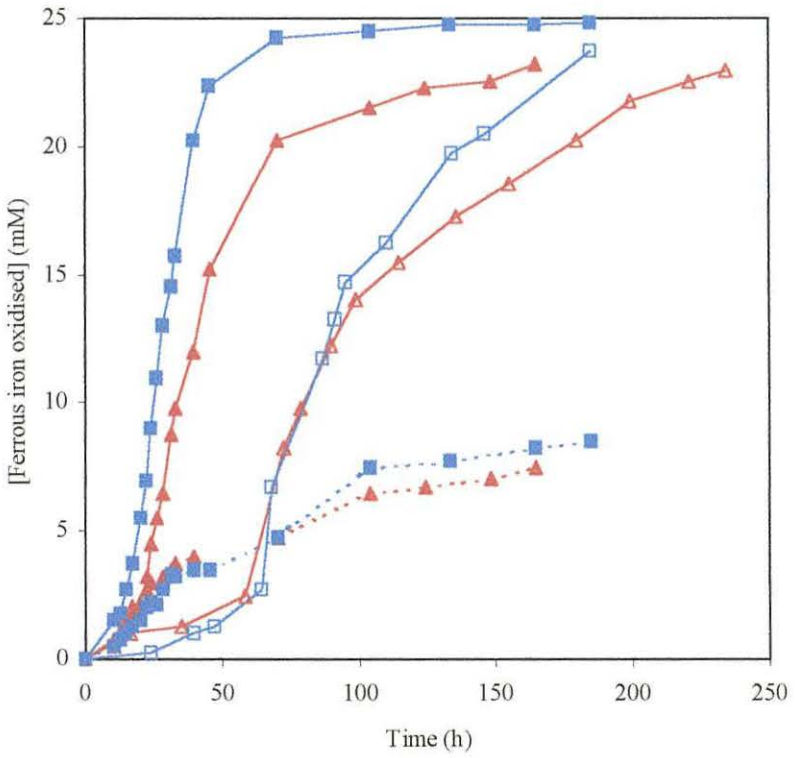


Fig. 4.3: Effects of the addition of 0.02% (w/v) yeast extract (solid symbols) or 2.5 mM tetrathionate (opened symbols) in 25 mM ferrous iron (dotted lines) medium on the oxidation of ferrous iron by RIV-14 (▲△) and L-15 (■□).

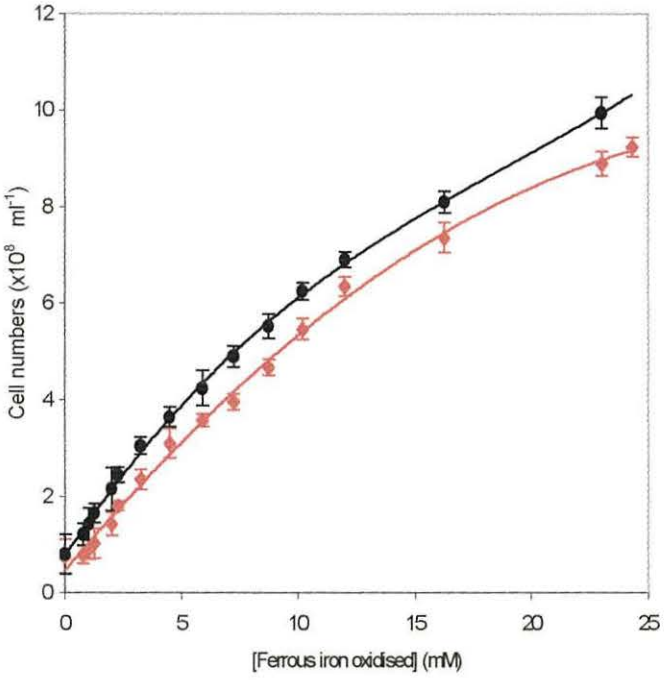


Fig 4.4: Relationship between growth and iron oxidation by isolates RIV-14 (◆) and L-15 (●) grown in 30 mM ferrous sulphate/yeast extract medium, pH 1.9, at 30°C.

4.3.2 Effect of tetrathionate on ferrous iron oxidation

4.3.2.1 Methodology

Pure cultures of RIV-14 and L-15 were grown in 100 ml of sterile 10 mM ferrous iron medium (section 2.2.1.1) pH 1.8, supplemented with 0.02% (w/v) yeast extract at 30°C with shaking (150 r.p.m.), in 250 ml shake flasks. The cells were harvested at late exponential phase by centrifugation (10,000 r.p.m., 15 minutes at 4°C), rinsed twice and finally resuspended in 5 ml of sterile pH 2.0 basal salts (section 2.1.1). The cell suspensions were then inoculated (2%) into duplicate 250 ml Erlenmeyer flasks containing sterile 20 mM ferrous iron medium, supplemented with various concentrations of potassium tetrathionate. The final concentrations of tetrathionate were: 0.001, 0.01, 0.1, 1 and 2 mM. Control cultures containing 20 mM ferrous iron alone were also prepared. The cultures were incubated with shaking (150 r.p.m.) at 30°C.

Samples were removed regularly and concentrations of ferrous iron monitored titrimetrically using ceric sulphate (section 2.6.1.2). From semi-logarithmic plots of ferrous iron oxidised against time, culture doubling times at various concentration of tetrathionate were determined.

4.3.2.2 Results

The requirement, of both isolates, for tetrathionate to promote autotrophic growth on ferrous iron was confirmed. The current experiment showed that the effect of different tetrathionate concentrations on iron-oxidation by both RIV-14 and L-15 was very similar (Fig 4.5). A minimum concentration of 10μm was sufficient to promote complete oxidation of 25 mM ferrous iron. However, oxidation of ferrous iron was found to be highly stimulated at concentrations of ≥ 0.1 mM tetrathionate, as indicated by the significant decreases ($P<0.01$, respectively) in culture doubling times, compared with the minimum concentration of 10 μM tetrathionate (Table 4.2).

Table 4.2 : Effects of tetrathionate concentration on cultures doubling times (t_d) on the oxidation of 25 mM ferrous iron by isolates RIV-14 and L-15. The values indicated are from duplicate experiments.

	Mean culture doubling times, t_d (h)	
Tetrathionate (mM)	RIV-14	L-15
0.001	n.d	n.d
0.01	27.6 ± 1.82 *a*	24.8 ± 0.45 *b*
0.1	18.2 ± 0.73 *c*	15.1 ± 2.05 *d*
1.0	15.6 ± 0.73 *d*	11.7 ± 1.01 *e*
2.0	12.8 ± 0.85 *e*	11.5 ± 0.95 *e*

n.d: culture doubling time was not determined due to the limited oxidation of ferrous iron displayed with both isolates.
a - *e*,: values not followed by the same letter are significantly different ($P < 0.01$)

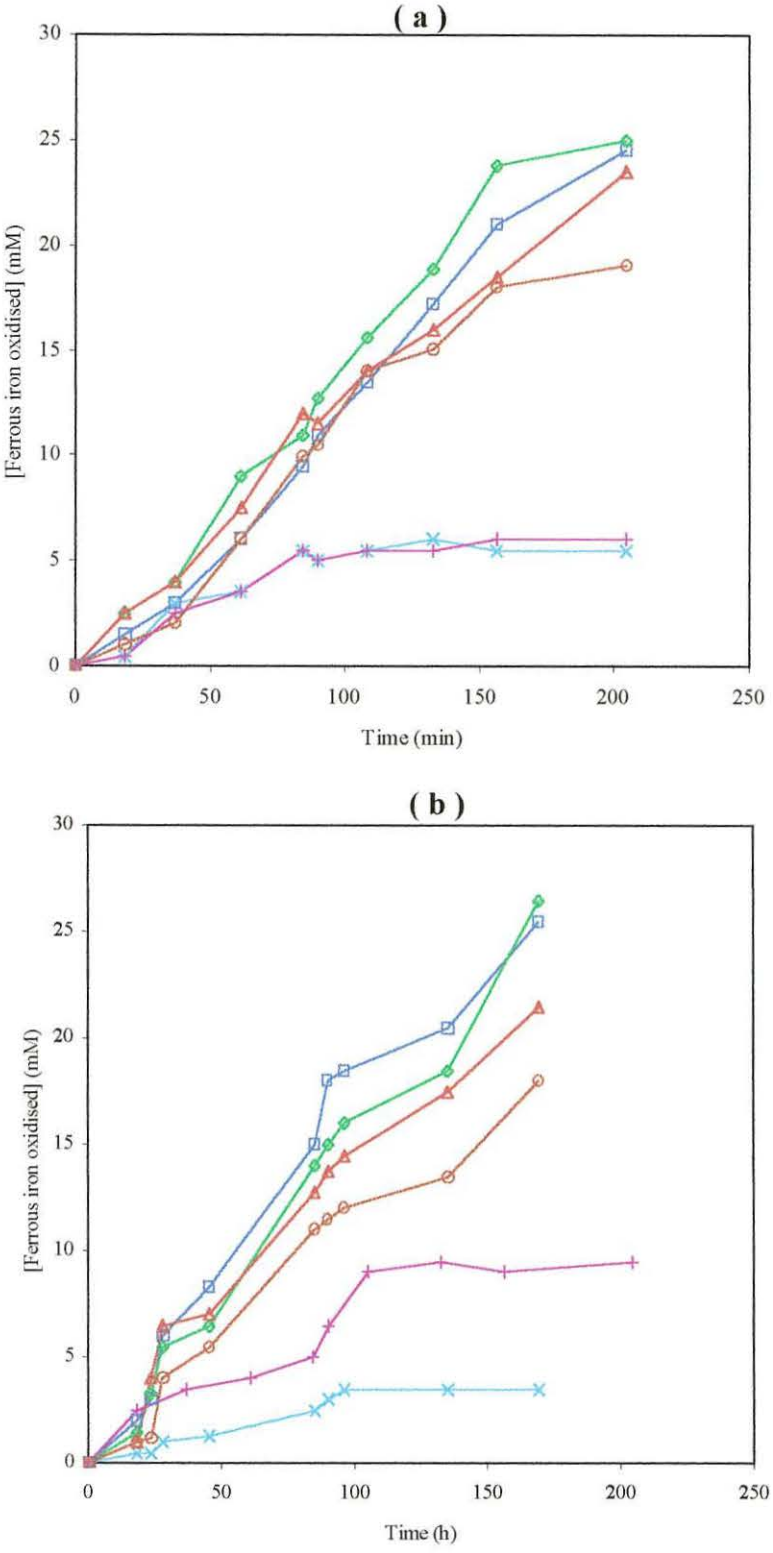


Fig. 4.5: Ferrous iron oxidation by (a) RIV-14 and (b) L-15, grown in medium containing 25 mM ferrous sulphate (×) or the same medium amended with 0.001 mM (+), 0.01 mM (○), 0.1 mM (△), 1 mM (□) and 2 mM (◇) of potassium tetrathionate.

4.3.3 Reduction of ferric iron

This experiment was designed to test the ability of the mesophilic, Gram-positive Montserrat isolates to use soluble ferric iron as an alternative electron acceptor to oxygen. Ferric iron reduction was tested under conditions where the bacteria were grown anaerobically in media supplemented with either an organic (glycerol) or an inorganic (tetrathionate) electron donor

4.3.3.1 Methodology

Isolates RIV-14 and L-15 were inoculated (from yeast extract-grown cultures) into sterile 50 ml medium containing 10mM glycerol/10 mM ferrous sulphate/0.5 mM potassium tetrathionate (section 2.2.1.3) or 10 mM ferrous sulphate/5 mM potassium tetrathionate (section 2.2.1.1) adjusted at pH 1.8, in 100 ml shake flasks. Cultures were incubated with shaking (120 r.p.m.) at 30°C.

Bacteria grown in glycerol/ferrous iron/tetrathionate and ferrous iron/tetrathionate media were harvested by centrifugation (10,000 r.p.m., for 15 minutes at 4°C), rinsed twice with sterile pH 2.0 basal salts (section 2.2.1.3) and finally resuspended in basal salts solution. The cell suspensions were then inoculated (1% v/v; in duplicate) in universal bottles containing sterile 20 ml basal salts, pH 1.8, supplemented with different concentrations of ferric iron, and amended with either 10 mM glycerol and 0.5 mM tetrathionate, or 5 mM tetrathionate alone, to provide heterotrophic and autotrophic growth conditions, respectively. The concentration of ferric iron added was 0, 5, 10, 15 and 25 mM, from a filter-sterilised 1 M, pH 1.7 stock solution. In order to establish strict anaerobic conditions, the iron-free medium was deoxygenated with nitrogen gas (N_2), prior to autoclaving. A portable oxygen meter was used to indicate the level of oxygen during N_2-sparging. Control cultures containing either 25 mM ferrous iron or no added iron were also prepared. Cultures were incubated under anaerobic conditions (in Oxoid

anaerobic jars) at 30°C, until the ferric iron was completely reduced as indicated by the bleaching of these cultures.

Reduction of ferric iron was determined by measuring concentrations of ferrous iron at the end of the incubation period using the ferrozine assay (section 2.6.1.3). Microbial biomass was determined by measuring the optical densities at 600 nm of harvested and resuspended cells as well as by cell counting using the Thoma counting chamber (section 2.3.2.2).

4.3.3.2 Results

Under strictly anaerobic conditions, both acidophilic iron-oxidising isolates, RIV-14 and L-15, were found to be capable of reducing ferric iron, using either glycerol or tetrathionate as electron donor (Fig. 4.6). Evidence of ferric iron reduction was more pronounced in the heterotrophic cultures containing glycerol where similar reducing capacity was displayed by both bacteria (all of the 25 mM ferric iron was reduced to ferrous iron; Fig. 4.6a). However more limited iron reduction was observed in autotrophic cultures, where tetrathionate was the electron donor. This was particularly the case with isolate L-15, where less than 10 mM ferric iron was reduced compared with *ca.* 14 mM ferric iron reduced with strain RIV-14, during the 3 week incubation period (Fig. 4.6b). No ferric iron reduction (< 2 mM) was detected in cell-free control cultures.

Biomass yields, measured either as optical densities of resuspended bacteria or from cell counts, were directly correlated with the amount of ferric iron reduced. Growth of isolates in either glycerol- or tetrathionate-containing medium was confirmed (Thoma cell counts), though less was detected in the latter. These gave definitive evidence that ferric iron reduction could support the growth of the Gram-positive mesophilic, iron-oxidising Montserrat isolates under anaerobic conditions. The biomass yields in the anaerobic cultures (autotrophic and heterotrophic) of L-15 which contained 25 mM

ferrous iron were similar to those obtained in control cultures to which no iron had been added. However, RIV-14 produced greater cell numbers (almost equivalent to the culture with 10 mM ferric iron; 1.2 x 10^8 cell/ml) in autotrophic cultures to which 25 mM ferrous sulphate was added than in those containing no added iron (7.8 x 10^6 cells/ml). There was no evidence of ferrous iron oxidation in these cultures, confirming that anaerobic growth conditions were maintained throughout the experiment.

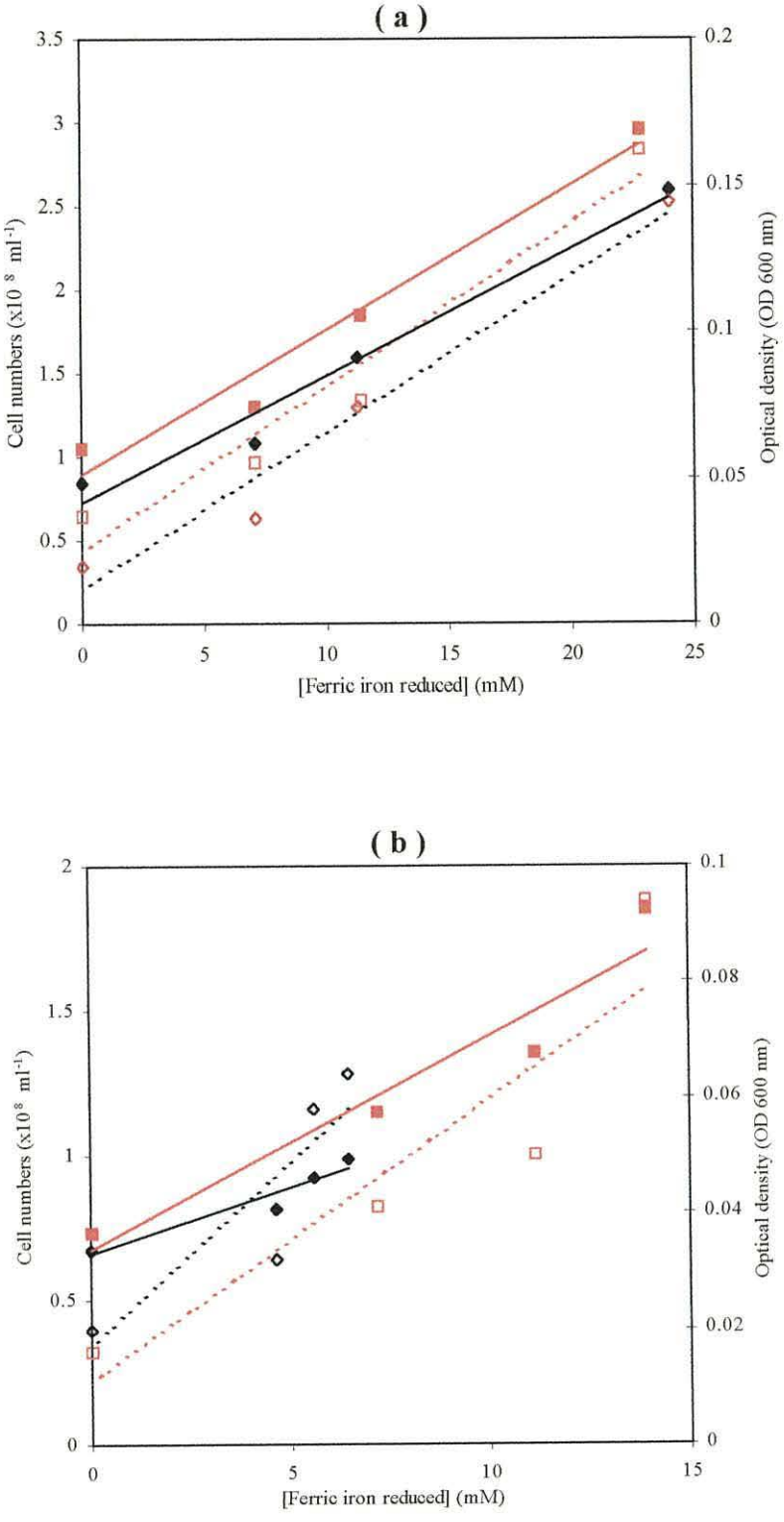


Fig. 4.6: Ferric iron reduction by RIV-14 (■□) and L-15 (◆◇), grown anaerobically in medium containing various concentrations of ferric iron, amended with either (a) 10 mM glycerol + 0.5 mM tetrathionate or (b) 5 mM tetrathionate. Cell counts are shown as solid lines/solid symbols, and optical density measurements as broken lines/open symbols

4.3.4 Specific rates of iron oxidation

4.3.4.1 Methodology

The specific rates of iron oxidation by the Gram-positive Montserrat isolates were evaluated using a variety of media which supporting their growth. Pure cultures of isolates RIV-14 and L-15 were grown in 250 ml Erlenmeyer flasks containing 100 ml of sterile 20 mM ferrous iron / 0.02% (w/v) yeast extract medium (section 2.2.1.1); pH 1.8 with shaking (150 r.p.m.) at 30°C. In the late exponential growth phase, cells were harvested by centrifugation (10,000 r.p.m., for 15 minutes at 4°C), washed twice with sterile pH 2.0 (with H_2SO_4) distilled water and finally resuspended in 30 ml of sterile, acidified distilled water.

The cell suspensions were then inoculated (1% v/v) into: (a) 2 l shake flasks containing 1 l of sterile 25 mM ferrous iron medium (section 2.2.1.1), supplemented with either (i) 0.02% (w/v) yeast extract, or (ii) 10 mM glycerol plus 0.5 mM tetrathionate, or (iii) 2.5 mM tetrathionate, pH 1.5; (b) 500 ml Duran bottles containing 500 ml medium (pH 1.5) amended with 25 mM ferric iron, 10 mM glycerol and 0.5 mM tetrathionate; this medium was deoxygenated with nitrogen before autoclaving. Cultures were incubated at 35° (RIV-14) and 37°C (L-15) with or without shaking (150 r.p.m.) for cultures grown on ferrous and ferric iron, respectively. Cells were harvested at the mid-exponential growth phase (or when complete reduction of ferric iron was observed) by centrifugation (10,000 r.p.m., for 15 minutes at 4°C), rinsed, and finally resuspended in a volume of sterile pH 1.5 basal salts (section 2.2.1). The cell suspensions contained $10^9 - 10^{10}$ bacterial/ml.

The specific rates of iron oxidation were measured in 10 ml aliquots of 'reaction mixture' (in triplicate), which comprised 5% (v/v) inoculum, basal salts / trace elements (section 2.2.1.1) and 5 mM ferrous sulphate as the substrate (pH 1.5). The inoculated basal salts solutions were equilibrated in a water bath set at the respective temperature

optima (35°C for RIV-14 and 37°C for L-15, as described in section 4.6.1.2) prior to addition of the substrate (ferrous iron). Cultures were incubated with continuous aeration throughout the experimental period. Samples were removed as soon after the addition of ferrous iron and regularly every 5 to 10 minutes interval, for up to 60 minutes. Concentrations of ferrous iron were measured using the ferrozine assay (section 2.6.1.3) and the amount of protein in the cell suspensions was determined using the Bradford assay (section 2.6.5).

4.3.4.2 Results

In these experiment, linear relationships between ferrous iron oxidised and time were observed with both Montserrat isolates with time were observed (Fig. 4.7a and 4.7b). In some cases lag periods were detected before iron oxidation commenced (Fig. 4.8c and 4.8d).

Specific iron oxidation rates of the Gram-positive iron-oxidisers grown under different conditions are given in Table 4.3, together with those of previously studied strains of the heterotrophic iron-oxidising mesophiles "*Ferromicrobium acidophilum*" (T23) and "*Alicyclobacillus*"-like strain SLC2, and the thermoacidophilic *Sulfobacillus* sp. (strain YTF1; Bridge, 1995) and *S. thermosulfidooxidans* (strain TH1; Nicolau, 1996). In general, the specific rates of iron oxidation by both isolates RIV-14 and L-15 grown aerobically were significantly greater ($P<0.01$ or $P<0.001$) than when the cells were grown anaerobically. Similar iron-oxidising activity was found for both isolates when grown aerobically on ferrous iron/tetrathionate medium. However, L-15 differed from RIV-14 in displaying significantly higher rates of specific iron oxidation when grown aerobically in ferrous iron / yeast extract medium. Anaerobically-grown RIV-14 and L-15 displayed very low rates of iron-oxidation compared with aerobically-grown

cultures. Similar results were also found with *Sulfobacillus* YTF-1 grown aerobically and microaerobically on ferric iron (Bridge, 1995).

The effects of various growth media on the specific iron oxidation rates were similar for both isolates. Significantly greater specific iron oxidation rates were observed in cells grown autotrophically on ferrous iron with tetrathionate than for those grown on ferrous iron in the presence of an organic substrate (either glycerol or yeast extract). The specific iron oxidation rates of both RIV-14 and L-15 grown aerobically in the presence of either organic substrate was greater than those of the heterotrophic iron oxidisers (T23 and SLC2) and the moderately thermophilic *Sulfobacillus* spp. (YTF1 and TH3) (Bridge, 1995; Bacelar-Nicolau, 1996) though the experiment was carried out under slightly different growth conditions (see Table 4.3). However, the effects of oxygen on the specific rates of iron oxidation in the Gram-positive Montserrat isolates were similar to those noted for the previously studied heterotrophic / mixotrophic iron-oxidisers.

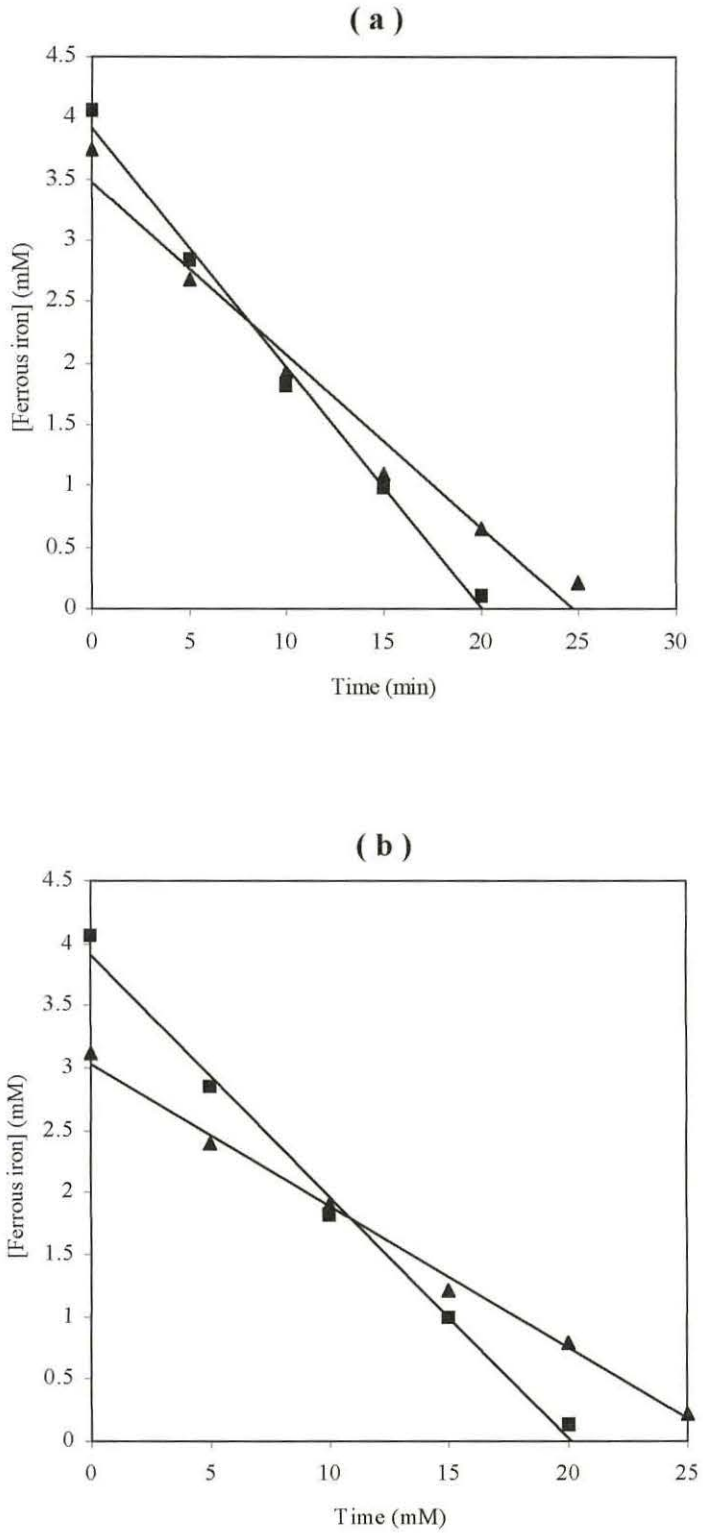


Fig. 4.7: Specific rates of ferrous iron oxidation by RIV-14 (▲) and L-15 (■), grown aerobically in 25 mM ferrous sulphate medium supplemented with either (a) 0.02% (w/v) yeast extract or (b) 2.5 mM tetrathionate. Experiments were carried out in triplicate of 10 ml reaction mixtures, with the cell suspension of either RIV-14 or L-15 containing: (a) 0.044 and 0.020 or (b) 0.013 and 0.015 mg protein, respectively.

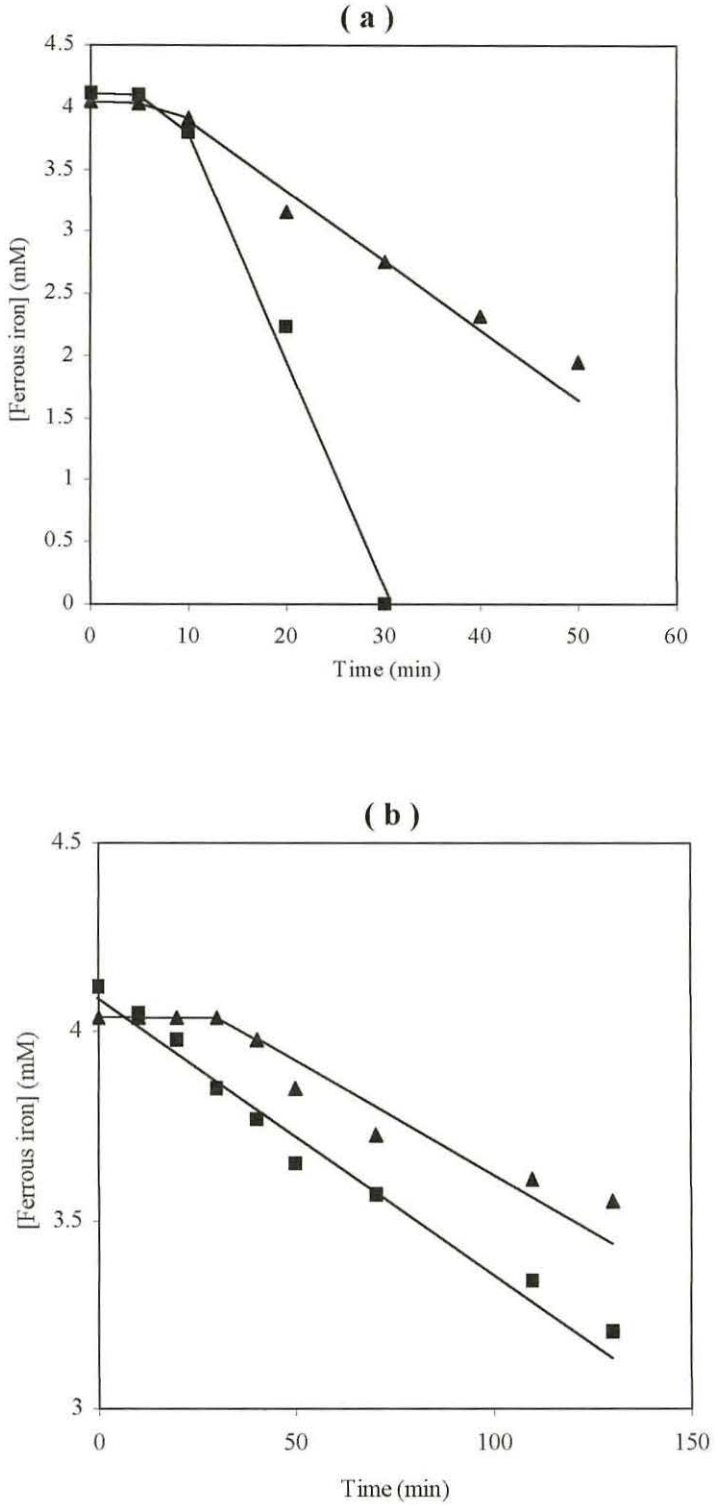


Fig. 4.8: Specific rates of ferrous iron oxidation by RIV-14 (▲) and L-15 (■), grown aerobically in medium supplemented with (a) 25 mM ferrous sulphate + 10 mM glycerol + 0.5 mM tetrathionate or anaerobically in medium containing (b) 25 mM ferric sulphate + 10 mM glycerol + 0.5 mM tetrathionate. Experiments were carried out in triplicate 10 ml reaction mixtures, with the cell suspension of either RIV-14 or L-15 containing: (a) 0.057 and 0.048 or (b) 0.060 and 0.056 mg protein, respectively.

Table 4.3: Specific rates of iron oxidation by Gram-positive iron-oxidising mesophilic isolates in comparison with that of heterotrophic iron-oxidising mesophiles and moderately thermophilic *Sulfobacillus* spp.. Isolates RIV-14 and L-15 were grown in various media containing Fe^{2+} (under aerobic conditions) and Fe^{3+} (under anaerobic conditions). The effect of growth conditions on the iron-oxidising activity were assessed in 3 replicate experiments.

Bacteria	Growth conditions	Specific rates (μmoles Fe oxidised/minute/mg protein)
RIV-14	Fe^{2+} / tetrathionate (aerobic)	147 ± 7.64 *a*
	Fe^{2+} / YE (aerobic)	29.3 ± 2.61 *b*
	Fe^{2+} / glycerol / tetrathionate (aerobic)	7.61 ± 0.21 *d*
	Fe^{3+} / glycerol / tetrathionate (anaerobic)	0.67 ± 0.06 *e*
L-15	Fe^{2+} / tetrathionate (aerobic)	137 ± 7.64 *a*
	Fe^{2+} / YE (aerobic)	105 ± 5.72 *c*
	Fe^{2+} / glycerol / tetrathionate (aerobic)	28.0 ± 4.59 *d*
	Fe^{3+} / glycerol / tetrathionate (anaerobic)	1.39 ± 0.19 *e*
"*Alicyclobacillus*"-like sp. (strain SLC2)[(a)]	25 mM Fe^{2+} (aerobic)	0.94 ± 0.07
	1 mM Fe^{3+} (microaerobic)	0.20 ± 0.10
"*F. acidophilum*" [(a)] (strain T-23)	25 mM Fe^{2+} (aerobic)	3.85 ± 0.25
	1 mM Fe^{3+} (microaerobic)	0.98 ± 0.23
S. thermosulfidooxidans[(a)] (strain TH1)	50 mM Fe^{2+}(aerobic)	2.06 ± 0.91
Sulfobacillus sp. (strain YTF1) [(b)]	Fe^{2+}/ glycerol / YE (aerobic)	10.14 ± 4.53
	Fe^{3+} / glycerol / YE (microaerobic)	6.79 ± 1.66

[(a)]: Bacelar-Nicolau, (1996); specific iron oxidation rates were determined at pH 2.0, with cells grown in Fe / 0.02% (w/v) yeast extract / 10 mM glycerol medium, incubated aerobically with Fe^{2+} or microaerobically with Fe^{3+} at 30°C

[(b)]: Bridge, (1995): specific rates of iron reduction were determined with cells grown under oxidising and reducing conditions in medium containing yeast extract/glycerol with either ferrous or ferric iron, respectively

a - *e* : values not followed by the same letter are significantly different ($P < 0.001$)

4.3.5 Specific rates of iron reduction

4.3.5.1 Methodology

Bacteria were grown in 100 ml shake flasks containing 50 ml heterotrophic basal salts (section 2.2.1.3); pH 1.8 supplemented with 10 mM glycerol, 1 mM tetrathionate and 10 mM ferrous iron and incubated at 35°C with shaking (120 r.p.m.). Cells were harvested at the late exponential phase by centrifugation (10,000 r.p.m. for 15 minutes at 4°C) and resuspended in 10 ml of sterile heterotrophic basal salts (section 2.2.1.3), pH 1.8. Whole cell suspensions of RIV-14 and L-15 were then inoculated into: (a) 500 ml Duran bottles, containing sterile 500 ml heterotrophic basal salts (pH 1.5) supplemented with 25 mM ferric iron, 10 mM glycerol and 0.5 mM tetrathionate; the medium was deoxygenated with nitrogen (N_2) prior to autoclaving. (b) 1 litre Erlenmeyer flasks containing 500 ml heterotrophic basal salts (pH 1.5) supplemented with 25 mM ferrous iron, 10 mM glycerol and 0.5 mM tetrathionate. Cultures were incubated at 35°C (RIV-14) and 37°C (L-15) with or without shaking (150 r.p.m) for cultures grown with ferrous or ferric iron, respectively. Bacteria were harvested at late exponential phase, or when complete reduction of ferric iron was observed, by centrifugation (10,000 r.p.m. for 15 minutes at 4°C), and resuspended in a volume of sterile pH 1.5 heterotrophic basal salts. Bacterial numbers in the cell suspensions estimated using the Thoma counting chamber, and were around 10^9 to 10^{10} cells/ml.

This experiment was carried out in triplicate 30 ml serum bottles containing 25 ml of pH 1.5 heterotrophic basal salts, initially gassed with nitrogen, sealed with tightly fitting rubber stoppers and secured with metal caps, prior to autoclaving. Cell suspensions of RIV-14 and L-15 were then inoculated (10 % v/v) into the serum bottles and equilibrated at the respective temperature optima before 5 mM ferric sulphate and 10 mM glycerol were added. Reduction of ferric iron was monitored by measuring

concentrations of ferrous iron using the ferrozine assay (section 2.6.1.3). Analyses of protein in the cell suspensions was determined using the Bradford assay (section 2.6.5).

4.3.5.2 Results

Ferric iron reduction by anaerobic cell suspensions of RIV14 and L-14 are shown in Fig 4.9. Similar rates of iron reduction by RIV-14 and L-15, were observed (Table 4.4). The data indicate that enhanced Fe^{3+} reduction activity occurred with cells grown anaerobically. However, reasonable rates of iron reduction were also detected with cells grown under oxidising condition, though these were significantly less than those for cells grown under reducing conditions ($P<0.001$). In contrast to these results, other studies have found that specific ferric iron reduction by the moderately thermophilic iron-oxidiser *Sulfobacillus* YTF1 was apparently lowered when cells were grown under reducing conditions (Bridge, 1995). Specific rates of ferric iron reduction for aerobically grown *Sulfobacillus* YTF1 was similar to that of the Montserrat isolates, particularly isolate L-15.

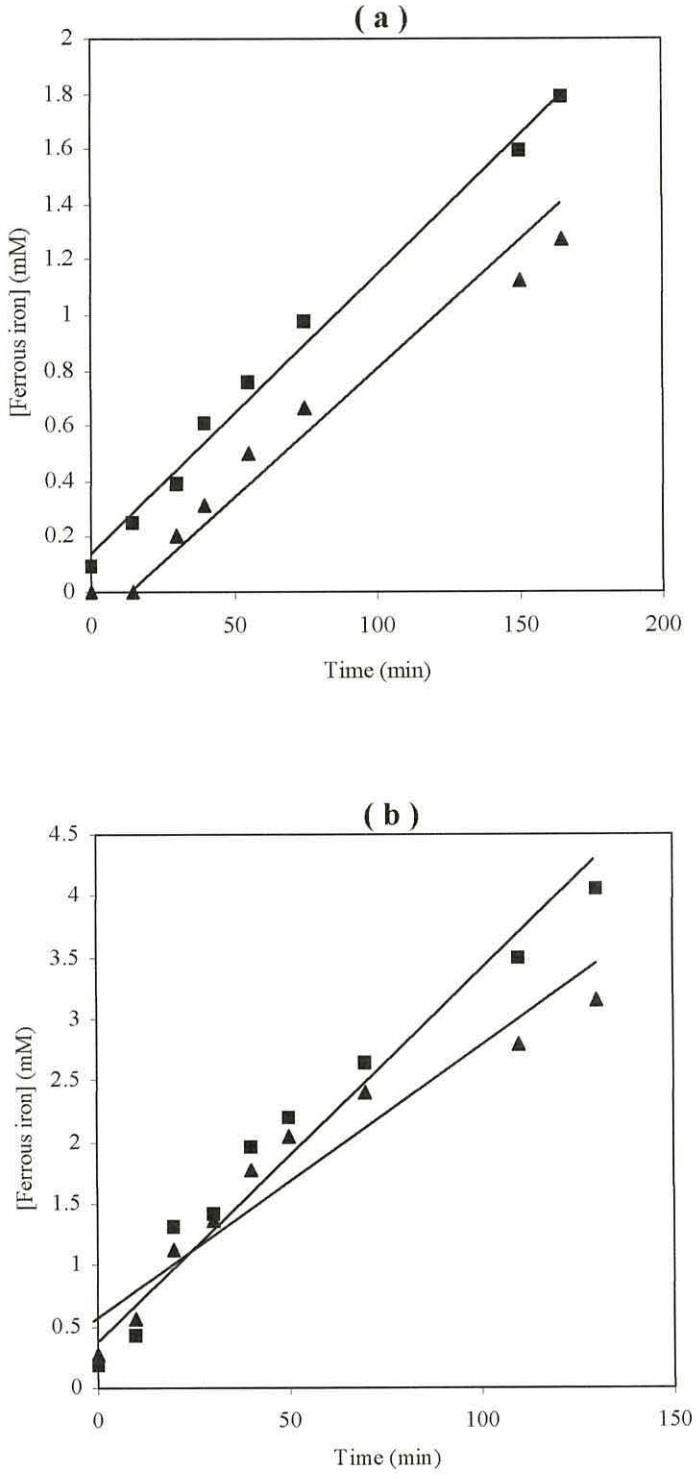


Fig. 4.9: Specific rates of ferric iron reduction by RIV-14 (▲) and L-15 (■), grown (a) aerobically in medium containing 25 mM ferrous sulphate + 10 mM glycerol + 0.5 mM tetrathionate and (b) anaerobically in 25 mM ferric sulphate + 10 mM glycerol + 0.5 mM tetrathionate.

Table 4.4: Specific rates of ferric iron reduction by Gram-positive iron-oxidising mesophilic isolates compared with the moderate thermophile *Sulfobacillus* YTF1. Isolates RIV-14 and L-15 were grown aerobically in 10 mM glycerol/0.5 mM tetrathionate medium containing 25 mM Fe^{2+} and anaerobically in a similar medium containing 25 mM Fe^{3+}. Specific rates were determined from 3 replicates (mean ± SD).

Bacteria	Growth conditions	Specific rates (μmoles Fe oxidise/minute/mg protein)
RIV-14	Fe^{2+} / glycerol / tetrathionate (aerobic)	2.49 ± 0.21 *a*
	Fe^{3+} / glycerol / tetrathionate (anaerobic)	7.14 ± 0.60 *b*
L-15	Fe^{2+} / glycerol / tetrathionate (aerobic)	3.43 ± 0.37 *a*
	Fe^{3+} / glycerol / tetrathionate (anaerobic)	7.21 ± 0.68 *b*
Sulfobacillus sp. (strain YTF1)[(a)]	Fe^{2+} / YE / glycerol (aerobic)	3.50 ± 1.66
	Fe^{3+} / YE / glycerol (microaerobic)	0.57 ± 0.27

[(a)]: Bridge, (1995): specific rates of iron reduction was determined with cells grown under oxidising and reducing conditions in medium containing yeast extract/glycerol with either ferrous or ferric iron, respectively

a, b: values not followed by the same letter are significantly different ($P < 0.01$)

4.4 Elemental sulphur oxidation by Gram-positive Montserrat isolates

4.4.1 Methodology

Isolates RIV-14 and L-15 were grown in 10 mM ferrous sulphate / 0.02 % (w/v) yeast extract (section 2.2.1.1) medium, pH 1.9, and cells were harvested at the late exponential phase by centrifugation (10,000 r.p.m. for 15 minutes at 4°C), rinsed twice and finally resuspended in 5 ml of sterile, pH 2.0 distilled water. The cell suspensions were then inoculated (1% v/v) into duplicate 100 ml shake flasks containing 50 ml of medium (section 2.2.1.2), initially set at pH 3.0, and supplemented with approximately 1.6 g/l flowable elemental sulphur (section 2.2.1.2), or with 1.6 g/l sulphur plus 0.02% (w/v) yeast extract. The effects of ferrous iron on bacterial sulphur oxidation were also tested by growing the bacteria in the sulphur medium supplemented with 1, 2, 5 and 10 mM ferrous sulphate. Cultures were grown in a shaking incubator, at 30°C. Samples

were removed regularly and sulphur oxidation was monitored by measuring the reduction of pH throughout the experimental period. Uninoculated media were also prepared and run simultaneously as controls to monitor any abiotic sulphur oxidation in the presence and absence of yeast extract. The purity of the cultures was checked at the end of the experiment by streaking onto ferrous iron/tetrathionate overlay plates (section 2.2.2.2). Growth of the bacteria was confirmed by Thoma cell counts (section 2.3.2.2).

4.4.2 Results

In contrast to the Gram-negative Montserrat isolates (section 3.4.1), the Gram-positive iron-oxidising acidophilic isolates RIV-14 and L-15 displayed only limited oxidation of elemental sulphur in either yeast extract-amended or non-amended media (Fig. 4.10). However, both isolates showed greater sulphur oxidation in the yeast extract-amended medium, particularly isolate RIV-14, though at a very slow rate. Negligible sulphur oxidation was detected in the autotrophic (organic-free) cultures where pH reduction was only ~0.2 units (from pH 2.98 to 2.75) throughout the experimental period (*ca.* 16 days), which was similar to that of the control (uninoculated) medium. Addition of various concentration of ferrous sulphate to the organic-free, sulphur-containing medium did not stimulate sulphur oxidation activity by either isolate. Similar patterns were displayed with those grown in the absence of ferrous iron (data not shown). Phase-contrast observation confirmed that both isolates had grown in the yeast extract-containing medium (final cell numbers, 9.6×10^7 cell/ml) whereas in yeast extract-free medium, cells were highly sporulated (inactive) and had not increased in numbers (i.e. were similar to the number of cells at time zero, $\sim 2 \times 10^7$ cell/ml).

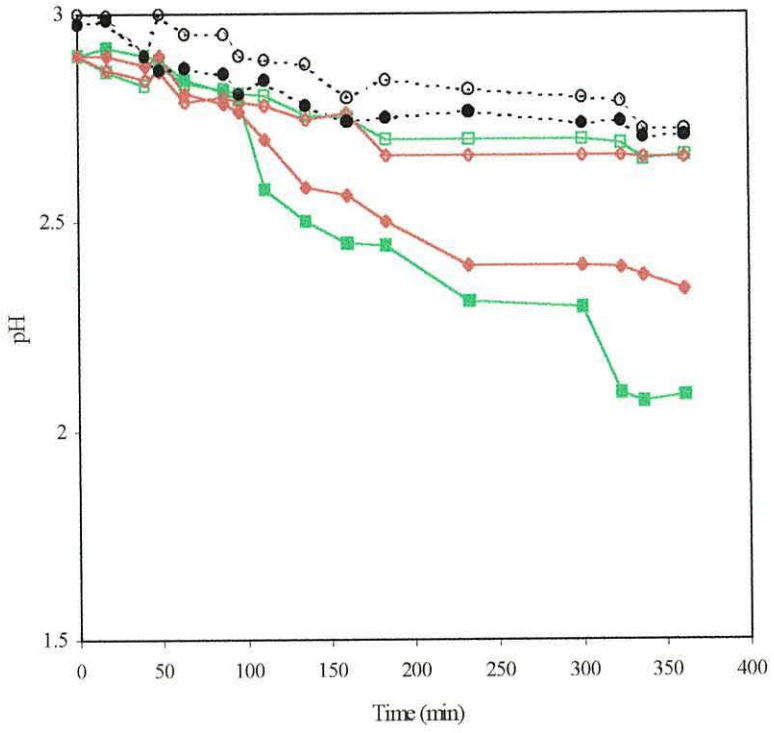


Fig. 4.10: Elemental sulphur oxidation by RIV-14 (■□) and L-15 (◆◇) in the media containing 1.6 g/l elemental sulphur (FS°) initially adjusted at pH 3.0, amended (solid symbols) or not (opened symbols) with 0.02% (w/v) yeast extract. Uninoculated control cultures are also showed (●○; broken lines)

4.5 Utilisation of organic compounds

From their initial isolation, the Gram-positive mesophilic isolates, RIV-14 and L-15 had been routinely maintained and grown in yeast extract-containing ferrous sulphate medium, which was found to be a satisfactory growth medium for the bacteria. It had been found (section 4.3.3.1) that the isolates were able to use glycerol in place of yeast extract, but the addition of a reduced sulphur compound (such as tetrathionate) was then essential. It was of interest to test whether other organic compounds could be metabolised by these bacteria.

4.5.1 Growth on various defined organic substrates

4.5.1.1 Methodology

Isolates RIV-14 and L-15 were grown in 10 mM glycerol medium (section 2.2.1.3) supplemented with 5 mM ferrous sulphate and 0.5 mM potassium tetrathionate, pH 1.8 at 30°C. Bacteria were harvested by centrifugation (10,000 r.p.m. for 15 minutes at 4°C) washed, and resuspended in pH 2.0 basal salts solution (section 2.2.1.3). The cells were then inoculated (2% v/v) into 5 mM ferrous iron / 0.5 mM tetrathionate liquid medium (section 2.2.1.3), pH 1.8, supplemented with one of the following organic substrates: (i) Sugars (at 5 mM final concentration of each): glucose, fructose, ribose, arabinose, mannose, xylose galactose, glucosamine, glucuronic acid and galacturonic acid. (ii) Alcohols: glycerol (10mM), mannitol (5mM), ethanol (15 mM) and methanol (20 mM), (iii) Organic acids: citric acid, acetic acid, succinic acid, pyruvic acid and glutamic acid (10 mM final concentration except for glutamic acid, added at 5mM) (iv) Others: phenol (1 mM), benzyl alcohol (1 mM), and glycine (10mM). The concentration of each organic substrate added was calculated to give similar amounts of organic carbon in the complete growth media. Various concentrations of acetic acid were also used (0.25, 0.5, 0.1 and 1 mM) in this study. Growth on organic acids was also tested in media initially adjusted to pH 2.25.

Two control cultures prepared were; (i) 5 mM ferrous sulphate + 0.5 mM tetrathionate (base-line control) and (ii) 5 mM ferrous sulphate + 0.5 mM tetrathionate + 10 mM glycerol (positive control). All organic acids were added as their sodium salts, though at the media pH in which the experiments were conducted these would be present in their undissociated forms, and therefore will be referred to through this chapter as acids (e.g. acetic acid, citric acid, etc.). All cultures were set up in replicate universal bottles, containing 5 ml of medium, which were incubated at 30°C, shaken (150 r.p.m.) for up to 10 days. Growth was assessed by measuring the optical densities at 600nm.

One millilitre culture aliquots were centrifuged (10,000 r.p.m. for 5 minutes) and the resulting pellets were resuspended in the same volume of distilled water prior to recording optical densities.

4.5.1.2 Results

Growth of RIV-14 and L-15 on a variety of single organic compounds is shown in Table 4.5. Bacterial growth was recorded semi-quantitatively, based on the comparison between cultures optical densities (ODs) and ODs of the base-line control cultures (in Fe^{2+}/tetrathionate medium; 0.015 for RIV-14 and 0.019 for L-15) as: (i) +: ODs were 25% more than the base-line controls, (ii) ++: ODs were 0.025 units more than the base-line controls, (iii) +++: ODs were 0.1 units more than the base-line controls (iv) I: growth was inhibited by the substrate, indicated by a decrease in ODs of 25% lower than the base-line controls, and (v) -: no growth was observed, indicated by less than 25% increase or decrease of cultures ODs compared to the base-line controls. In general, isolate L-15 displayed better heterotrophic growth than isolate RIV-14, which was indicated by the ability of the former to growth on a greater variety of organic substrates and often to achieve higher cell densities.

None of the organic substrates used was found to be at an inhibitory concentration for isolate L-15, but growth of RIV-14 was inhibited by the presence of 5mM arabinose and 10 mM of either acetic or succinic acid. Isolate RIV-14 was found to be more sensitive to organic acids, in that 1 mM acetic acid was enough to suppress its growth, though it grew on acetic acid at concentrations between 0.1 – 0.25 mM. Glycerol supported the growth of RIV-14 to a greater extent than the other organic substrates which gave positive results. It was also found that growth of isolate L-15 on organic acids was more successful in media poised at pH 2.25 than at pH 1.8, though this was not the case with RIV-14.

Table 4.5: Utilisation of defined organic compounds by the Gram-positive iron-oxidising Montserrat isolates, in media supplemented with 5 mM ferrous sulphate and 0.5 mM tetrathionate.

Substrates	Isolates	
	RIV-14	L-15
i) Sugars		
Glucose	+	+++
Fructose	-	+
Ribose	+	++
Arabinose	I	-
Mannose	+	+
Xylose	-	+
Galactose	-	+
Glucosamine	-	+
Glucuronic acid	-	-
Galacturonic acid	-	-
ii) Alcohols		
Glycerol	+++	+++
Mannitol	-	+
Ethanol	-	-
Methanol	-	+
iii) Acids		
Citric acid	++	++
Acetic acid: 10mM	I	- / (+)
0.1 mM	+	+
0.25 mM	+	+
0.5 mM	+	+
1.0 mM	-	+
Succinic acid	I	- / (+)
Pyruvic acid	-	- / (++)
Glutamic acid	++	+ / (++)
iv) Others		
Benzyl alcohol	-	-
Phenol	-	+
Glycine	-	+

+++ : OD 0.1 unit > base-line control
++ : OD 0.025 unit > base-line control
+ : OD 25% > base-line control
- : OD < 25% greater or less than the base-line control
I : Inhibitory ; OD > 25% lower than the base-line control
'( )' : growth evaluated in the media initially set at pH 2.25

4.5.2 Growth in glycerol medium

4.5.2.1 Methodology

Isolates RIV-14 and L-15 were grown in 100 ml shake flasks containing 50 ml of heterotrophic basal salts (section 2.2.1.3), pH 1.8 to which 10 mM glycerol, 5 mM ferrous sulphate and 0.5 mM tetrathionate was added. The cultures were incubated at 30°C, shaken at 120 r.p.m. When grown, the bacteria were inoculated (2% v/v) into duplicate 250 ml Erlenmeyer flasks containing 100 ml of the heterotrophic basal salts (pH 1.8), supplemented with 10 mM glycerol, 0.5 mM tetrathionate and various concentrations of ferrous sulphate (1, 5 and 10 mM) or 10 mM ferric sulphate in place of ferrous sulphate. Bacteria were also grown in medium containing 1 mM ferrous sulphate plus 5 mM tetrathionate, and the same medium to which 10 mM glycerol was added.

The cultures were incubated in an orbital shaking incubator, at 30°C. Samples were taken regularly and bacterial growth was measured spectrophotometrically, from optical densities (600 nm) of the resuspended cells in basal salts solution (pH 2.0). Phase-contrast observation was occasionally performed to check for any sign of stress, such as sporulation.

4.5.2.2 Results

Inclusion of ferrous iron in the growth medium enhanced bacterial growth. However, isolates L-15 and RIV-14 displayed different responses towards the concentrations of ferrous iron added to the media. The maximum biomass yield was found with isolate RIV-14 in 1 mM ferrous sulphate / glycerol / tetrathionate medium (Fig. 4.11a). In contrast, isolate L-15 showed maximum growth yields in the medium which contained the highest concentration of ferrous iron tested (10 mM) (Fig. 4.11b).

The requirement of iron by RIV-14 and L-15 could be fulfilled by either ferrous or ferric iron. Growth yields of both isolates in glycerol/tetrathionate medium

supplemented with 10 mM ferric sulphate were similar to corresponding cultures containing 10 mM ferrous sulphate. However, growth rates tended to be slower when ferric sulphate was used as an iron source (~ 9.8 and 12.5 h in ferrous iron/glycerol medium; 13 and 15.8 h in ferric iron/glycerol medium, for RIV-14 and L-15, respectively) and protracted (2 – 3 days) lag periods were also observed, particularly with RIV-14.

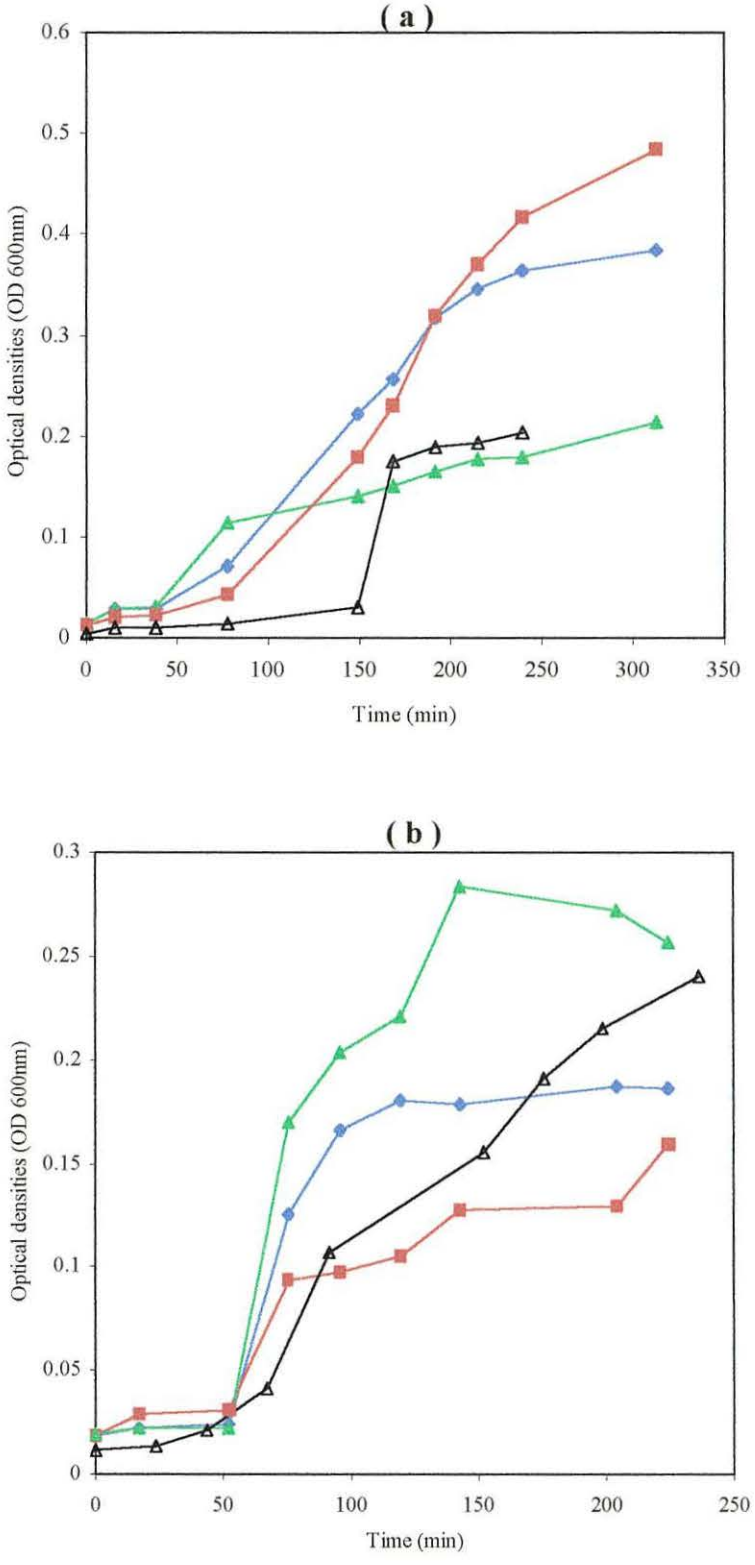


Fig. 4.11: Growth of (a) RIV-14 and (b) L-15, in medium containing 10 mM glycerol + 0.5 mM tetrathionate, amended with either 1 mM (■), 5 mM (◆) or 10 mM (▲) ferrous sulphate; or 10 mM ferric sulphate (△).

4.6 Controlled (bioreactor) growth of the Gram-positive isolates

Optimisation of bacterial growth conditions is an important part of characterisation studies. Temperature and pH are the two fundamental parameters that affect bacterial growth, and were studied for the Gram-positive isolates RIV-14 and L-15, (section 4.2.2). These experiments were carried out in the bioreactor unit described in section 3.5.1.1.

4.6.1 Effect of temperature on iron oxidation

4.6.1.1 Methodology

Bacteria were grown in 250 ml Erlenmeyer flasks containing 100 ml of sterile 20 mM ferrous iron medium (section 2.2.1.1), supplemented with 0.02% (w/v) yeast extract, adjusted to pH 1.8. The cultures were incubated at 30°C, shaken at 150 r.p.m..

Before the inoculum (10 % v/v) was introduced into the bioreactor vessel, the sterile 0.02 % (w/v) yeast extract-containing basal salts (section 2.2.1) was adjusted to pH 1.8, and amended with 25 mM ferrous sulphate. Cultures of each isolate were grown until ferrous iron oxidation was complete. In subsequent experiments, 20% (v/v) of the residual culture in the bioreactor was used as the inoculum and fresh ferrous iron / yeast extract medium added. The culture (working volume of 1 or 1.5 l) were run in batch mode, continuously aerated (0.5 l/minute) and stirred (150 r.p.m.), while pH was automatically controlled (pH 1.8 $\pm$ 0.1) throughout the incubation period, using H_2SO_4 and NaOH (1 M of each). Temperature was set at 25°, 30°, 33°, 35°, 37°, 40° and 43°C, and each experiment was carried out in duplicate. Iron oxidation by the bacteria was monitored regularly by withdrawing samples and measuring the concentration of residual ferrous iron titrimetrically using 1 mM potassium permanganate in sulphuric acid (section 2.6.1.1). Semi-logarithmic plots of the concentration of ferrous iron oxidised against time were used to calculate doubling times under each condition.

4.5.2.3 Results

Based on the cultures doubling times (t_d) of iron oxidation, the optimum temperature for growth was detected. The specific growth rates (μ) of the iron-oxidising isolates were calculated ($\ln 2/t_d$) based on calculated doubling time at the respective temperatures. The relationship between the specific growth rates and temperature indicated that the temperature optima for RIV-14 and L-15 were 33 and 37°C respectively (Fig 4.12). Very rapid oxidation of ferrous iron was detected at these temperatures, with an equivalent doubling time of as low as 2.5 h for each isolate. Isolate L-15 was more thermotolerant with an observed temperature maximum of 43°C compared with 37°C for RIV-14.

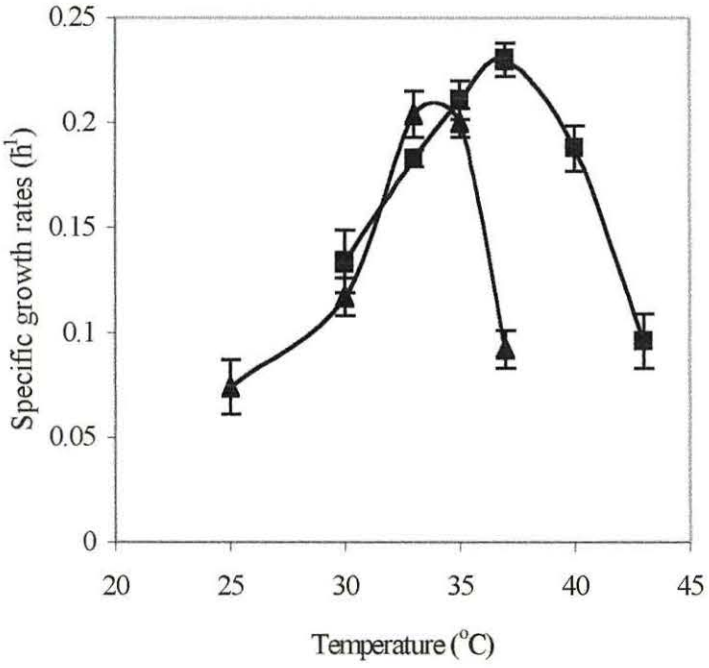


Fig. 4.12: Specific growth rates of RIV-14 (▲) and L-15 (■) determined, based on rates of ferrous iron oxidation as a factor of temperature, at pH 1.8.

4.6.2 Effect of pH on iron oxidation

4.6.2.1 Methodology

The effect of pH on growth of the iron-oxidising isolates was assessed using the same bioreactor described in section 4.6.1.1, except that the temperature was fixed at the respective optimum value for each isolate while the pH was varied.

The bacteria were grown in shake flasks, as previously described in section 4.6.1.1. The bioreactor, containing sterile 20 mM ferrous iron / 0.02% (w/v) yeast extract medium (section 2.2.1.1), pH 1.8, was equilibrated at 35° (RIV-14) and 37°C (L-15) prior to inoculation (10 % v/v) with the fresh cultures. In subsequent experiments, 20 % (v/v) of the residual culture in the bioreactor was used as inoculum and fresh 25 mM ferrous iron / 0.02% (w/v) yeast extract medium added to make up a final volume of between 1 and 1.5 litre. The pH of growth medium was set at 1.0, 1.2, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9 and 2.0. Growth at pH 0.8 was tested with isolate L-15. Samples were withdrawn at regular intervals and ferrous iron concentrations were measured by titration using 1 mM potassium permanganate (section 2.6.1.1). Culture doubling times (t_d) were determined using semi-logarithmic plots of iron oxidation versus time and growth rates were calculated from the equation $\mu = 2/td$.

4.5.2.4 Results

Both isolates were tolerant to extremely acidic conditions being capable of growth at pH 1.0 with equivalent doubling times of 6.5 (RIV-14) and 6.8 h (L-15), which is similar to that reported for *T. ferrooxidans* at its optimum pH (~pH 2.0). The optimum pH for growth were pH 1.5 (RIV-14) and 1.6 (L-15) (Fig. 4.13). Isolate L-15 appeared to be more sensitive to higher pH (pH 2.0) than RIV-14, though it was capable of growing in medium set at pH 0.8 with an equivalent doubling time of 24.5 h. This was

also found to be the lowest pH limit for L-15, as ferrous iron oxidation was not detected when the bacterium was grown in medium set at pH 0.7.

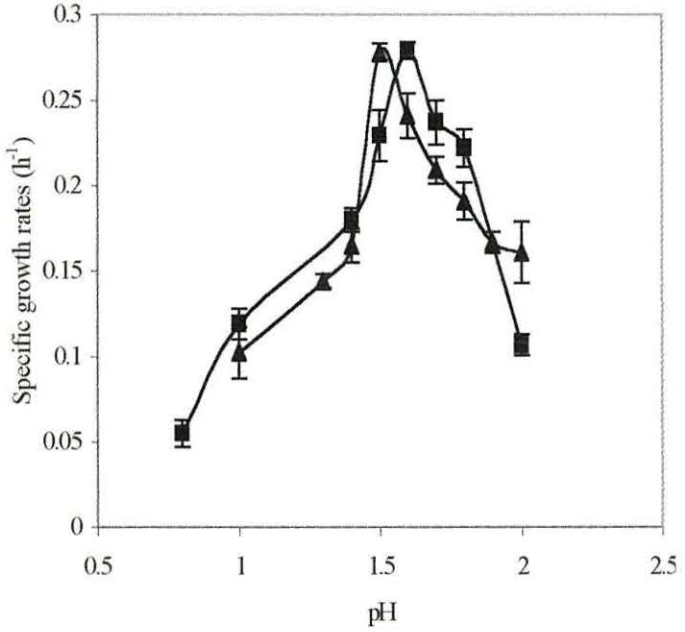


Fig. 4.13: Specific growth rates of RIV-14 (▲) and L-15 (■) based on rates of ferrous iron oxidation as a factor of pH, incubated at 35° and 37°C, respectively.

4.6 Tolerance to some heavy metals

4.6.1 Methodology

The tolerance of the Gram-positive iron-oxidising mesophiles to some heavy metals was tested and compared with that of the type strain of *T. ferrooxidans* (ATCC 23270). Isolates RIV-14 and L-15 were pre-grown in medium containing 10 mM ferrous iron and 0.02% (w/v) yeast extract, pH 1.9, at 30°C. The cells were harvested by centrifugation (10,000 r.p.m. for 15 minutes at 4°C), washed and resuspended in a volume of basal salts (section 2.2.1) adjusted to pH 1.9. The cell suspensions of each isolate were then inoculated (1% v/v) into 100 ml shake flasks containing 50 ml of 10 mM ferrous sulphate / 0.002% (w/v) yeast extract medium; pH 1.9. The medium was supplemented with various concentrations of copper, zinc, molybdenum, ferrous and

ferric iron, in duplicate. All metals were added as their sulphate salts, with the exception of molybdenum, which was added as sodium molybdate. Metal concentrations used were 5, 10, 25, 50, 100, 200 and 300 mM for copper and zinc, 0.05, 0.1, 0.2, 0.5 and 1.0 mM for molybdate, 100, 200, 300 and 500 mM for ferrous iron and 10, 25, 50, and 100 mM for ferric iron.

Cultures were incubated, unshaken, at 30°C for up to 10 days. Tolerance to the metals tested was determined by measuring the oxidation of ferrous iron by the bacteria throughout the incubation period. Ferrous iron concentrations were measured, titrimetrically, using potassium permanganate (section 2.6.1.1). The effects of elevated concentrations of ferrous and ferric iron on growth rates were also assessed. Semi-logarithmic plot of iron oxidation versus time was used to calculate culture doubling times.

4.7.1 Results

Elevated concentrations of heavy metals are common in environments where mineral leaching processes occur and this may affect growth and survival of some acidophilic bacteria. The minimum concentrations of the metals (MIC), which inhibited iron oxidation by RIV-14 and L-15 are summarised in Table 4.6.

Table 4.6: Tolerance of isolates RIV-14 and L-15 to some heavy metals, stated as the minimum inhibitory concentration of the metals (MIC), and comparison with the type strain of *T. ferrooxidans* (ATCC 23270)

Metals / Bacteria	Minimum inhibitory concentration (mM)		
	T. ferrooxidans	RIV-14	L-15
Fe^{2+}	>500	500	500
Fe^{3+}	>100	>100	100
Cu^{2+}	>300	100	100
Zn^{2+}	>300	300	>300
MoO_4^{2-}	0.2	0.2	0.2

'>' means that the minimum concentration required to inhibit bacterial growth was higher than the value indicated, and was not determined.

In general, isolates RIV-14 and L-15 were highly tolerant to elevated concentrations of the cationic metals tested, though to a lesser extent than *T. ferrooxidans*. The sensitivity of the Montserrrat isolates to copper was greater than that of *T. ferrooxidans*, and only partial oxidation of ferrous iron was observed (10 – 15% oxidation) by the former isolates in the presence of 500 mM copper. Zinc (Zn^{2+}) and ferrous iron were tolerated by all three iron-oxidisers at very elevated concentrations. Similar tolerance was noted with the molybdate anion (MoO_4^{2-}), which has been reported to be highly toxic to acidophilic bacteria at between 0.05 to 0.1 mM (Barrett *et al.*, 1993). Identical response in terms of iron oxidation (15%) was displayed by both Montserrat isolates and by *T. ferrooxidans* at 0.2 mM MoO_4^{2-}. The effect of increasing ferrous and ferric iron concentrations on bacterial growth was to increase culture doubling times (Fig. 4.14)

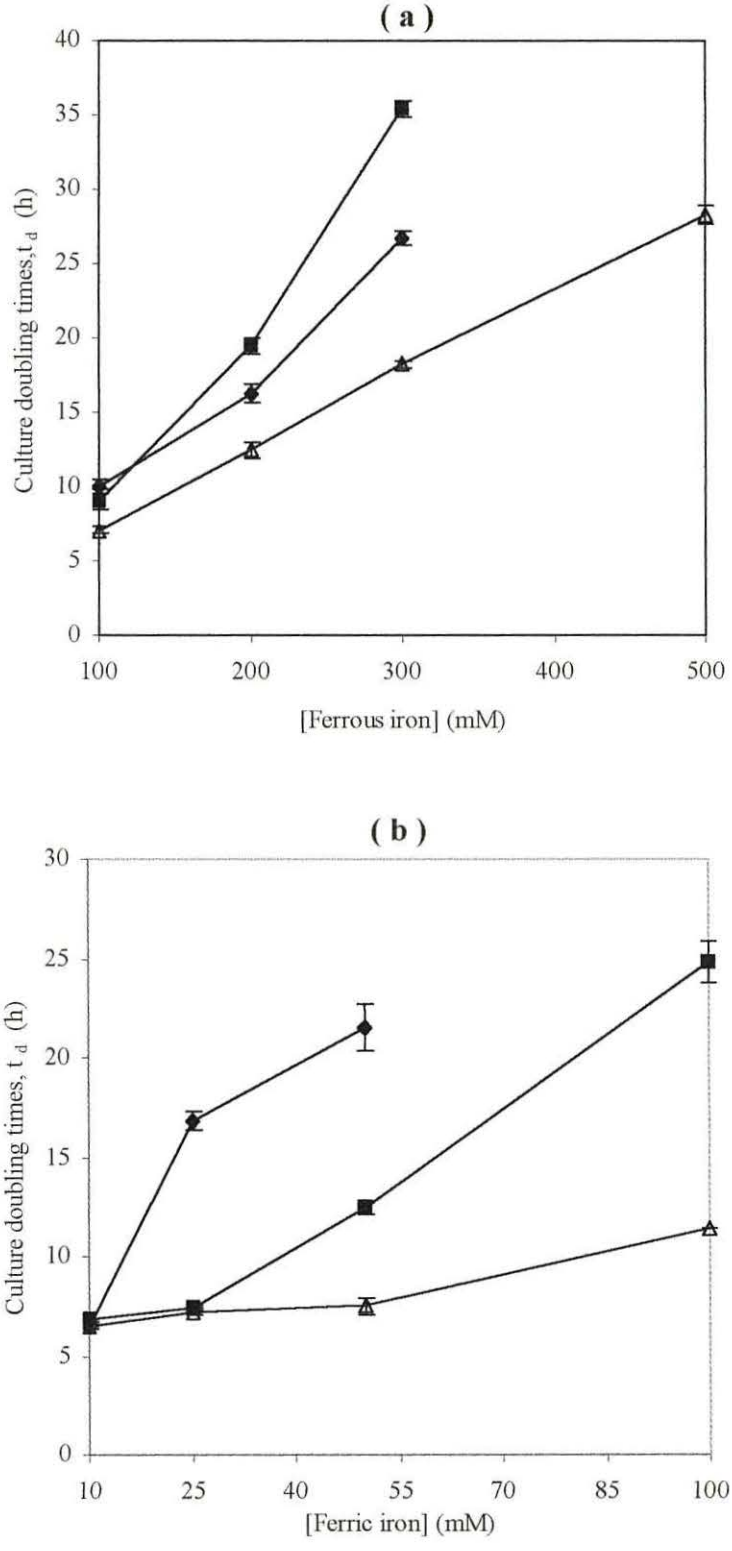


Fig. 4.14: Effect of (a) ferrous iron and (b) ferric iron on the growth of RIV-14 (■), L-15 (◆) and *T. ferrooxidans* (△), as indicated by the culture doubling times based on the oxidation of 25 mM ferrous iron.

4.8 Fixation of carbon dioxide

4.8.1 CO_2 fixation in autotrophic and mixotrophic cultures

4.8.1.1 Methodology

The ability of isolates RIV-14 and L-15 to incorporate carbon dioxide (CO_2) was studied by growing the bacteria in 25 mM ferrous iron medium (section 2.2.1.1), pH 1.9 containing $^{14}CO_2$, in the presence of either 0.02% (w/v) yeast extract, (Fe^{2+} / YE) or 2.5 mM potassium tetrathionate (Fe^{2+} / tet).

The bacteria were grown in 10 mM ferrous iron / 0.02% (w/v) yeast extract medium. Cultures were harvested during late exponential phase of growth by centrifugation (10,000 r.p.m. for 15 minutes at 4°C), washed twice and resuspended in sterile acidified (pH 2.0) basal salts solution (section 2.2.1); this procedure was intended to remove all soluble organic compounds from the media. Cell suspensions were inoculated (2% v/v) into duplicate 100 ml Erlenmeyer flasks containing 50 ml medium (Fe^{2+} / YE or Fe^{2+} / tet). The flasks were then sealed (Suba seals) and the cultures were supplemented with 1% (v/v) of 0.5 M $NaH[^{14}C]O_3$ (specific activity 11,640 dpm/µmol; Amersham International). Cultures were incubated in a rotary shaker (150 r.p.m.) at 30°C and carbon dioxide incorporation by the bacteria was monitored, as described in section 2.7. Iron oxidation was monitored by potassium permanganate titration (section 2.6.1.1). Cell numbers were counted using the Thoma chamber at the end of incubation. The relationship between the two processes was determined by plotting CO_2 fixed (nmoles/ml) against the concentration of ferrous iron oxidised (µmoles/ml), as described by Eccleston *et al.* (1985).

4.8.1.2 Results

Isolates RIV-14 and L-15 were able to fix carbon dioxide (Fig. 4.15). However, in the presence of 0.02% (w/v) yeast extract, carbon dioxide fixation was suppressed,

particularly by isolate RIV-14. Partial suppression of carbon dioxide fixation was displayed by isolate L-15 grown in organic-containing medium, through increasing amounts of carbon dioxide were incorporated during the later phases of iron oxidation. However in both cases, organic carbon appeared to be the preferable source, as showed by the suppression of carbon dioxide fixation in the early growth phase, compared with autotrophic cultures.

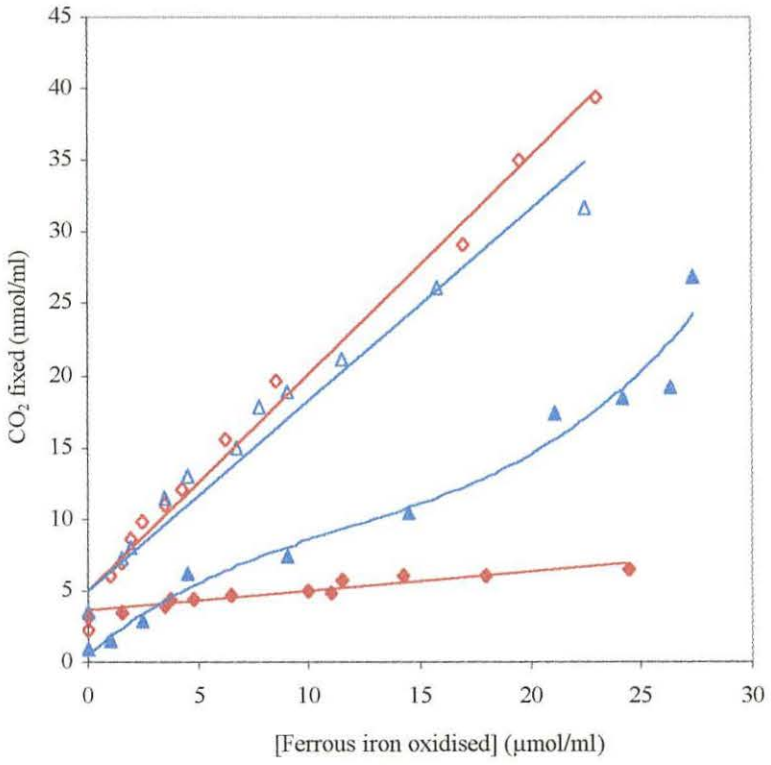


Fig. 4.15: Carbon dioxide incorporation by RIV-14 (◆◇) and L-15 (▲△), grown in medium containing 25 mM ferrous sulphate amended with either 0.02% (w/v) yeast extract (solid symbols) or 2.5 mM tetrathionate (opened symbols) in the presence of $NaH[^{14}C]O_3$.

The effect of yeast extract on fixation of carbon dioxide was determined on a unit cell basis. The biomass yields of both isolates RIV-14 and L-15 were estimated at the end of iron oxidation as 2.0×10^8 and 1.15×10^8 cells/ml in the presence of yeast extract, and 2.31×10^6 and 1.16×10^6 in the absence of yeast extract (autotrophic cultures). On this basis, it was clear that yeast extract addition had greatly diminished the amount of carbon dioxide fixed by both isolates (Table 4.7).

Table 4.7: Carbon dioxide incorporation by isolates RIV-14 and L-15, grown in 25 mM ferrous sulphate medium to which either 0.02% (w/v) yeast extract or 2.5 mM tetrathionate was added.

Bacteria / Medium	CO_2- C fixed (g C/cell)	
	Fe^{2+}/YE	Fe^{2+}/tetrathionate
RIV-14	6.45×10^{-16}	2.05×10^{-13}
L-15	7.21×10^{-16}	2.07×10^{-13}

4.8.2 Effect of yeast extract concentration on carbon dioxide fixation by isolate L-15

4.8.2.1 Methodology

In view of the pattern of carbon dioxide incorporation by isolate L-15 grown in yeast extract-containing medium (section 4.8.1.2), an experiment was carried out to investigate further the effect of various concentrations of yeast extract on carbon dioxide fixation activity by this bacterium.

Isolate L-15 was grown in 10 mM ferrous sulphate / 0.02% (w/v) yeast extract medium (section 2.2.1.1), set at pH 1.9, and inoculated (2% v/v) into duplicate 100 ml shake flasks containing 50 ml of 25 mM ferrous sulphate medium (section 2.2.1.1) pH 1.9, amended with various concentration of yeast extract (0.01, 0.02, 0.05 and 0.1%,

w/v). The flasks were sealed, supplemented with 1% (v/v) of 0.5 M NaH[^{14}C]O_3 and incubated at 30°C with shaking, at 150 r.p.m.. The remaining experimental protocol was as described in section 2.7.

Samples were taken regularly to monitor carbon dioxide fixation and ferrous iron oxidation by the bacterium. The relationship between the two processes was then determined by plotting the amount of carbon dioxide fixed against concentration of ferrous iron oxidised.

4.8.2.2 Results

Suppression of carbon dioxide fixation activity by isolate L-15 was found to be directly correlated with the original concentration of yeast extract in the growth medium. Complete inhibition of carbon dioxide fixation was found when 0.1% (w/v) yeast extract was added to the medium (Fig. 4.16).

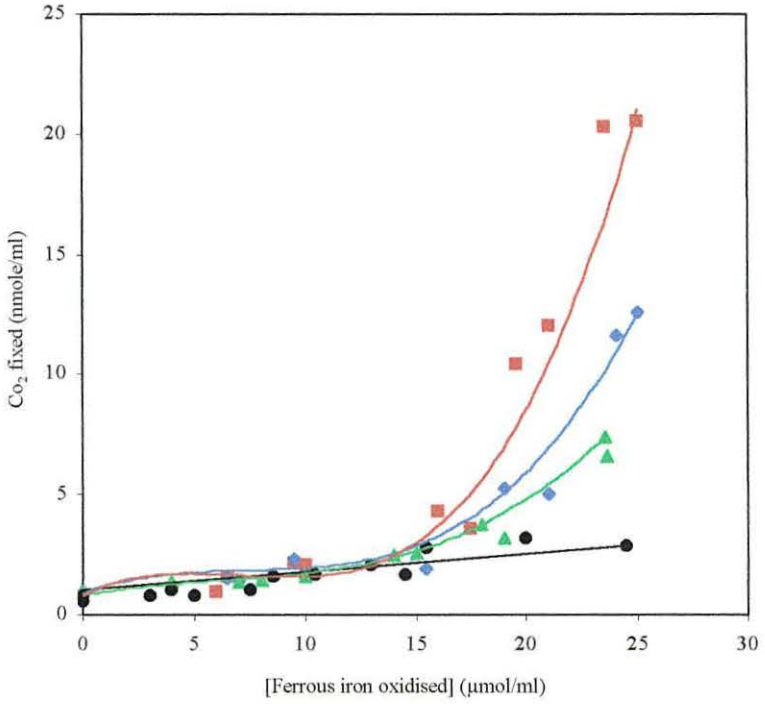


Fig. 4.16: Carbon dioxide incorporation by L-15 grown in 25 mM ferrous sulphate medium, amended with 0.01% (■), 0.02% (◆), 0.05% (▲) and 0.1% (●); (w/v) yeast extract in the presence of NaH[^{14}C]O_3

4.9 Molecular characterisation of the Gram-positive mesophilic isolates

4.9.1 Phylogeny based on the comparative analysis of 16S rRNA gene sequences

The PCR amplification and cloning of the 16S rRNA gene fragment of the Gram-positive Montserrat isolates, RIV-14 and L-15 was carried out by Dr. F. F. Roberto of the Idaho National Engineering and Environment Laboratory (INEEL), Idaho Falls, U.S.A. A similar procedure to that described in section 2.9 was followed with the exception that the reverse and forward primers used were different.

4.9.1.1 Methodology

Cell suspensions of isolates RIV-14 and L-15 were centrifuged at 10,000 r.p.m for 5 minutes and the resulting pellets were washed twice in sterile deionised water. The final washed cell pellets were resuspended in 20 µl of 0.05 M NaOH, 0.25% SDS and lysed by heating for 15 minutes at 95°C. The cell lysate was diluted with 200 µl of water and cell debris was pelleted by centrifugation (10,000 r.p.m. for 5 minutes). A 4 µl aliquot of this crude DNA extract was then used in a subsequent 50 µl PCR reaction mix. The 16S rRNA gene fragments were amplified from genomic DNA using forward (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse (5'-GGYTACCTTGTTACGACTT-3') PCR primers, corresponding to *E. coli* ribosomal RNA sequence position 8 – 27 and 1510 – 1492, respectively. These primers were those described by Kane *et al.* (1993). The amplified 16S rRNA gene sequences were cloned into the pCR2.1 vector (Invitrogen, Inc., Carlsbad, CA, USA) using the TOPO TA cloning kit according to the manufacturer's instruction, as stated in section 2.9.1. Authentic clones were verified by restriction enzyme digestion to confirm that inserts were of the expected size (section 2.9.3).

Positive transformants were grown overnight in ampicillin-containing LB liquid medium and plasmid DNA was extracted and purified (section 2.9.4), followed by

sequencing (section 2.9.4.1). Sequence data were analysed and a phylogenetic tree was constructed, as described in section 2.9.4.2.

4.9.1.2 Results

Near full-length 16S rRNA gene sequences were obtained from both Gram-positive isolates RIV-14 (1431 bp) and L-15 (1432 bp). Comparison of 16S rRNA sequences with the Genbank database sequences using BLAST revealed a close phylogenetic relationship with other moderately thermophilic acidophilic members of the genus *Sulfobacillus,* and the closest being *S. thermosulfidooxidans* with 94% sequence identity. Binary level comparison (BLAST of 2 sequences) between the 16S rDNA sequence of RIV-14 and L-15 indicate 97% sequence identity. Using the alignment of the 16S rRNA sequences from RIV-14, L-15 and other selected bacteria from the Genbank databases, a phylogenetic tree was constructed (Fig. 4.17) which showed the taxonomic position of the mesophilic Gram-positive isolates within the cluster of moderately thermophilic acidophiles.

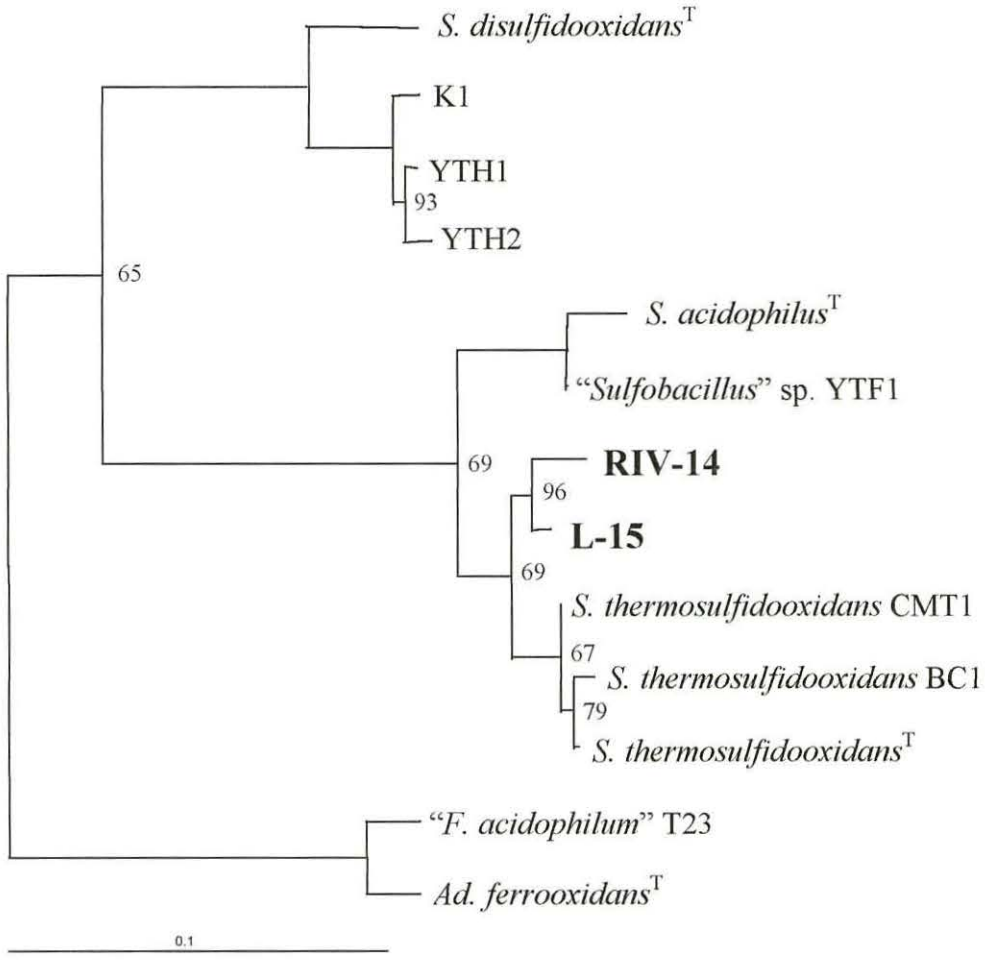


Fig 4.17: Phylogenetic relationship of *Sulfobacillus* strain RIV-14 and L-15 to other selected moderately thermophilic and mesophilic acidophiles based on the comparative analysis of the 16S rRNA sequences contained in the Genbank. Branch lengths are proportional to the calculated evolutionary distances. The tree was rooted using *Korarchaeote* as an outgroup. The bar indicates 0.1 nucleotide substitutions per site.

4.9.2 Analysis of DNA base (G + C) composition

4.9.2.1 Methodology

Pure cultures of RIV-14 and L-15 were grown in 500 ml liquid medium (section 2.2.1.1) poised at pH 1.8, supplemented with 10 mM ferrous sulphate and 0.02% (w/v) yeast extract, and incubated at 35°C. Cells were harvested during the late exponential growth phase by centrifugation (10,000 r.p.m. for 10 minutes. at 4°C) and washed with TE buffer (10 mM Tris-HCL + 1 mM EDTA; pH 8.0).

Chromosomal DNA was then extracted from the cell pellets of RIV-14 and L-15, following the procedure described in section 2.8, and were subsequently purified by caesium chloride gradient centrifugation (section 2.8.2). The G + C content of the chromosomal DNA was determined by melting point analysis in 0.1X SSC as described by Marmur and Doty (1962; section 2.8).

4.9.2.2 Results

The DNA base composition of the Gram-positive Montserrat isolates are shown in Table 4.8, together with the G + C values for the moderate thermophiles *S. acidophilus* and *S. thermosulfidooxidans*.

Table 4.8: DNA base composition of the isolates RIV-14 and L-15 and comparison with other known species of *Sulfobacillus*.

Bacteria	Mol % (G + C)
S. acidophilus (strain NAL)[1]	55.0 – 57.0
S. thermosulfidooxidans (strain VKM 1269)[2]	53.6 – 53.9
Sulfobacillus sp. (YTF1)	61.9
RIV-14	55.8
L-15	57.5

[1] : Norris *et al.* (1996); [2] : Durand (1996)

4.10 Discussion

In the preliminary characterisation of the original isolates from Montserrat, RIV-14 and L-15 were classed together as 'iron-oxidising heterotrophic acidophiles' (Johnson, unpublished data). Because of the interest in acidophilic bacteria which may be used for commercial oxidation of mineral sulphides, further detailed characterisation of these bacterial strains was carried out. In the present study both isolates RIV-14 and L-15 oxidised ferrous iron when grown in liquid and on solid media, and the relationship between iron oxidation and growth was confirmed. However, the two isolates were not "*Ferromicrobium*" spp. as was originally supposed. Experimental work described in the present chapter confirmed that isolates RIV-14 and L-15:

- are Gram-positive, spore-forming eubacteria;
- are able to grow autotrophically, heterotrophically and chemolithoheterotrophically;
- have a nutritional requirement for reduced sulphur, which may be provided as inorganic (tetrathionate) or organic (as in yeast extract) sulphur;
- are able to fix carbon dioxide, through CO_2 fixation is suppressed when organic carbon is provided;
- are able to obtain energy from the oxidation of ferrous iron, though are less well adept at growing on elemental sulphur;
- are facultative anaerobes, being capable of coupling the oxidation of an organic (glycerol) or inorganic (tetrathionate) electron donor to the reduction of ferric iron in the absence of oxygen;
- are obligately acidophilic (displaying the greatest tolerance to proton acidity of any iron-oxidising eubacterium yet characterised) and are mesophiles;

- are phylogenetically most closely related to bacteria of the genus *Sulfobacillus*, (previously described as moderately thermophilic acidophiles)
- other work (Chapter 6) has confirmed that both isolates can catalyse the oxidative dissolution of pyrite.

Morphological characteristics of microorganisms are generally too simple to serve as a basis for a genuine and reliable classification and identification, though some degree of differentiation may be possible. Cells of the iron-oxidising Montserrat isolates RIV-14 and L-15 were very similar to each other. Both were straight rods, found as single cells, pairs or short chains (generally up to 5 – 8 individual rods), with dimensions of 2.3 - 3 μm x 0.6 – 0.7 μm, respectively. Cells of both isolates were motile when grown in ferrous iron / yeast extract medium, and endospores were observed when bacteria were grown under adverse nutritional or environmental conditions. It is important to stress that the reported cell sizes were the average values of cells from cultures grown under the same conditions and at the same phase of growth. It has been reported that cell sizes of acidophilic bacteria vary with the incubation time and these bacteria may lose more than 50% of the cellular volume with protracted incubation (Bacelar-Nicolau, 1996). Cell morphologies of the bacteria were also observed to be subject to variation in response to growth conditions. Growth of the bacteria with glycerol as the sole organic substrate gave rise to shorter, more swollen rods, particularly with isolate RIV-14, and cell motility was, for both isolates, less obvious than in ferrous iron / yeast extract medium. Variations in cell morphologies in relation to growth conditions has also been noted for other iron- and/or sulphur-oxidising acidophilic bacteria, including several moderately thermophilic strains of *Sulfobacillus* (Norris *et al.*, 1980; Norris *et al.*, 1996).

Both Gram-positive isolates were confirmed as mesophiles, as evident from their optimum growth temperature of 35°C (RIV-14) and 37°C (L-15), and their inability to grow at 45°C. The two isolates also shared similar capacities for iron metabolism, as shown by their abilities to metabolise ferrous or ferric iron, when grown in an appropriate medium under aerobic or anaerobic conditions. Growth of the bacteria on ferrous iron was stimulated by the presence of either yeast extract (complex organic substrate) or tetrathionate, and they failed to grow in the absence of either one of these.

The fact that both isolates grow only on the ferrous iron/tetrathionate overlay solid medium served as an early indication of their requirement for reduced sulphur compounds. This could be related to the environment from which they were isolated (volcanic sites), which were tend to be rich in sulphur as well as ferrous and ferric iron (Atkinson *et al.*, 2000). The requirement of a reduced sulphur compound (which was satisfied by adding tetrathionate at a minimum concentration of 10 μM) for autotrophic growth on ferrous iron parallels that reported for a number of moderately thermophilic acidophiles, such as *S. acidophilus* and *S. thermosulfidooxidans* (Norris *et al.*, 1996; Krasil'nikova *et al.*, 1998). However, tetrathionate was not required when the Montserrat isolates were grown in medium containing yeast extract, presumably because the end products of assimilatory sulphate reduction (sulphur-containing amino acids and vitamins) are present in this product. More rapid oxidation of ferrous iron was displayed when yeast extract, rather than tetrathionate, was added to the ferrous iron medium. This was evident from culture doubling times, based on iron oxidation, which were statistically different ($P<0.001$) in the respective media. However, it was noted that growth rates in inorganic medium (initial $\mu \sim 0.04\ h^{-1}$) were substantially increased (μ 0.07 – 0.06 h) following several transfers through ferrous iron / tetrathionate medium.

Yeast extract is a complex mixture of organic materials. However, both bacteria were also capable of growth in defined organic substrates (such as glycerol), though again it was found that there was a requirement for a reduced inorganic sulphur compound; RISC (0.5 mM tetrathionate was used in this case) further suggesting that the bacteria are not capable of assimilatory reduction of sulphate. Furthermore, growth of these bacteria was found to be highly dependent on the concentration of ferrous iron present in the medium. This was evident by an assessment of bacterial growth in glycerol-containing medium, in that the biomass yield was observed to be related to concentration of ferrous iron, though the two isolates responded very differently, with L-15 showing a positive correlation and RIV-14 a negative correlation. The iron-dependent metabolism of these bacteria was, however, found to be independent of the ionic form (Fe^{2+} or Fe^{3+}) provided. Both isolates were able to grow in ferric iron/glycerol/tetrathionate medium and produced similar biomass yields to those grown in ferrous iron/glycerol/tetrathionate medium, though growth rates were greater in the ferrous iron-containing medium. Furthermore, there was evidence that both isolates were not capable of heterotrophic growth in the iron-free media, indicating a strict requirement for iron, again similar to *S. acidophilus* and *S. thermosulfidooxidans* (Norris *et. al.*, 1996). Iron is essential for virtually all living organisms though often at very low concentrations; a similar requirement for elevated concentrations of iron has also been reported for other mesophilic acidophilic bacteria (Johnson *et al.*, 1992; Lobos *et al.*, 1986).

In addition to being able to use ferrous iron as an electron donor, both isolates could also use ferric iron as an electron acceptor. Both RIV-14 and L-15, were capable of growth in oxygen-free medium in the presence of ferric iron, thus indicating that they

are facultative anaerobes. Unlike most obligately heterotrophic acidophilic bacteria (mainly those of the genus *Acidiphilium*; Johnson and McGinness, 1991), the Gram-positive mesophiles from Montserrat required strictly anoxic conditions to promote ferric iron reduction. Isolates RIV-14 and L-15 were capable of coupling ferric iron reduction to the oxidation of an organic (glycerol) or inorganic (tetrathionate) electron donor, though growth and iron oxidation were better in heterotrophic cultures. This was evident from the observed rapid iron reduction for both isolates grown in glycerol medium (2 weeks for complete reduction of 25 mM ferric iron) whereas incomplete reduction and lower biomass yields (even with a protracted incubation period of 5 weeks) was observed in tetrathionate medium.

There was some evidence which indicated tetrathionate disproportionation by isolate RIV-14, though not by L-15. In anaerobic cultures containing 5 mM tetrathionate and 25 mM ferrous iron, isolate RIV-14 displayed growth yields similar to those in 5 mM tetrathionate.25 mM ferric iron medium. Since there was no other possible electron acceptor (such as nitrate) in the medium, and RIV-14 (as L-15) does not reduce sulphate or ferment organic substrates (no growth of either was observed in anaerobic glycerol / ferrous iron cultures) and strictly anoxic conditions were maintained, the inference was that growth was occurring by disproportionation of tetrathionate. Disproportionation is a unique form of energy metabolism reported for autotrophic sulphate-reducing bacteria (SRB), that are capable of using sulphur compounds of intermediate oxidation state as both electron donors and electron acceptors (Thamdrup *et al*, 1993; Loveley and Phillips, 1994; Fuseler *et al.*, 1996). In the bacterially-mediated disproportionation reaction, sulphur and RISCs (e.g. thiosulphate, sulphite, tetrathionate, etc) are fractionated into two new compounds, one of which is more oxidised (sulphate; SO_4^{2-}) and another one of which is more reduced (hydrogen sulphide; H_2S) than the original substrate (Canfield and

Thamdrup, 1994; Cypionka *et al.*, 1998). In contrast to the SRB, the acidophilic iron-oxidising isolate RIV-14 lacks the ability to reduce sulphate. Disproportionation of tetrathionate would theoretically result in an increase in sulphate and sulphide concentrations, which should be directly related to a simultaneous decrease in tetrathionate concentration, as; $S_4O_6^{2-} + 2H_2O \rightarrow 2SO_4^{2-} + 2H_2S$. However, in this case additional and more thorough experimental work is required to prove without doubt that RIV-14 is capable of disproportionating tetrathionate.

The study of Fe^{2+}-oxidising and Fe^{3+}-reducing activities undertaken with isolates RIV-14 and L-15 has clearly demonstrated that the specific rates of these activities are significantly affected by the various conditions under which cultures are grown. Comparison of the rates of iron oxidation and reduction for cells cultured under different growth conditions was undertaken to determine the independent effects of oxygen and form of iron (ferric and ferrous) on the bacterial oxido-reduction activities.

Variations in the specific rates of Fe^{2+}-oxidising activities were found for both RIV-14 and L-15 grown under different conditions (type of medium and oxygen availability). Highest specific rates of Fe^{2+} oxidation were found for autotrophic- and aerobically-grown cells. The reason for this is possibly a greater amount of 'iron-oxidase' produced when ferrous iron is the sole energy source, and a greater diversity of proteins produced by the bacteria during growth in organic-containing medium. In the presence of organic compounds, both isolates metabolised the available organic substrate(s) for growth; this would require the synthesis of substantial amounts of additional proteins (enzymes, carriers etc) involved in the catabolism of these organic substrate(s). In contrast, when ferrous iron was provided as sole energy source, the bacteria would be predicted to synthesise relatively greater amounts of the 'iron-oxidase'

and other proteins involved specifically in ferrous iron oxidation. The specific iron oxidation rates of L-15 (105 and 28 μmoles/minute/mg protein) and RIV-14 (28.31 and 7.61 μmoles/minute/mg protein) grown aerobically in either yeast extract or glycerol-containing medium were significantly greater than those reported for the moderately thermophilic iron-oxidiser *S. thermosulfidooxidans*, (strain TH1; 2.06 μmoles/minute/mg protein; Bacelar-Nicolau, 1996) grown aerobically in 50 mM Fe^{2+} medium supplemented with 10 mM glycerol and 0.02% (w/v) yeast extract. The results also showed that the effects of organic substrates on specific iron oxidation rates were more pronounced for isolate RIV-14 than for L-15.

Results from the specific Fe^{2+} oxidation experiments indicated that the synthesis of Fe^{2+}-oxidising systems was suppressed in cells grown under anaerobic conditions. The observed 'constitutive' levels of Fe^{2+}-oxidising activities for RIV-14 and L-15 were significantly lower in cells grown under anoxic conditions (0.67 and 1.39 μmoles/minute/mg protein, respectively) than those determined for aerobically-grown cells (7.61 and 28.0 μmoles/minute/mg protein, respectively). Similar effects on Fe^{2+}-oxidising activities were also found with some strains of heterotrophic mesophiles (e.g. strain SLC2, and "*F. acidophilum*" T-23; Table 4.4) grown under aerobic or microaerobic conditions (Bacelar-Nicolau, 1996).

The study of specific Fe^{3+}-reduction undertaken with isolates RIV-14 and L-15 showed that the 'constitutive' level of Fe^{3+}-reducing activity (2.49 and 3.43 μmoles/minute/mg protein, respectively) in cells grown under aerobic conditions (in Fe^{2+}/glycerol/ tetrathionate medium) was higher than that of the Fe^{2+}-oxidising activity (0.67 and 1.39 μmoles/minute/mg protein, respectively) found in cells grown under anoxic conditions (in Fe^{3+}/glycerol/tetrathionate medium). However, Fe^{3+}-reduction by both isolates was found to be inducible in anaerobic conditions, as shown by the

significant increase ($P < 0.001$ respectively) of specific iron reduction rates compared to cells grown aerobically. Maximum rates of Fe^{3+}-reduction were much less than those of maximum Fe^{2+}-oxidation for both RIV-14 and L-15.

One possibility is that the Fe^{2+}-oxidising and Fe^{3+}-reducing systems in these isolates are actually a single system, as in the postulated 'sulphur:ferric ion-oxidoreductase (SFORase)', described in *T. ferrooxidans* by Sugio *et al* (1985; 1987; 1992a,b). However, in view of the different responses of the 'iron oxidase' and 'iron reductase' systems in isolates RIV-14 and L-15 to growth conditions (particularly in response to culture aeration) it seems probable that the systems are separate and distinct in these isolates. In contrast to RIV-14 and L-15, other studies have indicated that iron oxido-reduction in *Sulfobacillus* YTF1 is carried out by the same system. Greater rates of both Fe^{2+} oxidation and Fe^{3+} reduction were displayed by cells grown aerobically (10.14 and 3.50 μmoles/minute/mg protein, respectively) than microaerobically (6.79 and 0.57 μmoles/minute/mg protein; Bridge, 1995).

The ability of the Gram-positive Montserrat isolates to oxidise elemental sulphur was very limited even when yeast extract was included in the medium, as indicated by the relatively weak acid production in both cultures throughout a protracted incubation period. It is possible that this was due to the nature of the reduced (elemental) sulphur used as substrate in this study. It is conceivable that tetrathionate is more favourable substrate for growth of these bacteria. Successful anaerobic growth using tetrathionate as electron donor was observed for both RIV-14 and L-15. Tetrathionate has been claimed to be the main energy source for *S. thermosulfidooxidans* during its autotrophic growth as revealed by the enzymatic assessment of sulphur metabolism (Krasil'nikova *et al.*, 1998). Variable sulphur and tetrathionate oxidation activities have also been reported for three

strains of moderately thermophilic *Sulfobacillus* (ALV, BC and K; Wood and Kelly, 1983) and several mesophilic acidophiles including *T. ferrooxidans* and *A. acidophilum* (Norris *et al.*, 1986).

Both Gram-positive Montserrat isolates were found to be tolerant of extremely acidic conditions, particularly isolate L-15, as observed by growth in media poised at pH 1.0 or below. Tolerance of such low pH could facilitate the use of these bacteria for processing sulphidic ores using extremely acidic liquors. This would be advantageous, for example, in minimising the production of secondary ferric iron precipitates during bioleaching. In addition, data on tolerance to the heavy metals tested showed that these were comparable to those of iron-oxidising mesophiles and moderately thermophiles, and greater than that of most heterotrophic acidophiles (Mahapatra and Banerjee, 1996; Said, 1990).

Metabolic flexibility of isolates RIV-14 and L-15 was also apparent on the basis of their carbon metabolisms. The isolates were found to be capable of autotrophic growth in 25 mM Fe^{2+}/ 2.5 mM tetrathionate medium, obtaining energy from iron oxidation, and cellular carbon from atmospheric carbon dioxide (CO_2), i.e. chemolithoautotrophic metabolism. This was confirmed by the observations that CO_2 fixation was coupled to ferrous iron oxidation during growth of both isolates.

In contrast, growth of RIV-14 and L-15 in ferric sulphate /glycerol/tetrathionate medium under aerobic conditions showed that these isolates were capable of heterotrophic growth, using glycerol as the sole source of both carbon and energy. Ferric iron was not reduced by these isolates under aerobic conditions, and so energy acquisition from the re-oxidation of ferrous iron was not a possibility under these

conditions. Both RIV-14 and L-15 were also found to be able to grow as chemolithoheterotrophs. This was evident by the pattern of CO_2 fixation displayed by both isolates during growth in 25 mM ferrous iron / 0.02% (w/v) yeast extract medium. As with to autotrophic metabolism, growth was supported by energy from iron oxidation but, in contrast, organic materials, rather than inorganic CO_2, were used as carbon source. In the presence of yeast extract, CO_2 fixation by both isolates was suppressed, though this suppression was found to be dependent on the amount of organic carbon present in the culture. This was indicated by the ability of L-15 to switch its carbon metabolism to carbon dioxide fixation when the organic carbon source became depleted. A similar chemolithoheterotrophic metabolism was also reported for the moderately thermophilic iron-oxidising acidophiles *S. thermosulfidooxidans,* (Zakharchuk *et al.*, 1994) and *S. acidophilus* (Wood and Kelly, 1983). However, unlike the moderately thermophilic acidophiles, there was no evidence of mixotrophic metabolism (i.e. simultaneous fixation of CO_2 fixation and organic carbon) by the mesophilic isolates RIV-14 and L-15.

Switching of carbon metabolisms from an organic to an inorganic source was, however, more apparent with isolate L-15, which required greater concentrations of yeast extract (0.1%) to suppress CO_2 fixation during oxidation of 25 mM ferrous iron than did RIV-14 (0.02%). This also indicated that L-15 was more adept at using yeast extract for more rapid biosynthesis than RIV-14. In addition, higher yields of L-15 grown in ferrous sulphate / yeast extract medium (2.54×10^8 cells/ml) gave further indication that this isolate was more 'heterotropically inclined' than RIV-14 (1.21×10^8 cells/ml) in the same medium. In contrast, more efficient autotrophic growth was displayed by RIV-14 than L-15 in Fe^{2+}/tetrathionate medium (yields of 2.31×10^7 and 1.16×10^7 cells/ml for RIV-14 and L-15, respectively) though in both cases, final cell numbers were much smaller than those observed in heterotrophic media.

The metabolic flexibility displayed by isolates RIV-14 and L-15 when grown in various growth conditions is summarised in Fig. 4.19. The advantages of such metabolic versatility may be perceived as a key factor in survival and competition in of these bacteris in natural environments.

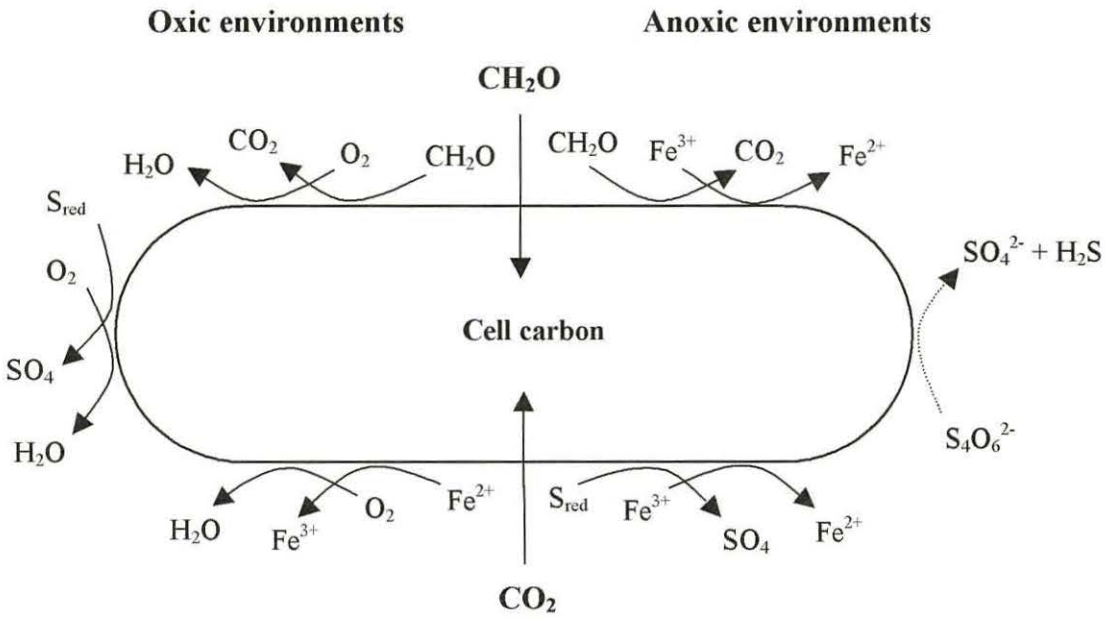


Fig. 4.18: Diagramatic representation of metabolic flexibility in the novel Gram-positive mesophilic acidophilic *Sulfobacillus*-like strains RIV-14 and L-15, either in oxic or anoxic environmental conditions. The broken line indicates preliminary evidence for disproportionation of tetrathionate by RIV-14.

Based upon most of the morphological and physiological characteristics (rod-shaped, Gram-positive, sporulating iron-oxidising acidophiles) it appeared that both RIV-14 and L-15 belong to the genus *Sulfobacillus*. However these isolates, unlike other described iron-oxidising acidophilic members of the genus, have an optimum growth temperature of 35°C (RIV-14) and 37°C (L-15), and thus represent the first mesophilic

Gram-positive iron-oxidising acidophilic bacteria to be fully characterised. The Gram-positive acidophile *S. disulfidooxidans* (Dufresne *et al.*, 1996) is also mesophilic but, in contrast to the Montserrat isolates, does not oxidise ferrous iron. Also its phylogenetic positioning (as a genuine *Sulfobacillus* species.) has been open to question (Chapter 5). Comparative 16S rRNA gene sequence analyses, using the BLAST system, also revealed that both Montserrat isolates were clustered with members of the genus *Sulfobacillus,* with *S. thermosulfidooxidans* as the nearest phylogenetic neighbour (94% DNA identity with both RIV-14 and L-15). Phylogenetically, RIV-14 and L-15 were closely related to each other on the basis of 16S rRNA gene sequences, with 97% similarity, though this value is low enough to consider them as two distinct species. The results obtained from the mol% G + C determinations showed that RIV-14 and L-15 have G + C values of 55.8% and 57.5%, respectively. However, further work (DNA homology analysis etc.) is necessary to confirm fully the taxonomic positions of these isolates.

Despite the similarities displayed by both RIV-14 and L-15 throughout these studies, there were several physiological traits that distinguished these isolates from each other:

- both are obligately acidophiles, but L-15 was found to be more acidophilic than RIV-14. This was evidenced by the ability of L-15 to grow at pH 0.8, while RIV-14 did not (pH minimum 1.0). Growth of L-15 under extremely acidic conditions was also confirmed by the large number of viable cells observed in the pyrite oxidation experiment, at pH 0.8 (Chapter 6);
- L-15 was more thermotolerant with an observed temperature maximum of 43°C, compared to 37°C for RIV-14

- both isolates could grow heterotrophically, but L-15 was more 'heterotrophically inclined';
- CO_2 fixation by RIV-14 was found to be completely suppressed by the presence of 0.02% (w/v) yeast extract, though 0.1% (w/v) yeast extract was required to completely suppress CO_2 fixation by L-15;
- smaller specific Fe^{2+} oxidation rates were found with RIV-14 than L-15, when grown in the presence of organic compounds;
- isolate L-15 was capable of more efficient leaching of pyrite than RIV-14;
- growth of both isolates under chemolithoheterotrophic conditions was related to ferrous iron concentrations though L-15 showed a positive correlation and RIV-14 a negative correlation with increasing concentrations of ferrous iron;
- although both isolates were facultative anaerobes, RIV-14 displayed a greater propensity for reducing ferric iron. Furthermore, there was some evidence that RIV-14 (but not L-15) is able to grow anaerobically by disproportionation of tetrathionate.

(5)

Characterisation of a Novel Moderately Thermophilic Iron-Oxidising "*Alicyclobacillus*"-like bacterium

5.1 Introduction

Over the past two decades, several unusual thermoacidophilic *Bacillus* species have been isolated and characterised, though the evolutionary and taxonomic position of these isolates remained unresolved until biomolecular methods based on 16S rRNA gene sequence analysis became widely used. Wisotzkey *et al.* (1992) proposed the creation of a new genus, *Alicyclobacillus*, consisting of obligately acidophilic thermophiles which could be separated from other *Bacillus* species by their 16S rDNA sequences and by the presence of ω-alicyclic fatty acids in their cell membranes. Consequently three species, *Bacillus acidocaldarius* (Darland and Brock, 1971), *B. acidoterrestris* and *B. cycloheptanicus* (Deinhard *et al.*, 1987b) were transferred to the genus *Alicyclobacillus*. Based on phylogenetic and fatty acid composition characteristics, a fourth species, *Alicyclobacillus hesperidum*, was recently described; this bacterium is more closely related to *Al. acidoterrestris* than to other *Alicyclobacillus* spp..

All four species of *Alicyclobacillus* are straight, rod-shaped bacteria, which form endospores under adverse environmental or nutritional conditions, and are acidophilic aerobes. A wide range of organic compounds can be used as the sole source of carbon and energy for growth, and the bacteria are obligately heterotrophic. However, they are not as metabolically versatile as *Sulfobacillus* spp. as none of these species are reported to be able to respire on inorganic compounds such as iron or sulphur, which are commonly abundant in bioleaching environments. *Alicyclobacillus* spp. have therefore not been considered in the context of bioleaching of sulphidic minerals. However, two strains of *Alicyclobacillus*-like bacteria (YTH1 and YTH2) isolated from Yellowstone

National Park, Wyoming were capable of ferric iron reduction under microaerobic conditions (Johnson *et al.*, 2000). In addition, some mesophilic iron-oxidising isolates (coded SLC1, SLC2 and SLC66) have also been identified as *Alicyclobacillus*-like bacteria (Johnson, unpublished data). In both cases, there may be a possible role of these bacteria for mineral bioleaching operations.

In the current study, a moderately thermophilic acidophilic bacterium coded GSM was isolated. Analysis of the 16S rRNA gene sequence indicated a relationship to the genus *Alicyclobacillus*. Further characterisations of the isolate was carried out in order to distinguish this isolate from the previously reported species of *Alicyclobacillus* and other acidophilic bacteria.

5.1.1 Bacteria origin and sites of sampling

Samples of weathered regolith (spoil) were collected by two members of the (then) United States Bureau of Mine (USBM), Mr L.J. Froisland and Mr. W.W. White, as part of their activity during a visit to the Golden Sunlight Mine in Whitehall, Montana, during December 1994. Samples, taken in sterile containers, were sent to Dr. D.B. Johnson at the University of Wales, Bangor, in the United Kingdom for isolation and identification of indigenous acidophilic bacteria.

Massive relocation of waste dump materials had been carried out at the Golden Sunlight Mine to alleviate pressure and stop movement of old slide materials which caused distortion in mill configuration and forced a shutdown of processing activity. The relocated Golden Sunlight Mine waste dump rock was generating heat throughout the entire dump, suggesting that it would be an interesting niche for thermophilic bacteria. The dump was composed of layers of waste rock ranging from few inches to 1 – 2 ft. thick, which lay at an angle and varied in texture, particle size and colour. Variations in

these layers were considered to imply either differences in mineral oxidation at different points within the dump, or differences in the origin of the waste rock.

Samples were taken from a total of four pits, labelled as pit 22, 23, 25 and 26; however, only samples from pit 26 will be referred to in this report. Pit 26 lay at an elevation of 552 ft on the top of the dump and was the unexcavated surface of the waste rock dump. A 15 ft. deep cut was made at the top of the first 60 ft. highwall of the dump and revealed the materials along the sides of this cut, which had been in place for about 6 years. Layers in this pit were horizontal and very little angle was apparent. Samples were taken from each layer (of different depth) and all layers were numbered from the bottom of the pit, as summarised below:

Layer 26-1: Gray layer about 1 ft. thick at the bottom of the pit, which contained massive sulphide and large amounts of fine materials.

Layer 26-2: Yellow-brown layer about 3 ft. thick containing lots of small rock particles (1" x 2" with some 3" x 4" rocks), which appeared to be barren shale rock with some sulphide minerals.

Layer 26-3: Yellow-gray layer about 1 ft. thick containing more sulphide minerals than 26-2 but less than 26-1. Small rock particles (<1") dominated this layer.

Layer 26-4: Red layer about 6" thick, but thinning in spots to almost nothing.

Layer 26-5: Gray layer about 9" thick containing course shale rock (between 1" to 3"). More sulphide mineralisation in this layer than 26-4 with the sulphide occurring as fracture filling shale. Open structure with voids between the rocks.

Layer 26-6: Brown layer about 1 ft. thick containing latite porphyry and shale rocks. Smaller rock particles than in 26-5

Layer 26-7: Yellow layer about 1.5 ft. thick.

Layer 26-8: Gray shale layer on top of dump about 6 ft. thick.

5.2 Purification and characterisation of acidophilic bacteria from Golden Sunlight Mine

5.2.1 Isolation and purification

5.2.1.1 Methodology

The dilution spread-plate method was used for isolation of some iron-oxidising acidophiles present in Golden Sunlight Mine spoil samples. Sample of spoil from each layer (section 5.1.1) were firstly sieved to remove large rock particles in the soils. One to two grams of each sample was added to a 250 ml Erlenmeyer flask, containing sterile 200 ml basal salts solution, initially adjusted to pH 2.5 (section 2 2.1), swirled to disperse the soil and homogenised for 30 seconds. The suspensions were then serially diluted (10^{-1}, 10^{-2}, 10^{-3}) in the same basal salts solution. Commencing at the highest dilution, a 100 µl aliquots were spread onto the solid media (in duplicate), specially formulated to support growth of acidophilic bacteria (section 2.2.2). All plates were incubated at 30°C for up to 4 weeks.

Plates were examined and preliminary identification of isolates made on the basis of colony morphologies and cell characteristics. Using the stereo scan microscope (section 2.4.1.1), bacterial colonies that developed on the solid media were counted, and phase-contrast microscope (section 2.4.1.2) was used to record cellular characteristics. Isolates were purified by repeated single colony isolation, onto 10 mM ferrous sulphate / 0.02% (w/v) yeast extract liquid medium (section 2.2.1.1), pH 2.0, incubated at 30°C. Purity of cultures was checked periodically by streaking liquid cultures onto solid media (section 2.2.2),

5.2.1.2 Results

The majority of colonies formed on the overlay solid media were observed as iron-encrusted colonies, which indicated iron oxidation by the acidophilc isolates. However there were also some non iron-oxidising acidophiles observed on the sulphur overlay (FeSo) plates, whose colonies differed from those of the iron-oxidisers in being gelatinous, off-white colonies, showing no evidence of ferric iron staining. Colonies of these 'non iron-oxidising heterotrophic' isolates were also found on Fe/YE non-overlay plates. Six acidophilic isolates were selected, and preliminary identification of these is given in Table 5.1. However, five out of the six isolates were lost during the purification procedures and the single isolate which survived (GSM 3) was the only one studied in more detail. This isolate is referred to throughout this chapter as 'GSM'.

Table 5.1: Some morphological characteristic of the acidophilic bacteria isolated from various pits of waste dump rock in the Golden Sunlight Mine, Montana.

Pit	Isolation routes	Isolate	Cell morphology	Endospores	Iron oxidation	Preliminary identification
26-2	Fe/YE plate → Fe/YE liquid medium	GSM2	Straight rods	-	-	Acidophilic heterotrophs
26-3	FeSo plate → Fe/YE liquid medium	GSM3	Straight filamentous rods	+	+	*Sulfobacillus*-like
26-5	Fe/YE plate → Fe/YE liquid medium	GSM5	Straight rods	+	-	*Alicyclobacillus*-like
26-6	FeSo plate → Fe/YE liquid medium	GSM6	Straight rods	+	-	*Alicyclobacillus*-like
26-7	FeSo plate → Fe/YE liquid medium	GSM7	Straight rods	+	+	*Sulfobacillus*-like
26-8	FeTSB0 plate → Fe liquid medium	GSM8	Straight filamentous rods	+	+	*Sulfobacillus*-like

5.2.2 Characterisation of isolate GSM on solid and liquid media

5.2.2.1 Methodology

A pure culture of isolate GSM was grown in 10 mM ferrous sulphate liquid medium (section 2.2.1.1), pH 2.0, supplemented with 0.02% yeast extract, at 30°C. The active pure culture was inoculated into 100 ml shake flasks containing 50 ml of 10 mM ferrous iron medium (section 2.2.1.1), pH 2.0, supplemented (or not) with 0.02% (w/v) yeast extract, and the same culture was also streaked onto ferrous iron and ferrous iron/tetrathionate overlay and yeast extract non-overlay solid media (section 2.2.2). Cultures in liquid and on solid media were incubated at various temperatures (30°, 37° and 45°C).

Growth was observed by the oxidation of ferrous iron in the liquid media (*ca* 2 – 3 days) and colony formation on plates (*ca* 7 – 21 days). Cellular and colony characteristics were observed using phase-contrast (section 2.4.1.2) and stereo microscopes (section 2.4.1.1), respectively. A specimen for scanning electron microscope (section 2.4.2) observation was also prepared.

5.2.2.2 Results

In this preliminary characterisation, GSM was found to be capable of growing at all temperatures tested (30°, 35° and 45°C), both on solid and in liquid media. Despite being isolated at 30°C, growth at 45°C indicated that GSM was a thermophile. In order to obtain a pure culture of GSM, single colony isolation (from 45°C plates) was repeated (incubated at 30°C and 37°C for comparison) and the purity was confirmed by comparing the colonies. Similar colonies were found at lower temperature (30° and 37°C), as at 45°C. Better growth was observed in yeast extract-containing than in organic-free liquid medium.

Phase-contrast observation showed that GSM was a non-motile, straight rod-shaped bacterium, with approximate dimensions of 2.6 ± 0.35 μm length and 0.74 ± 0.13 μm width, as shown by scanning electron micrographs (Fig 5.1). This bacterium also formed endospores, most notably when grown in organic-free medium. The spores were oval in shape and located at the ends of the individual rods (Fig 5.1).

Isolate GSM was capable of growing on both overlay (Feo and FeSo) and non-overlay (Fe/YE) solid media. Colonies grown on different solid media displayed different characteristics, especially those on non-overlay plates. On ferrous sulphate-overlay (Feo) plates, GSM initially grew as small (*ca* $\geq$ 0.5 mm) heavily ferric iron-stained colonies after 3 to 4 days incubation at 45°C. With prolonged incubation, the

colonies grew larger (ca 1 mm) and became increasingly raised and covered with ferric iron precipitates on the surface of individual colonies (Fig. 5.2a). On ferrous sulphate/tetrathionate overlay (FeSo) plates, GSM initially (3-5 days) grew as flat round, off-white gelatinous colonies with slight brown-staining in the centre. These colonies became more raised (domed), and ferric-encrusted during extended incubation (7-10 days) at 45°C, similar to those grown on Feo plates. GSM grown on Fe/YE non-overlay plates was readily distinguished by the formation of flat, round colonies with irregular edges which were off-white and gelatinous during early growth (3 - 4 days), though ferric iron staining was evident in the centre of colonies with prolonged incubation (7 – 10 days) at 45°C (Fig 5.2b)

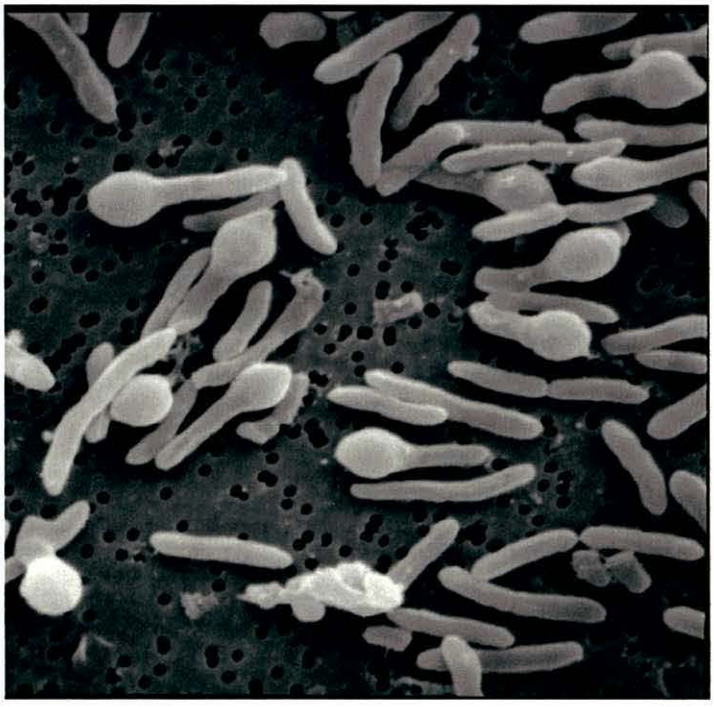
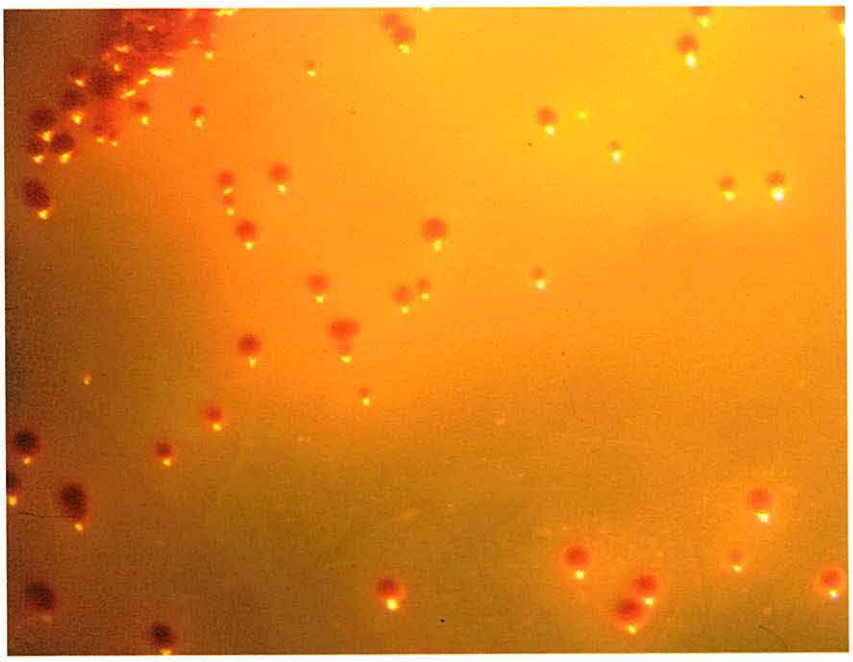


Fig. 5.1: Scanning electron micrograph of GSM grown in basal salts (pH 1.8), containing 10 mM glycerol, 1 mM ferrous sulphate and 0.5 mM tetrathionate at 4°C. Cells were observed during the late exponential phase

(a)



(b)

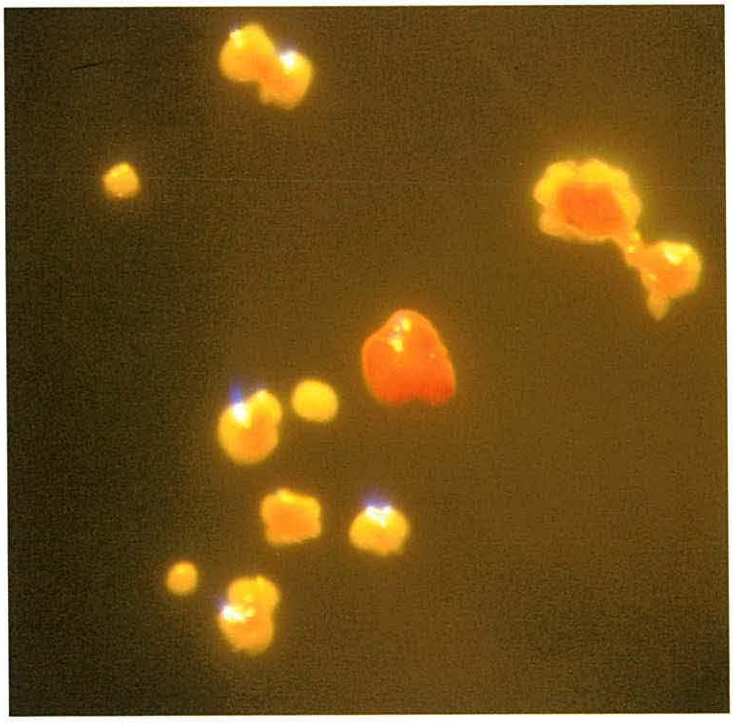


Fig. 5.2: Colonies of GSM grown on (a) Feo overlay, (b) Fe/YE non overlay solid media. Plates were incubated at 45°C for up to 14 days.

5.3 Utilisation of defined organic compounds by isolate GSM

The heterotrophic (or mixotrophic) nature of GSM was indicated from its initial isolation, where satisfactory growth occurred in yeast extract-containing medium rather than in its absence. In a separate test, GSM was found to grow on glycerol, though growth was greatly enhanced by the inclusion of tetrathionate and ferrous iron at very low concentration (0.5 – 1 mM). It was of interest to test the capability of this isolate to utilise defined organic compounds, rather than yeast extract, to support its growth.

5.3.1 Growth on various organic substrates

5.3.1.1 Methodology

A pure culture of GSM was grown in a 100 ml shake flask containing 50 ml of sterile heterotrophic basal salts (section 2.2.1.3), pH 1.8, supplemented with 10 mM glycerol, 1 mM ferrous iron and 0.5 mM potassium tetrathionate, incubated at 45°C. Cells were harvested by centrifugation (10,000 r.p.m., 10 min, 4°C) washed and resuspended in sterile pH 2 basal salts. GSM was then inoculated (2% v/v) into universal bottles containing 5 ml of the heterotrophic basal salts (pH 1.8) containing 1 mM ferrous iron and 0.5 mM tetrathionate and the medium was supplemented with one of the following organic substrates: (i) Sugars (at 5 mM final concentration each): glucose, fructose, ribose, arabinose, mannose, xylose, galactose, glucosamine, glucuronic acid and galacturonic acid, (ii) Alcohols: glycerol (10 mM), mannitol (5 mM), ethanol (15 mM) and methanol (20 mM), (iii) Organic acids: citric acid, acetic acid , succinic acid, pyruvic acid and glutamic acid (10 mM final concentration except for glutamic acid, added at 5 mM), (iv) others: phenol (1 mM), benzyl alcohol (1 mM) and glycine (10 mM). The concentration of each organic substrate added was calculated to give similar amounts of organic carbon in the complete growth media.

Cultures grown in ferrous iron / tetrathionate medium and the same medium amended with 10 mM glycerol were used as the base-line and positive controls, respectively, and all cultures were set up in duplicate. Cultures were incubated in a shaking incubator (150 r.p.m.) at 45°C, for up to 7 days. Growth was assessed by measuring the optical density (OD) at 600 nm against a distilled water blank, and results were recorded as positive or negative growth by comparing the OD values with those of the reference cultures.

5.3.1.2 Results

Most organic substrates tested were found to be capable of supporting the growth of GSM, as summarised in Table 5.2. Bacterial growth was recorded semi-quantitatively, based on the comparison between culture optical densities (ODs) of the base-line control culture in ferrous iron / tetrathionate medium (OD = 0.021): (i) +: ODs were 25% greater than the base-line control, (ii) ++: ODs were 0.025 units more than the base-line control, (iii) +++: ODs were 0.1 units more than the base-line control, (iv) I: growth was inhibited by the substrate, indicated by ≥ 25 decrease in ODs compared to the base-line control, and (v) -: no growth was observed, indicated by less than 25% increase or decrease of culture ODs compared to the base-line control.

All of the sugars tested, with the exception of arabinose, supported the growth of GSM. Growth was found to be inhibited by both ethanol and methanol. GSM was also capable of utilising a range of organic acids for growth, particularly citric and glutamic acids. Growth of GSM was observed in medium containing 1.5 mM acetic acid though not in 10 mM acetic acid, indicating that this acid (and also succinic acid) was toxic to the bacterium at the higher concentration.

Table 5.2: Utilisation of organic compounds by isolate GSM, in media supplemented with 1 mM ferrous iron and 0.5 mM tetrathionate (n = 2).

Substrates	Growth as compared with the experimental references
i) Sugars	
Glucose	+++
Fructose	+++
Ribose	++
Arabinose	-
Mannose	+++
Xylose	+++
Galactose	+++
Glucosamine	++
Glucuronic acid	++
Galacturonic acid	++
ii) Alcohols	
Glycerol	+++
Mannitol	++
Ethanol	I
Methanol	I
iii) Acids	
Citric acid	+++
Acetic acid: 10 mM	-
1.5 mM	++
Succinic acid	-
Pyruvic acid	++
Glutamic acid	+++
iv) Other compounds	
Benzyl alcohol	-
Phenol	-
Glycine	+

+++ : OD 0.1 unit > base-line control
++ : OD 0.025 unit > base–line control
+ : OD 25% > base-line control
- : OD < 25% greater or less than the baseline control
I : Inhibitory; OD > 25% lower than base-line control

5.4 Effect of some heavy metals on growth of GSM

5.4.1 Methodology

A pure culture of GSM was grown in a 100 ml Erlenmeyer flask containing 50 ml of 10 ml ferrous sulphate medium (section 2.2.1.1), pH 1.8, supplemented with 0.02% (w/v) yeast extract. During the late exponential growth phase, cells were harvested by centrifugation (10,000 r.p.m. for 10 min at 4°C) washed and resuspended in sterile pH 1.8 basal salts (section 2.2.1).

The cell suspension was then inoculated (1% w/v) into heterotrophic basal salts (section 2.2.1.3), pH 1.9, supplemented with 3 mM glucose, 2 mM ferrous sulphate and 1 mM potassium tetrathionate. The effect of heavy metal on growth of GSM on glucose was tested in the presence of various concentrations of copper and zinc (10, 25, 50, 100, 200 and 300 mM), both added as sulphate salts, and of molybdate (Na_2MoO_4: 0.05, 0.1, 0.2, 0.5 and 1.0 mM). Cultures were set up in duplicate universal bottles containing 5 ml of liquid medium. Two control cultures were also prepared by growing the bacterium in the medium containing 2 mM ferrous sulphate and 1 mM tetrathionate, amended (positive control) or not (base-line control) with 3 mM glucose. All cultures were incubated at 45°C with shaking (150 r.p.m.) for up to 14 days. Growth of GSM was confirmed by viable cell counts (section 2.3.3) on Fe/YE non overlay solid medium (section 2.2.2.3) and phase contrast microscopic observation was also carried out to examine cells for any morphological changes (sporulation etc.; section 2.4.1.2).

5.4.2 Results

In general, the sensitivity of GSM to the metallic ions tested was less than the previously studied mesophilic iron-oxidising heterotrophs, "*F. acidophilum*" T23 and isolate SLC2 (Bacelar-Nicolau, 1996), though it was much more sensitive to Cu^{2+} ions than the chemolithotrophic iron-oxidising mesophile, *T. ferrooxidans*. As with the

heterotrophic mesophiles, GSM was also found to be more tolerant to molybdate than *T. ferrooxidans*.

Tolerance of GSM to the metals tested is shown as the minimum inhibitory concentrations (MICs) in Table 5.3. The MICs were determined from viable cell counts of GSM using ferrous iron/yeast extract solid medium (Fig 5.3), compared with numbers in the positive control culture (6.3 x 10^8 cell/ml). Growth was found to be totally inhibited by 300 mM Cu^{2+} and 1 mM MoO_4^{2-}.

Table 5.3: Tolerance of GSM to some metals ion in comparison with *T. ferrooxidans* and two heterotrophic strains of iron-oxidising mesophiles

Bacteria	MIC (mM)		
	Cu^{2+}	Zn^{2+}	MoO_4^{2-}
GSM	>200	>300	>0.5
T. ferrooxidans (ATCC 23270)*	>300	>300	0.2
SLC2**	150	NA	0.4
"*F. acidophilum*" T23**	150	NA	0.8

*: this study, MIC was determined based on iron-oxidising activity
**: Bacelar-Nicolau, 1996)
NA: not attempted in the study
'>': means that the minimum concentration required to inhibit bacterial growth is greater than the value indicated (the highest concentration tested).

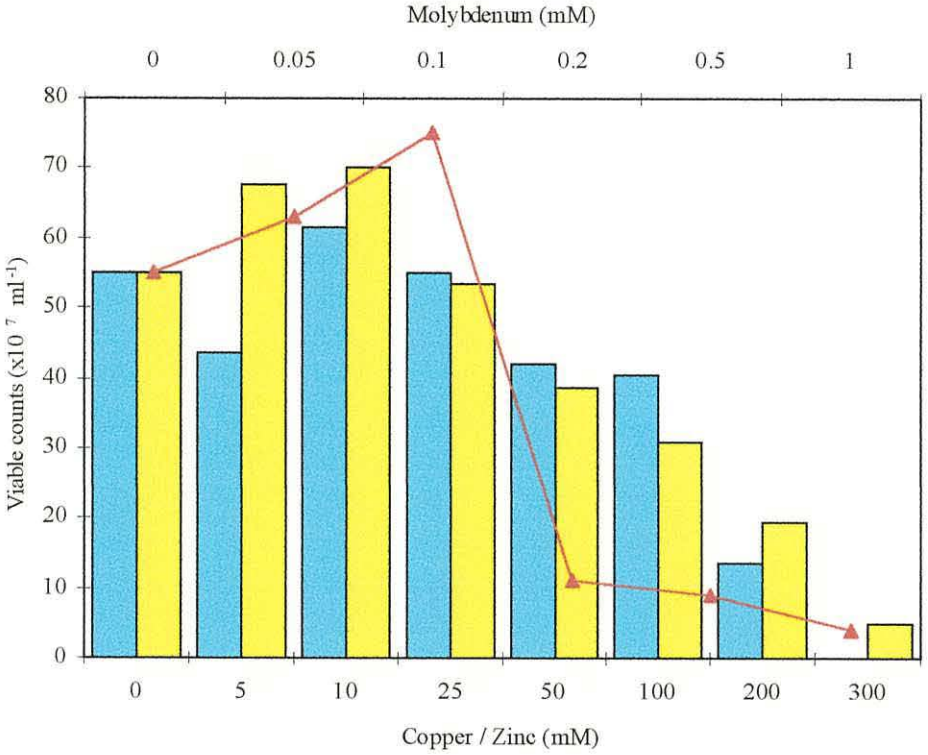


Fig. 5.3: Effects of some heavy metals on growth of the moderately thermophilic isolate GSM, tested by growing the bacterium on various concentration of copper ( ), zinc ( ) and molybdenum (▲). Growth yields (as viable cell counts) were assessed by plating onto Fe/YE solid medium. Cells numbers in the glycerol-free control culture were ~ 7 x 10^7 ml^{-1}

5.5 Aerobic and anaerobic growth of GSM on iron

Growth of GSM was stimulated by the addition of organic substrates to the growth medium (section 5.3.1.2). An experiment was carried out to study whether ferrous iron oxidation and organic substrate utilisation by GSM were coupled during growth. In addition, the ability of GSM to respire anaerobically using ferric iron as electron acceptor was assessed.

5.5.1 Oxidation of ferrous iron and organic substrate utilisation

5.5.1.1 Methodology

In this experiment, fructose was used as the sole organic substrate in ferrous iron containing medium. GSM was grown in 50 ml heterotrophic basal salts (section 2.2.1.3), pH 1.9, supplemented with 10 mM ferrous sulphate and 0.02% (w/v) yeast extract. Cells were harvested by centrifugation (10,000 r.p.m. for 15 min. at 4°C) at two different phases of growth; (i) mid-exponential phase, at which point there was no evidence of ferrous iron oxidation, and (ii) stationary phase, after complete oxidation of ferrous iron. Cells were washed and resuspended in sterile pH 2.0 basal salts (section 2.2.1). The two cell suspensions were used to inoculate (1% v/v) duplicate 100 ml shake flasks containing 50 ml of sterile heterotrophic basal salts (pH 1.9), supplemented with 10 mM fructose, 0.5 mM tetrathionate and 20 mM iron. Cultures were incubated at 45°C and continuously shaken at 150 r.p.m..

Samples were taken regularly to measure concentrations of ferrous iron (ferrozine assay; section 2.6.1.3) and fructose (anthrone assay; section 2.6.4). Biomass yields were determined as total cell counts using the Thoma bacteria counting chamber (section 2.3.2.2). Culture purity was also tested at the end of the experiment by plating onto yeast extract solid media (section 2.2.2.3).

5.5.1.2 Results

The two processes i.e. ferrous iron oxidation and fructose utilisation by GSM are not coupled. This was shown for both cultures inoculated with either cells harvested at the mid-exponential (Fig.5.4a) or the stationary (Fig 5.4b) growth phases. However, the strong correlation between growth of GSM and fructose utilisation indicated that these two processes were closely coupled.

This bacterium did not utilise both substrates simultaneously, and lag periods of either iron oxidation or fructose utilisation were detected. A long lag period of iron oxidation and immediate used of fructose was observed in cultures inoculated with cells harvested at the mid-exponential growth phase. In contrast, a completely reversed response was displayed in cultures where cells harvested at the stationary growth phase were used as an inoculum.

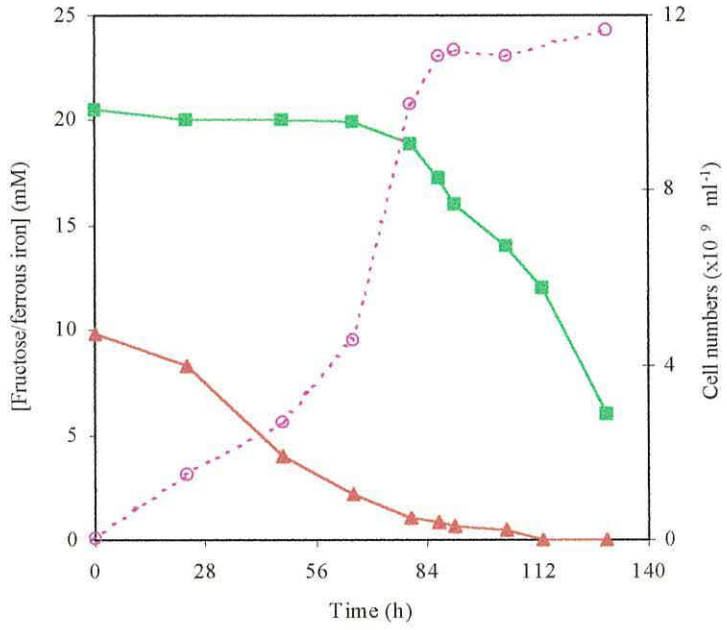


Fig 5.4a: Relationship between ferrous iron oxidation (■), fructose utilisation (▲) and growth (○; broken lines) of the moderately thermophilic isolate GSM, using cells harvested at the mid-exponential phase as inoculum.

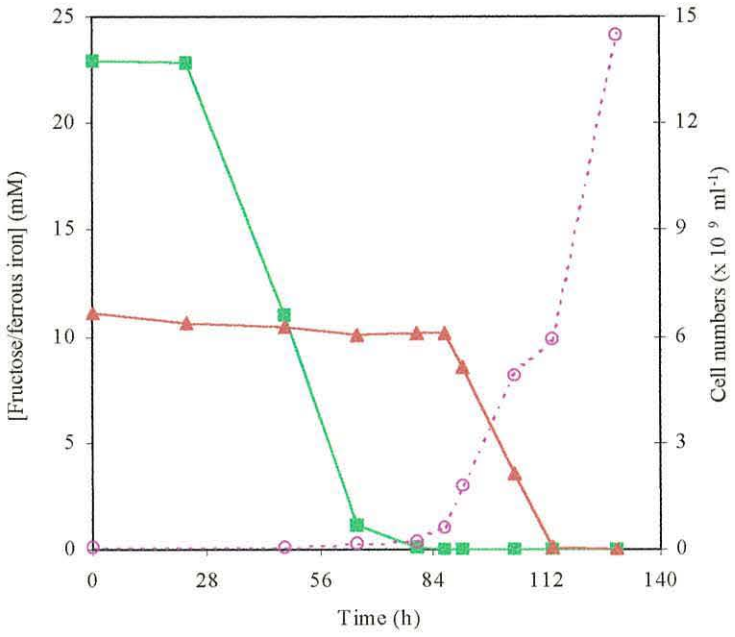


Fig 5.4b Relationship between ferrous iron oxidation (■), fructose utilisation (▲) and growth (○; broken lines) of the moderately thermophilic isolate GSM, using cells harvested at the stationary phase as inoculum.

5.5.2 Ferric iron reduction in anaerobic cultures

5.5.2.1 Methodology

Isolate GSM was grown in a 100 ml Erlenmeyer flask containing 50 ml of heterotrophic basal salts (section 2.2.1.3), pH 1.8, supplemented with 10 mM fructose, 1 mM ferrous sulphate and 0.5 mM tetrathionate, incubated at 45°C, unshaken. Cells were harvested during the late exponential growth phase by centrifugation (10,000 r.p.m. for 10 minutes at 4°C) and resuspended in sterile pH 1.8 basal salts.

The cell suspension was then inoculated (1% v/v) into serum bottles containing sterile 50 ml heterotrophic basal salts (section 2.2.1.3) pH 1.8, amended with 10 mM fructose, 0.5 mM tetrathionate and various concentrations of ferric iron (0, 5, 10 and 25 mM). In order to establish anaerobic conditions, the medium used in this experiment was deoxygenated by gassing with nitrogen (N_2) prior to heat sterilisation.

Duplicate cultures were incubated at 45°C. Ferric iron reduction was monitored by measuring concentrations of ferrous iron, using the ferrozine assay (section 2.5.1.3). Growth was determined by measuring optical densities at 600 nm (OD 600nm) and total cell counts using the Thoma bacterial counting chamber (section 2.3.2.2).

5.5.2.2 Results

Iron reduction was observed with GSM grown heterotrophically in the presence of ferric iron and fructose as electron donor. However, the capacity for anaerobic respiration was less than noted for the mesophilic Gram-positive Montserrat isolates, RIV-14 and L-15 (section 4.3.3.2). Incomplete reduction of ferric iron was observed with cultures containing 25 mM ferric sulphate, and smaller numbers of cell were also found with GSM (5.8×10^7 cell/ml) than for both RIV-14 (3×10^8 cell/ml) and L-15 (2.6×10^8 cell/ml), after a protracted incubation (~ 4 weeks). A linear relationship between growth and iron reduction was observed isolate GSM (Fig. 5.5).

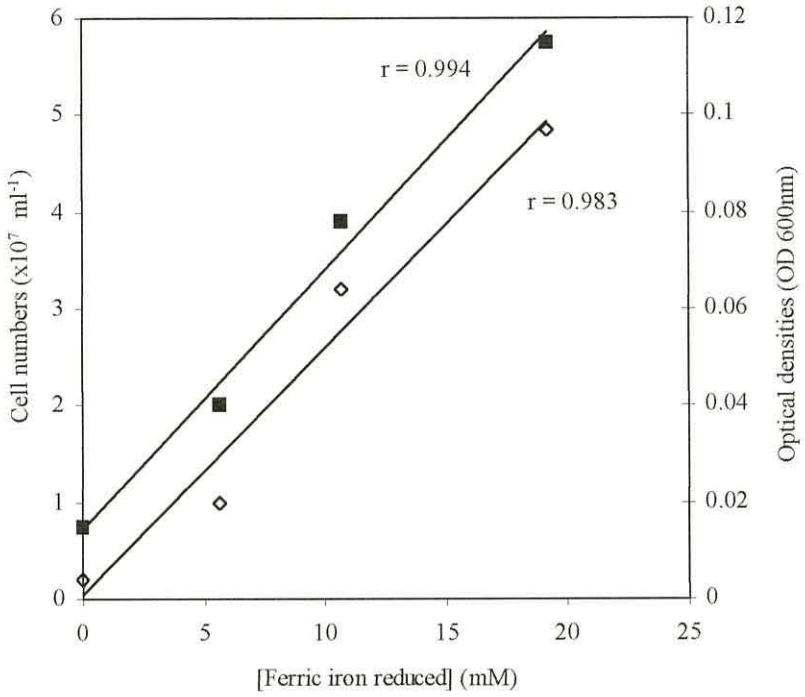


Fig. 5.5: Reduction of ferric iron by the moderately thermophilic iron-oxidising isolate GSM grown anaerobically on various concentrations of ferric iron, with fructose as electron donor. Growth of the bacterium was determined by measuring total cell numbers (◆) and optical densities at 600nm (◇)

5.6 Reduced inorganic sulphur compound oxidation by GSM

Two major facets of acidophilic bacteria in bioleaching processes are their capacities to oxidise iron and/or sulphur. The ability of GSM to oxidise iron had been demonstrated (section 5.4), and therefore an experiment was carried out to assess the ability of GSM to utilise inorganic sulphur as the main source of energy under two growth conditions (with and without the additional of glycerol).

5.6.1 Oxidation of elemental sulphur and tetrathionate

5.6.1.1 Methodology

A pure culture of GSM was grown in basal salts medium (section 2.2.1) pH 1.8, supplemented with 0.02% (w/v) yeast extract and 5 mM ferrous sulphate. During the late

exponential growth phase, cells were harvested by centrifugation (10,000 r.p.m. for 15 minutes at 4°C), washed and resuspended in sterile pH 2.0 basal salts solution.

The cell suspension was then inoculated (1% v/v) into duplicate 100 ml Erlenmeyer flasks containing sulphur medium (section 2.2.1.2) amended or not with 1 mM glycerol, and the medium adjusted at pH 3.0. Flasks contained either 0.4 g/l (≡ 12.5 mM) elemental sulphur or 2 mM tetrathionate. Cultures were incubated at 45°C, shaken at 150 r.p.m.. Samples were removed regularly and bacterial growth was estimated by cell counts using the Thoma bacteria counting chamber (section 2.3.2.2). Sulphur oxidation was monitored by measuring changes in pH throughout incubation. Concentrations of tetrathionate and thiosulphate were measured by cyanolysis, as described in section 2.6.3.

5.6.1.2 Results

Isolate GSM was able to oxidise both elemental sulphur and tetrathionate. In both cases, enhanced oxidation was observed in media containing 1 mM glycerol. Sulphur oxidation is an acid-producing reaction, which resulted in a reduction of pH during growth. In this experiment, elemental sulphur was not analytically monitored, though the concentration of sulphur was determined based on the stoichiometry:

$$S + H_2O + 1.5O_2 \rightarrow 2H^+ + SO_4^{2-} \qquad (5.1)$$

Based on reaction (5.1) equivalent sulphur concentrations were calculated from proton concentrations, derived from pH measurement ($pH = -\log_{10}[H^+]$). Complete oxidation of 12.5 mM sulphur was recorded in the organic-containing cultures while limited oxidation (4.7 mM) was detected in the autotrophically-grown culture (Fig. 5.6b). In both cases, tetrathionate was found to accumulate in the glycerol-free cultures throughout the experiment (Fig 5.6b). Growth was coupled with pH reduction, which was also found to

be significantly greater in the presence of glycerol, with about 10-fold increase in final cell numbers (Fig 5.6a).

A similar pattern of oxidation was observed in tetrathionate cultures, though negligible tetrathionate utilisation was detected in glycerol-free medium. In contrast, inclusion of glycerol resulted in rapid and complete utilisation of the reduced sulphur compound, which was coupled to bacterial growth (Fig 5.7a). Oxidation of tetrathionate resulted in the production of detectable amounts of thiosulphate. In the presence of glycerol, the amount of thiosulphate produced during growth declined during incubation (Fig. 5.7b). Reduction of pH as a consequence of tetrathionate oxidation was more apparent in the culture to which glycerol was added.

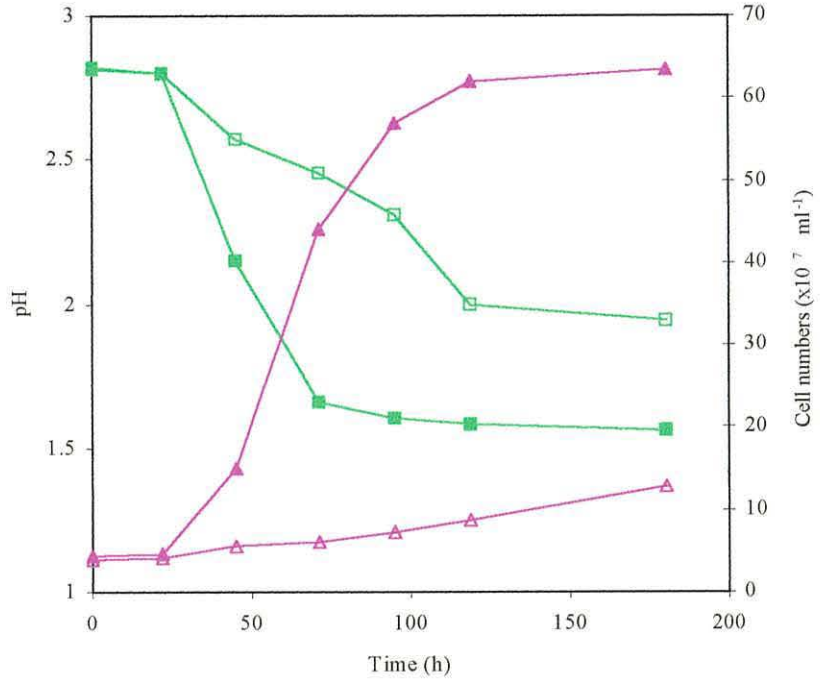


Fig 5.6a: Oxidation of elemental sulphur by GSM in the presence (solid symbols) and absence (open symbols) of 1 mM glycerol. Changes in culture pH (■□) and cell numbers (▲△) with time were recorded.

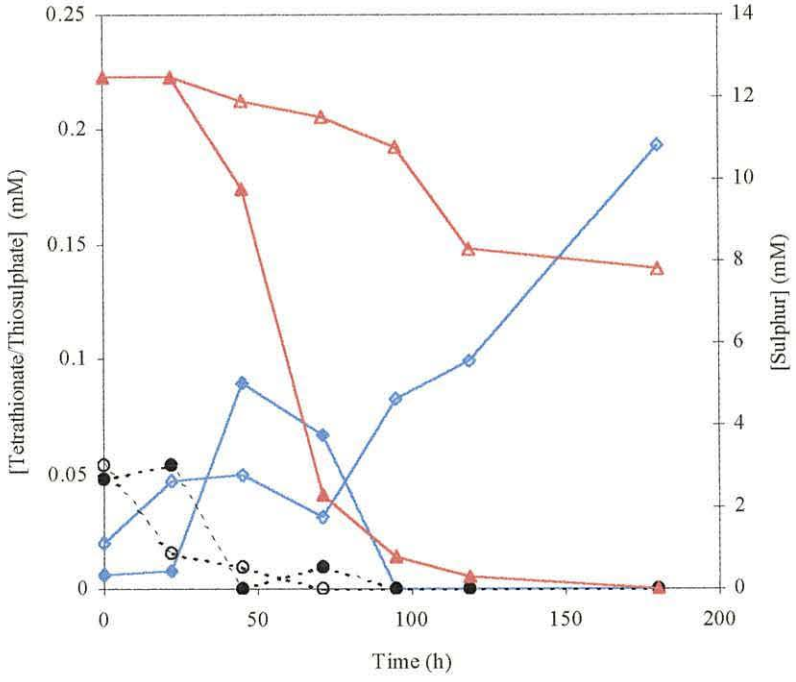


Fig. 5.6b: Tetrathionate (◆◇) and thiosulphate (●○; broken lines) produced as intermediates during elemental sulphur oxidation by GSM. Calculated data for sulphur concentrations are also shown (▲△). Solid symbols represent data from cultures containing 1 mM glycerol; open symbols, data from glycerol-free cultures

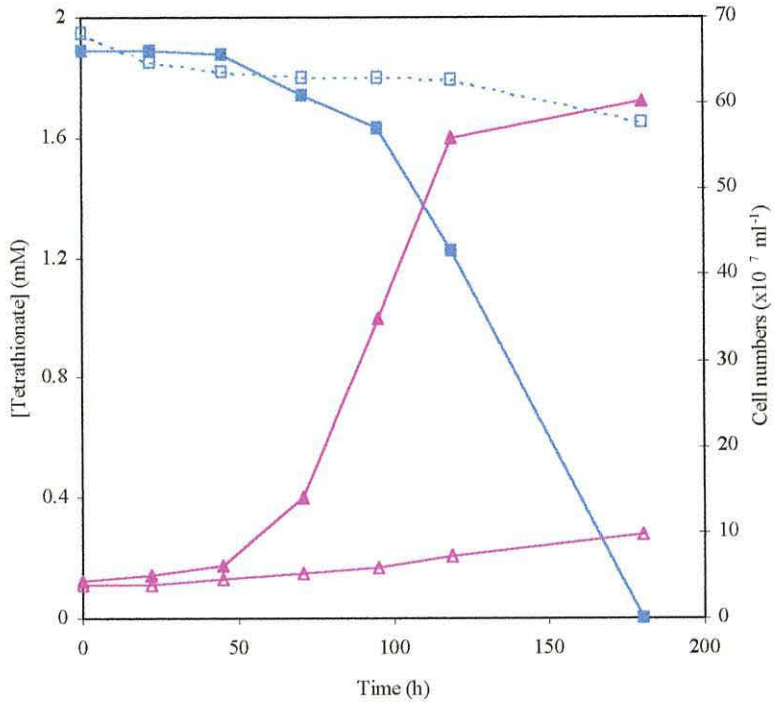


Fig 5.7a: Oxidation of tetrathionate by GSM in the presence (solid symbols) and absence (open symbols) of 1 mM glycerol. Key: Tetrathionate (■□; solid lines), cell numbers (▲△; broken lines).

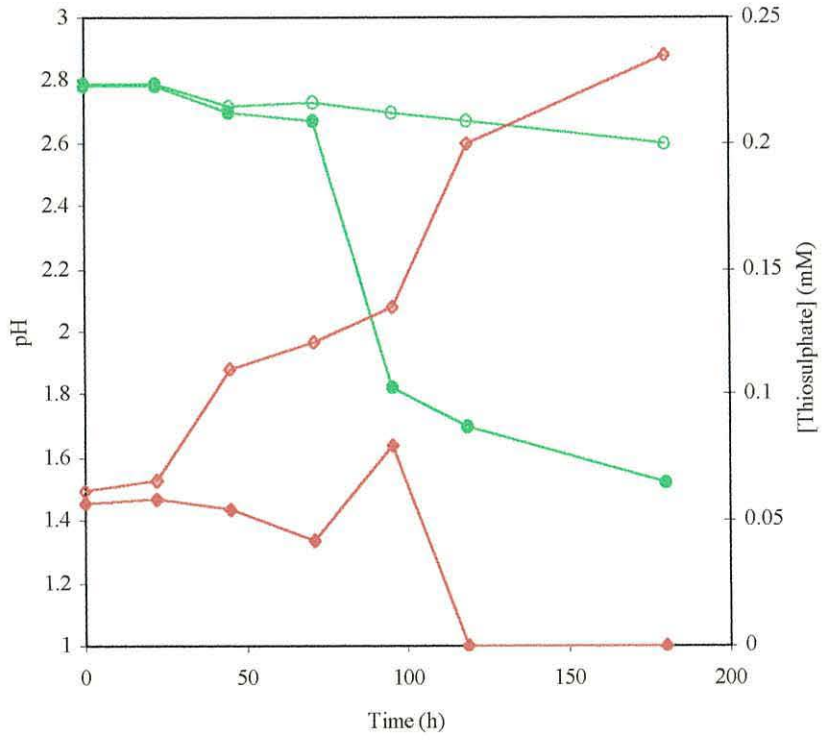


Fig. 5.7b: Production of thiosulphate (◇◆; broken lines) and changes in pH (●○) during tetrathionate oxidation in cultures of GSM supplemented (solid symbols) or not (open symbols) with 1 mM glycerol.

5.7 Carbon dioxide incorporation

5.7.1 Methodology

Isolate GSM was grown in a shake flask containing 50 ml of 10 mM ferrous sulphate medium (section 2.2.1.1) pH 1.8, supplemented with 0.02% (w/v) yeast extract. During the late exponential growth phase, cells were harvested by centrifugation (10,000 r.p.m. for 15 minutes at 4°C), washed twice and resuspended in a sterile pH 2.0 basal salts solution (section 2.2.1).

The cell suspension was then inoculated (1% v/v) into duplicate 100 ml Erlenmeyer flasks containing 50 ml of 25 mM ferrous sulphate medium (initially poised at pH 1.6), supplemented with either 0.02% (w/v) yeast extract or 2.5 mM tetrathionate. The inoculated flasks were sealed (Suba seals) and cultures were amended with 1% (v/v) of 0.5 M $NaH[^{14}C]O_3$ (Amersham Ltd) with a specific activity of 10,324 dpm/µmol. Cultures were incubated in a shaking (120 r.p.m.) incubator at 45°C.

Samples of cultures were taken regularly to measure ferrous iron concentrations (section 2.6.1.1 and 2.6.1.2) and carbon dioxide fixation (section 2.5). Biomass production was monitored by cell counts using the Thoma bacteria counting chamber (section 2.3.2.2). The relationship between the two processes was determined by plotting CO_2 fixed (nmoles/ml) against the concentration of ferrous iron oxidised (µmoles/ml).

5.7.2 Results

GSM was found to be capable of utilising either inorganic (carbon dioxide) or organic (yeast extract) carbon (Fig 5.8). In yeast extract-containing cultures, CO_2 fixation was initially suppressed, though accelerated during the latter phase of iron oxidation. This effect could be related to the depletion of organic substrate in the growth medium, resulting in switching of carbon assimilation by GSM from the organic compounds (yeast extract) to inorganic carbon dioxide.

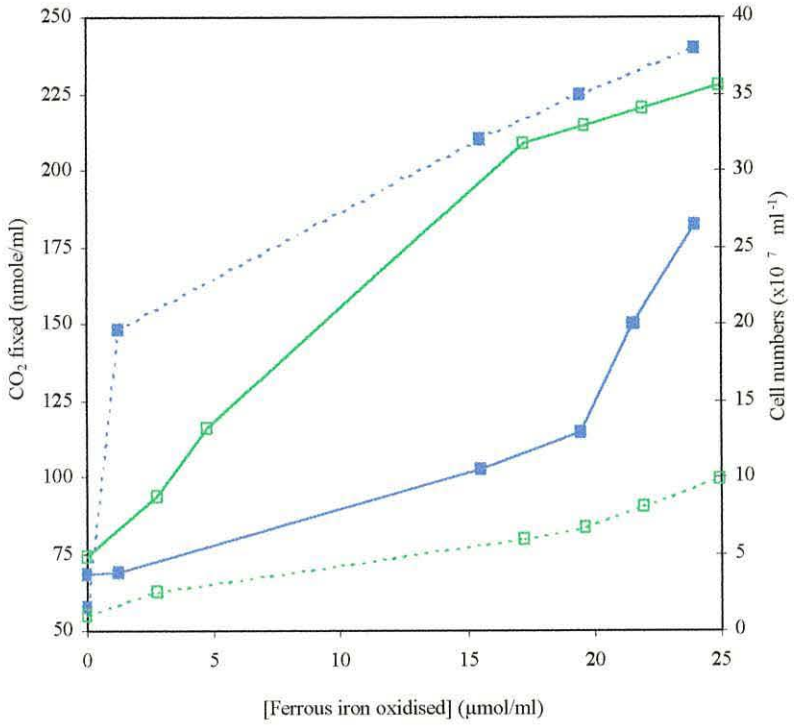


Fig. 5.8: Incorporation of carbon dioxide by isolate GSM grown in 25 mM ferrous sulphate medium supplemented with either 0.02% (w/v) yeast extract (■; solid lines) or 2.5 mM tetrathionate (□; solid lines). Bacterial numbers are indicated by the broken lines.

In contrast to the partial suppression observed in the presence of yeast extract GSM was able to meet its carbon requirement by fixing carbon dioxide in organic-free medium. In this case, GSM obtained its energy for growth from the oxidation of ferrous iron. Despite displaying efficient CO_2 fixation in the organic-free medium, the limited energy obtained from the oxidation of ferrous iron in the autotrophic cultures resulted in significantly lower biomass yields (approximately 4-fold) than in yeast extract-containing medium.

5.8 Controlled (bioreactor) growth of GSM

5.8.1 Effect of temperature on growth rates

5.8.1.1 Methodology

This experiment was carried out in a 2 l capacity bioreactor with a working volume of 1.5 l, (an Electrolab bioreactor fitted with a unit controller of temperature and pH). The pH was automatically controlled using 1 M solutions of sulphuric acid (H_2SO_4) and sodium hydroxide (NaOH).

GSM was grown in a 250 ml Erlenmeyer flask containing 150 ml of sterile heterotrophic basal salts (section 2.2.1.3); pH 1.9, supplemented with 10 mM fructose, 0.5 mM tetrathionate and 1 mM ferrous sulphate, incubated (shaken) at 45°C. The active culture was then inoculated (10% v/v) into the bioreactor vessel, which contained 1.4 l of sterile heterotrophic basal salts, initially equilibrated at 45°C; pH 1.9 and amended with the same concentration of fructose, tetrathionate and ferrous sulphate as in the inoculating culture. The culture was grown with continuous aeration (0.5 l/minute) and was stirred at 100 r.p.m., throughout the experiment. Temperature was set at 35°, 40°, 45°, 47° and 50°C, and each experiment was duplicated. In subsequent experiments, 10% of the residual culture volume was used as inoculum and fresh medium was added to make up a final volume of 1.5 l.

Samples were withdrawn at regular intervals and growth of GSM was monitored by measured the optical densities at 600 nm (OD 600nm). Semi-logarithmic plots of OD 600nm against time were to determine the growth rates of GSM at the various temperatures tested.

5.8.1.2 Results

Optimum growth of GSM was determined to be 45°C (Fig 5.9), with an equivalent culture doubling time of around 10.7 h. This confirmed that GSM is a moderate thermophile. However, the maximum and minimum temperatures supporting growth of GSM were not determined in this experiment, though the T_{max} is > 50°C.

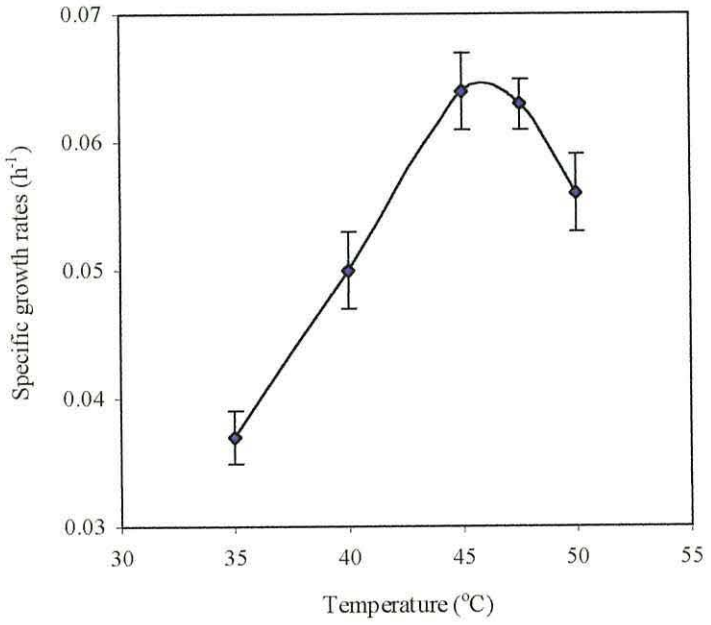


Fig. 5.9: Effect of temperature on the growth rates of isolate GSM based on optical densities at 600 nm of fructose-grown cultures, grown in a bioreactor at pH 1.8.

5.8.2 Effect of pH on growth rates

5.8.2.1 Methodology

Growth of GSM as a function of pH was studied using a similar set up to that described in section 5.7.1.1. Temperature was fixed at 45°C throughout this experiment, and growth of GSM was monitored at various pH values (between pH 1.6 – 2.0).

An active pure culture of GSM grown in a shake flask containing heterotrophic basal salts (section 2.1.1.3) pH 1.9, supplemented with fructose, tetrathionate and ferrous sulphate (section 5.7.1.1) was used as the inoculum. The bioreactor, containing 1.4 l of

medium, was equilibrated at 45°C and pH was controlled at 1.9 prior to inoculation (10% v/v).

Cultures were grown with continuous aeration (0.5 l/min) and agitation (150 r.p.m.). The same procedure was followed as in section 5.7.1.1, except that temperature was fixed at 45°C and pH was fixed at between pH 1.6 to 2.0, using 1 M of H_2SO_4 or 1 M NaOH. Experiments were carried out in duplicate and growth rates of GSM at the pH values were calculated as described earlier.

5.8.2.2 Results

The effect of pH on the growth rates of GSM grown in fructose medium was carried out at 45°C, which was the optimum temperature (section 5.7.1.2). Optimum growth was determined to be pH 1.8 with equivalent culture doubling time of around 8.3 h (Fig 5.10). However, this study did not attempt to determine the maximum and minimum pH that permitted growth of the bacterium.

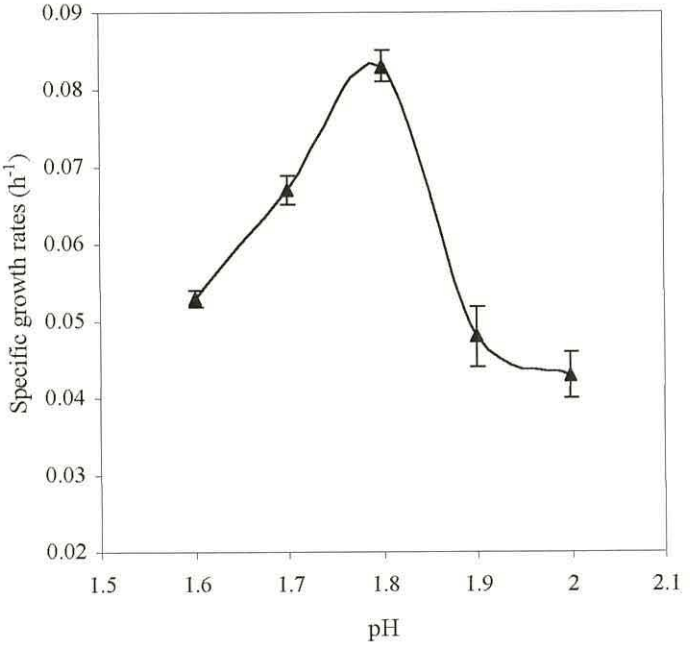


Fig. 5.10: Effects of pH on the growth rates of isolate GSM based on optical densities at 600 nm of fructose grown culture, studied in a bioreactor at 45°C.

5.9 Molecular characterisation of the moderately thermophilic isolate GSM

5.9.1 Phylogeny based on the comparative analysis of 16S rRNA gene sequence

Isolate GSM was grown in a 50 ml liquid medium (pH 1.8) supplemented with 10 mM fructose, 1 mM ferrous sulphate and 0.5 mM tetrathionate (section 2.2.1.3). Cells were harvested by centrifugation (10,000 r.p.m. for 10 min at 4°C) and washed in TE buffer (10 mM Tris-HCl + 1 mM EDTA; pH 8.0).

Chromosomal DNA (cDNA) extraction and purification from GSM was carried out following the method of Wilson (1987; section 2.8). PCR amplification of the 16S rRNA gene fragment was set up following the procedure described in section 2.9, using purified cDNA (0.5 – 1 µl) as the template. Cloning of the amplified 16S rRNA gene was carried out using the TOPO TA cloning kit (section 2.9.1), and positive clones were screened by RFLP (section 2.9.3) and PCR (section 2.9.2) analysis. A positive transformant was grown in a fresh LB liquid medium with ampicillin selection for plasmid DNA preparation (section 2.9.4) followed by sequencing (section 2.9.4.1). Sequence data were analysed and a phylogenetic tree was constructed, as described in section 2.9.4.2.

5.9.1.2 Results

A nearly complete sequence of the 16S rRNA gene (1451 bp) was obtained from the moderately thermophilic iron-oxidising isolate, GSM. Comparison of this sequence with other sequences in the Genbank indicated a close relationship to members of the genus *Alicyclobacillus*. According to the BLAST search, the closest relationship to GSM was the 16S rRNA gene from *Al. cycloheptanicus*, with which it had 93% sequence identity.

Using aligned 16S rRNA gene sequences of GSM and other bacteria, a phylogenetic tree was constructed (Fig 5.11). Included in the nearest cluster of bacteria

related to GSM were a putative iron-oxidising acidophilic bacterium, "*S. thermosulfidooxidans*" strain K1 (Karavaiko *et al.*, 2000), and iron-reducing isolates YTH1 and YTH2 (Johnson *et al.*, 2000).

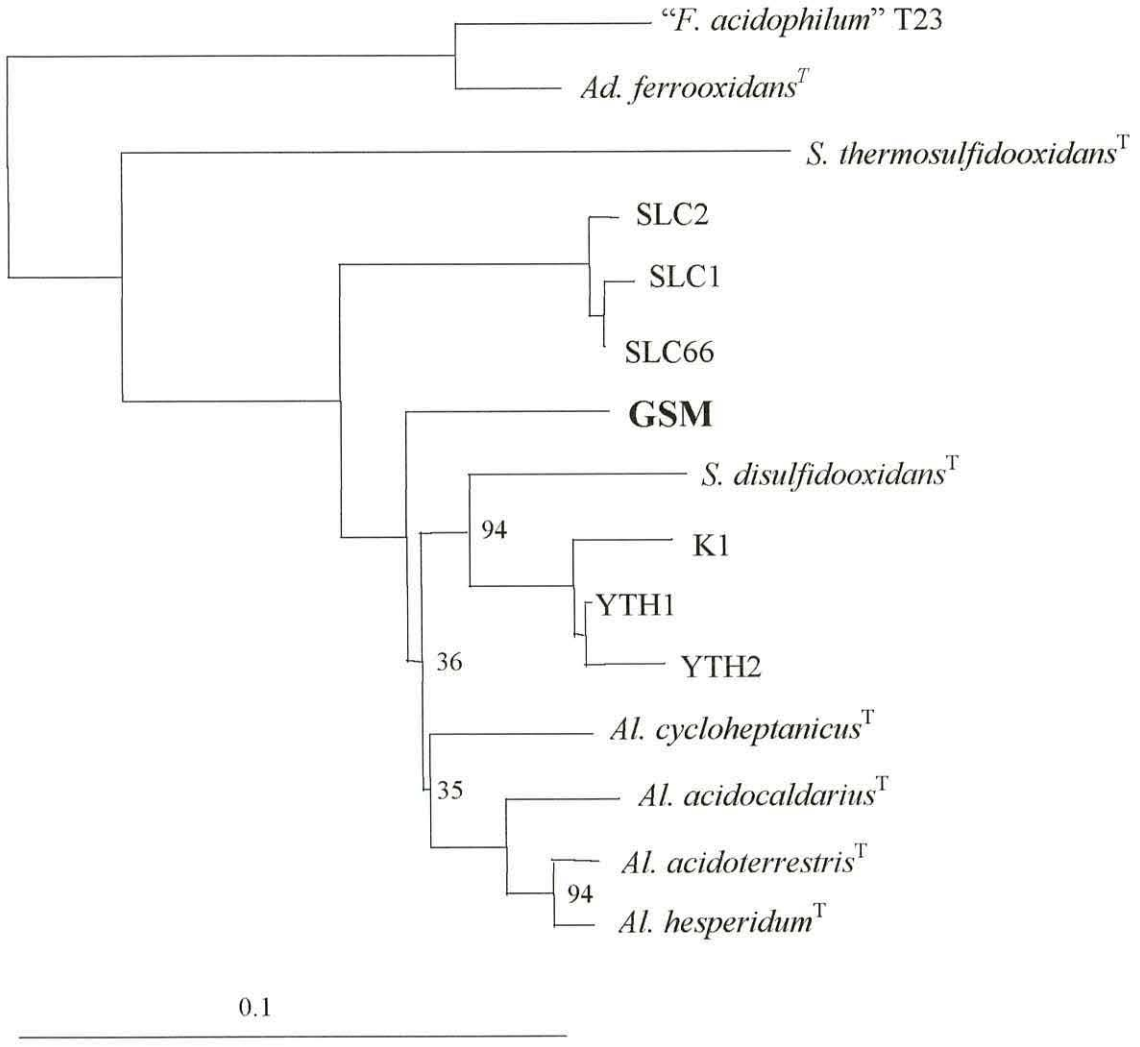


Fig 5.11: Phylogenetic relationship of *Alicyclobacillus*-like isolate GSM to other selected moderately thermophilic and mesophilic acidophilic bacteria based on the 16S rRNA sequences contained in the Genbank. Branch lengths are proportional to the calculated evolutionary distances and bootstrapping values were from 100 replicates. The tree was rooted using *Korarchaeote* as an outgroup. Bar, 0.1 nucleotide substitutions per site

5.9.2 Analysis of DNA base (G + C) composition

5.9.2.1 Methodology

Isolate GSM was grown in a 200 ml liquid medium set at pH 1.5 and supplemented with 10 mM fructose, 1 mM ferrous sulphate and 0.5 mM potassium tetrathionate. Cells were harvested during the late exponential growth phase by centrifugation (10,000 r.p.m. for 10 minutes at 4°C) and washed with TE buffer (10 mM Tris-HCl + 1 mM EDTA; pH 8.0).

Chromosomal DNA was then extracted from the bacterium using a proteinase K-SDS procedure as described by Wilson, (1987; section 2.6) and subsequently purified on CsCl gradients (section 2.7). The G + C content was determined by melting point analysis in 0.1X SSC as described by Marmur and Doty (1962; section 2.8).

5.9.2.2 Results

DNA base composition of isolate GSM was compared with those of previously characterised *Alicyclobacillus* spp., as summarised in Table 5.4. The G + C value of 54.9% for GSM was similar to that of *Al. cycloheptanicus* (its closest neighbour based on 16S rRNA gene sequence analysis).

Table 5.4: DNA base composition of the novel GSM isolate, and comparison with known species of *Alicyclobacillus*.

Bacteria	Mol % (G + C)
Al. acidocaldarius	61.2 – 62.2 [a]
Al. acidoterrestris	51.6 – 53.3 [b]
Al. cycloheptanicus	54.0 – 56.9 [c]
Al. hesperidum	53.3 [d]
GSM	54.9

[a]: Darland and Brock, (1971); [b]: Deinhard *et al.*, (1987a); [c]: Deinhard *et al.*, (1987b); [d]: Albuquerque *et al.*, (2000)

5.10 Determination of fatty acid compositions

Based on 16S rRNA gene sequence, GSM is closely related to the genus *Aicyclobacillus* though they exhibit very low 16S rDNA similarity (BLAST) (section 5.9). Since ω-alicyclic fatty acids are diagnostic for *Alicyclobacillus* spp., it was necessary to determine whether or not these fatty acids were present in isolate GSM.

5.10.1 Methodology

Isolate GSM was grown in 200 ml liquid media (section 2.2.1.3) supplemented with 10 mM glycerol, 1 mM ferrous sulphate and 0.5 mM potassium tetrathionate. Cells were harvested during the late exponential growth phase by centrifugation (10,000 r.p.m., 10 minutes at 4°C) and washed, first in 10 mM H_2SO_4 and finally in distilled water. The resulting pellet was freeze-dried and sent to the DSMZ, Braunschweig, Germany for fatty acids analysis. A cell pellet of the iron-reducing Yellowstone isolate YTH1 (Johnson et al., 2000) was also sent for fatty acid analysis. *Al. acidoterrestris*^{T} was used as reference bacterium by the DSMZ.

5.10.2 Results

No ω-alicyclic fatty acids were found in GSM extracts, though three fatty acids present were not identified. The iron-reducing isolate YTH1 was found, in contrast, to contain significant amounts of ω-cyclohexane-C17:0 and ω-cyclohexane-C19:0 fatty acids.

5.11 Discussion

Of the total of six 'strains' of acidophilic bacteria isolated from the waste dump rock sample (pit 26) of Golden Sunlight Mine, three were preliminarily identified as *Sulfobacillus*-like (iron-oxidising) and two as *Alicyclobacillus*-like (non iron-oxidising) isolates, classified species of which are predominantly moderately thermophilic acidophiles. Thermoacidophilic isolates might have been expected in view of the self-heating nature of the Golden Sunlight Mine waste dump rock. "*Sulfobacillus*"-like GSM (as initially identified; Table 5.1) was the single isolate which survived during repeated purification. It was originally isolated as an iron-oxidising colony on ferrous iron/tetrathionate overlay solid medium. Further characterisation of isolate GSM was carried out, partly due to an interest in its potential for bioleaching. Characterisation studies revealed that this bacterium:

- is able to oxidise ferrous iron in liquid and on solid media;
- does not couple ferrous iron oxidation and growth in the presence of organic compounds;
- is metabolically flexible, capable of autotrophic, heterotrophic and chemolithoheterotrophic growth;
- is a facultative anaerobe, able to grow anaerobically on ferric iron as electron acceptor;
- shows considerable tolerance to some heavy metals;
- has a 16S rRNA gene sequence which aligns it most closely with *Alicyclobacillus* spp.
- does not produce ω-fatty acids

The effects of temperature and pH on growth rates confirmed the thermoacidophilic nature of isolate GSM. Although the maximum and minimum

temperatures that supporting its growth were not determined, the temperature used (30°C) during its isolation indicated that the isolate was capable of growing at a wider range of temperature than the range tested (i.e. < 30° - > 55°C). Growth was determined as being optimum at 45°C, which is similar to characterised *Alicyclobacillus* spp. (48 - 60°C). However, GSM was apparently more acidophilic than known species of *Alicyclobacillus*. Evidence for this came from the optimum pH of 1.8 displayed by GSM which is lower than the type strains *Al. acidocaldarius* (~pH 3 – 4; Darland and Brock, 1971); *Al. acidoterrestris* (~ pH range 2.2 – 5.8; Deinhard *et al.*, 1987a) and *Al. cycloheptanicus* (~ pH 3.5 – 4.5; Deinhard *et al.*, 1987b).

Organic carbon utilisation by GSM resembled more closely that reported for *Alicyclobacillus* spp rather than for *Sulfobacillus* spp.. However, GSM was able to utilise a wider range of organic substrates, including several organic acids such as citric and glutamic acids which were reported to inhibit the growth of thermoacidophilic strains of *Al. acidocaldarius* (Darland and Brock, 1971). On the other hand, GSM displays a similar capacity to *Alicyclobacillus* spp. to metabolise sugars with the exception of arabinose. In contrast to *Alicyclobacillus* spp., both ferrous iron and reduced inorganic sulphur were required (at minimum concentrations of 100 and 500 μM, respectively) for heterotrophic growth of GSM. A requirement for iron has been reported for virtually all life forms, including mesophilic acidophilic heterotrophs of the genus *Acidiphilium* (e.g. *A. organovorum*; Lobos *et al.*, 1986) moderately thermophilic iron-oxidising sulfobacilli such as *S. acidophilus* (Norris *et al.*, 1996) and *S. thermosulfidooxidans* (Senyushkin *et al.*, 1997).

Isolate GSM was able to grow autotrophically in ferrous iron/tetrathionate medium. Reduced inorganic sulphur-dependent metabolism by GSM resembled that observed with the Gram-positive mesophilic isolates RIV-14 and L-15 (section 4.3), though in contrast, GSM appeared to grow more readily in autotrophic media than did the mesophiles. This was supported by the ability of GSM to grow in organic-free elemental sulphur medium, and by the greater cell yields ($7 - 9 \times 10^7$ cell/ml) for GSM compared to $1 - 3 \times 10^7$ cell/ml for RIV-14 and L-15, when grown in ferrous iron/tetrathionate medium. The requirement of a reduced sulphur supplement for both autotrophic and heterotrophic growth was also similar to that reported for moderately thermophilic iron-oxidising acidophiles, isolated from various leaching environments where soluble iron and reduced sulphur compounds tend to be abundant (Brierley, 1978; Norris and Barr, 1985).

Under anaerobic conditions, GSM was able to use ferric iron as a terminal electron acceptor. In anaerobic cultures, where fructose was provided as electron donor, ferric iron was reduced to ferrous iron. The correlation between ferric iron reduction and cell numbers showed that GSM could grow by anaerobic respiration, and is therefore a facultative anaerobe. However, the Gram-positive mesophiles RIV-14 and L-15 (section 4.3.3.2), and the moderately thermophilic acidophiles *Sulfobacillus* YTF1, *Sulfobacillus thermosulfidooxidans* and *Acidimicrobium ferrooxidans* (Bridge and Johnson, 1998) displayed more rapid and complete reduction of ferric iron in anaerobic condition. Anaerobic respiration (using ferric iron) by GSM resembled more closely that recorded for the *Alicyclobacillus*-like isolates YTH1 and YTH2 (Johnson *et al.*, 2000).

GSM was capable of oxidising both elemental sulphur and tetrathionate. In both cases, sulphur oxidation was enhanced by the addition of an organic compound

(glycerol) to the medium. RISC metabolism is generally more complicated than ferrous iron oxidation due to the diversity of enzymes involved in the metabolic pathways. Various species of sulphur (elemental sulphur, thiosulphate, tetrathionate, sulphate, sulphide and sulphite) which are products of sulphur metabolism are dependent on the type of sulphur compound used as an initial substrate (e.g. Smith and Strohl, 1991; Kelly *et al.*, 1997). However, sulphate is commonly the end product of sulphur compound metabolism by acidophilic bacteria and promotes acidification of the growth media. Sulphate production by GSM was not measured analytically though in all cases it was indicated by an increase in hydrogen ion concentration (reduction of pH) throughout the experimental period.

In contrast to elemental sulphur oxidation, a more limited ability to oxidise tetrathionate was displayed by GSM in autotrophic (organic-free) medium. In addition, tetrathionate was also found to accumulate during elemental sulphur oxidation. The limited capacity of GSM to oxidise tetrathionate is possibly due to the fact that tetrathionate is more toxic than elemental sulphur to acidophiles (Johnson, unpublished data). The longer lag period observed during tetrathionate oxidation could also be a reflection of tetrathionate toxicity. On the other hand, complete oxidation of both elemental sulphur and tetrathionate was observed in glycerol-supplemented medium. In this case, toxicity of tetrathionate was less apparent. From its initial isolation, GSM was found to grow better in the presence of organic compounds as in this case ($\sim 6.2 \times 10^8$ cell/ml in glycerol containing elemental sulphur or tetrathionate medium, and $\sim 1 \times 10^8$ cell/ml in inorganic elemental sulphur or tetrathionate medium).

Thiosulphate was produced during the oxidation of both elemental sulphur and tetrathionate, though more was detected during tetrathionate than during elemental sulphur oxidation. Thiosulphate is relatively unstable in acidic solutions, and tends to

decompose to sulphur and sulphite (Pronk *et al.*, 1990). Thiosulphate accumulated in organic-free tetrathionate medium, but not in glycerol-containing medium, where a sharp decline of pH was observed due to tetrathionate oxidation. In this case, depletion of thiosulphate could possibly have been due to increased acidity, which was found to be much greater in organic-containing (pH 1.56) than in organic-free medium (pH 2.64) at the end of the experimental period.

Acidophilic heterotrophic bacteria have been reported to be, in general, less tolerant to heavy metals than iron-oxidising acidophiles such as *T. ferrooxidans* (Johnson and Kelso, 1983; Said, 1990). However, GSM was found to display significant tolerance to copper, zinc and molybdate. Metal tolerance, combined with the ability of GSM to oxidise both ferrous iron and RISCs, indicated that it might be a useful bioleaching organism, which was confirmed in subsequent pyrite leaching experiments (Chapter 6).

From its initial isolation, GSM was identified as an iron oxidiser. However, iron oxidation was found not to be coupled to bacterial growth in heterotrophic media. A long lag before iron oxidation began was observed for cultures inoculated with cells harvested during the mid-exponential growth phase, i.e. prior to the onset of iron oxidation. In this case, no lag period was found for fructose utilisation. In contrast, lag-free and rapid oxidation of ferrous iron was observed in cultures inoculated with cells harvested during the late stationary growth phase, i.e. following complete oxidation of iron, and a long lag period occurred before fructose utilised. In both cases, growth was found to be coupled to fructose utilisation, as evidenced by increasing cell numbers during the period when fructose was metabolised. Based on these observations, it can be suggested that both iron-oxidising and organic substrate metabolising systems are

inducible in this bacterium. The induction and suppression of these systems appears to be effected by growth conditions as indicated by the lag periods observed in cultures inoculated with cells harvested during mid-exponential and late stationary growth phases. Greater growth was displayed at the expense of energy from fructose catabolism, reflecting the fact that relatively little energy is produced from the oxidation of ferrous iron (-91 kJ/mol) compared to that from fructose (-2830 kJ/mol) (Unden, 1999).

The metabolic versatility of GSM was evident from its ability to grow chemolithoautotrophically by fixing carbon dioxide and deriving energy from the oxidation of ferrous iron, confirmed by the observation that CO_2 fixation was coupled to iron oxidation during growth in 25 mM ferrous iron/2.5 mM tetrathionate medium. GSM was also found to be able to grow as a chemolithoheterotroph in ferrous iron/yeast extract medium. This was shown by the pattern of CO_2 fixation, which was suppressed during the early growth phase, indicating that organic materials were used preferentially in yeast extract-containing medium. As with chemolithoautotrophic metabolism, growth was supported by energy from iron oxidation but, in contrast, cellular carbon was assimilated from organic materials rather than inorganic CO_2. However, GSM was found to be capable of switching its carbon metabolism from an organic to an inorganic source, as indicated by temporary suppression of CO_2 fixation in yeast extract-containing medium. The increase in CO_2 fixation during the late growth phase in ferrous iron/yeast extract medium was most probably due to depletion of organic carbon in the medium. The regulatory carbon metabolism in GSM tended to avoid the energetically more expensive CO_2 fixation under favourable nutritional conditions and thus the proportion of carbon assimilated from CO_2 and organic substrates was highly dependent on the availability of pre-fixed organic carbon compounds. The pattern of carbon metabolism in GSM was similar to that observed with the Gram-positive mesophile L-15 (section 4.8.2.2), though

GSM was capable of more effective utilisation of organic compounds. In addition, the final cell numbers were 10-fold higher for GSM (2.45×10^9 cell/ml) than the mesophilic isolate L-15 (2.54×10^8 cell/ml) when grown in 25 mM ferrous iron / 0.02% yeast extract medium. This ability may be of great importance for the growth as well as the survival of this bacterium in its natural environment, as concentrations of dissolved organic carbon compounds may vary though are generally small in mineral leaching environments.

The moderate thermophile GSM is a Gram-positive spore-forming eubacterium that can oxidise both ferrous iron and sulphur. Accordingly, this isolate would appear to belong to the genus *Sulfobacillus*. However, its 16S rRNA gene sequence showed that GSM isolate is more closely related to *Alicyclobacillus* spp. than to *Sulfobacillus* spp. (Fig 5.11).

From a phylogenetic point of view, a major concern is whether GSM should be classified as a new species of the genus *Alicyclobacillus* or as a species of a novel genus. Three approaches were used in this study to distinguish GSM from *Alicyclobacillus* spp. and other iron-oxidising acidophilic bacteria: (i) biomolecular-based techniques, DNA base composition (G + C) and the comparative analysis of the 16S rRNA gene sequences analysis between GSM and other moderately thermophilic acidophiles (particularly *Alicyclobacillus* spp.); (ii) analysis of fatty acid composition of the cell membrane, and (iii) differences in physiological characteristics between GSM and other known moderately thermophilic iron-oxidising acidophiles.

Comparative analysis of the 16S rRNA gene sequences revealed that *Al. cycloheptanicus* is the closest relative to GSM (of sequenced eubacteria). The level of binary sequence similarity between the two strains is 93%, which would indicate that GSM is novel species of the genus *Alicyclobacillus*. However, many of the physiological

characteristics of GSM are very different to those of the four classified *Alicyclobacillus* spp. particularly with regard to its chemolithotrophy and metabolism of iron and sulphur. In addition, no characterised *Alicyclobacillus* sp. has been reported to grow under anaerobic conditions, whilst GSM can grow by using ferric iron as terminal electron acceptor in the absence of oxygen. The most closely related bacteria to GSM (*Al. cycloheptanicus*) was obtained from the Deutsche Sammlung von Microorganismen und Zellkulturen (DSMZ) and confirmed to be incapable of oxidising ferrous iron or of growth on ferrous iron or ferrous iron/tetrathionate overlay solid media (Johnson, unpublished data). In addition, it has also been confirmed that in contrast to *Alicyclobacillus* spp., isolate GSM does not contain ω-alicyclic fatty in its cell envelope. If GSM is to be classified as a novel *Alicyclobacillus* sp., then the genus description would have to be radically modified. The picture is further complicated by the positionings of the unclassified strains K1, and YTH1 and YTH2 (Fig. 5.11). The latter two Yellowstone isolates, although not able to oxidise ferrous iron, can bring about the dissimilatory reduction of ferric iron under anaerobic conditions (Johnson *et al.*, 2000). The situation with strain K1 is more ambiguous. This sequence was originally deposited as that of the putative type strain (VKM 1269) of *S. thermosulfidooxidans* (Tuorova *et al.*, 1994). However, it became clear that this was an error, when *S. thermosulfidooxidans* was resequenced by other groups (Durand, 1996) and its true phylogenetic position (e.g. relative to *S. acidophilus*) was established. Karavaiko *et al.* (2000) later confirmed that the sequence originally deposited as that of *S. thermosulfidooxidans* VKM 1269 was, in fact, that of another acidophile, strain K1. No physiological description was given of strain K1 (Karavaiko *et al.*, 2000) though earlier a strain labelled K1 had been described by the group as *S. thermosulfidooxidans* subsp. 'thermotolerans', i.e. an iron-oxidising moderate thermophile (Kovalenko and

Malakhova, 1983). It might well be, therefore, that iron-oxidising eubacteria, including GSM and strain K1, cluster more closely to *Alicyclobacillus* than to *Sulfobacillus*. This picture is further complicated by other, iron-oxidising *Alicyclobacillus*-like bacteria that have been characterised (strains SLC1, SLC2 and SLC66) though not yet classified (Johnson, unpublished data). These are readily distinguished from GSM and other *Alicyclobacillus* spp. in being mesophilic. The phylogenetic relationship between SLC isolates and *Alicyclobacillus* spp. are shown in Fig. 5.11. However, these isolates exhibit very low 16S rDNA sequence similarity (BLAST) with GSM and other moderately thermophilic acidophiles (88 – 89% DNA identity with *Alicyclobacillus* spp., suggesting that they could be classified as species of new genus, distinct from GSM.

(6)

Leaching of Sulphide Minerals by the Novel Mesophilic and Moderately Thermophilic Iron-Oxidising Acidophiles Isolated from Various Geothermal Environments

6.1 Introduction

Pyrite is a sulphidic mineral which varies in size; from 5 to > 400 μm, and shape from cubical to polyhedral (Evangelou, 1995). Pyrite is often found with coal and many other sulphidic minerals, which are associated with metals such as gold, copper and silver. The most reactive form of pyrite is framboidal and/or polyframboidal, due to their high specific surface and high porosity (Evangelou, 1995). It has been well documented that pyrite oxidation is a surface controlled reaction and therefore the large surface area of framboidal pyrite exposed to environmental and microbial degradation is conducive to faster rates of reactivity (Hoffmann *et al.*, 1981).

A variety of acidophilic bacteria have been detected and isolated from various acidic and/or geothermal environments that are capable of attacking sulphidic mineral (Ghauri, 1991; Johnson *et al.*, 1992; Pizarro *et al.*, 1996). As a result of microbial metabolism, insoluble metal deposits are converted to soluble metal sulphates. The solubilisation process is termed bioleaching and occurs naturally wherever suitable conditions are found for growth of mineral-oxidising bacteria. The bacteria that are most active in the degradation of sulphide minerals oxidise ferrous iron and/or sulphur in pure and mixed cultures. In commercial bioleaching operations, pure cultures are unlikely to be found, and combinations of microorganisms with different metabolic capacities might be more effective at accelerating mineral dissolution.

Two species of mesophilic acidophilic bacteria, *T. ferrooxidans* and *L. ferrooxidans*, have been implicated as being the most significant microorganisms

involved in sulphide mineral oxidation, although moderately thermophilic (or thermotolerant) bacteria and extremely thermophilic archaea are also known to be important in certain situations, such as self-heating coal spoils and bioleaching operations in which temperatures exceed 40°C (e.g. Rawlings, 1997; Rawlings and Silver, 1995; Johnson, 1995b). A requirement of enhanced carbon dioxide concentration has been reported for maximum growth of moderately thermophilic mineral oxidising *Sulfobacillus* spp. grown under autotrophic conditions, which has been perceived as a major drawback to their use in commercial bioleaching (Norris, 1990). However, subsequent reports had shown that some mixed cultures of moderately thermophiles do not have such requirements, thus makes the proposition of using these acidophiles in bioleaching operations much more attractive (Norris and Owen, 1993). For example, co-cultures of moderately thermophilic *Sulfobacillus thermosulfidooxidans* and *Ad. ferrooxidans* have been reported to be capable of more extensive ferrous iron oxidation than pure cultures under atmospheric CO_2, presumably due to the ability of the former to grow chemolithoheterotrophically by utilising fixed organic carbon released by the latter in mixed cultures (Clark and Norris, 1996). Furthermore, a requirement of yeast extract has been reported for successful pyrite leaching by the iron-oxidising heterotroph '*F. acidophilus*' strain T23 in pure culture. However, in mixed culture with the chemolithotrophic sulphur-oxidising *T. thiooxidans*, leaching was observed without the provision of organic compounds (Bacelar-Nicolau and Johnson, 1999).

This present study investigated the ability of the four Montserrat isolates (S-10, M-12, RIV-14 and L-15) and the moderately thermophilic isolate (GSM) to oxidise pyrite in pure cultures. Chalcopyrite was also used in some experiments. The type strains of *T. ferrooxidans* (ATCC 23270) and *L. ferrooxidans* (DSM 2705), were used as reference bacteria in some experiments.

6.2 Pyrite leaching in the presence and absence of yeast extract

6.2.1 Methodology

Shake flasks cultures were set up to study the effect of yeast extract on pyrite leaching by the four Montserrat isolates, S-10, M-12, RIV-14 and L-15. A reference culture of the type strain of *T. ferrooxidans* (ATCC 23270) and a sterile (cell-free) culture were also included in this experiment as controls. The bacteria were first adapted to oxidising pyrite by several transfers through 1% (w/v) pyrite medium (section 2.2.1.4).

Active cultures were inoculated (5% v/v) into 250 ml Erlenmeyer flasks (in triplicate), containing 100 ml of sterile 1% (w/v) pyrite medium, or the same medium supplemented with 0.02% (w/v) yeast extract, both poised at pH 2.0 prior to autoclaving. All cultures were incubated in an orbital shaker (150 r.p.m.) at 30°C. Samples were removed at regular intervals, to monitor total soluble iron in the leachates, and pH changes during incubation. For total iron, samples were prepared for AAS analyses as described in section 2.6.2. Culture purity was checked at the start and the end of the experiment by streaking on ferrous iron overlay (section 2.2.2.1), ferrous iron/tetrathionate overlay (section 2.2.2.2) and yeast extract plates (section 2.2.2.3).

6.2.2 Results

All four iron-oxidising mesophilic Montserrat isolates tested were capable of oxidising pyrite, both in the presence (Fig. 6.1a) and absence (Fig 6.1b) of yeast extract. Based on total soluble iron analysed, pyrite leaching by the Gram-negative isolates S-10 and M-12 was found to be slightly suppressed in yeast extract-containing media. In contrast, enhanced leaching was observed with the Gram-positive isolates RIV-14 and L-15 when yeast extract was present. In the yeast extract-free medium, a similar pattern

of pyrite oxidation was displayed by the Gram-negative isolates S-10 and M-12 as *T. ferrooxidans* (Fig. 6.1a).

Enhanced pyrite oxidation by both RIV-14 and L-15 in yeast extract-containing medium was evident by a greater amount of iron solubilised in the presence (final total soluble iron ~ 3.5 g/l for both) than in the absence (final total soluble iron ~ 1.5 and 2.5 g/l for RIV-14 and L-15,respectively) of yeast extract. A biphasic pattern of pyrite oxidation was also observed with both RIV-14 and L-15 grown in yeast extract-containing medium. Based on the final total soluble iron concentrations, isolate L-15 (2.5 g/l) displayed a greater capacity to oxidise pyrite than RIV-14 (1.6 g/l) in autotrophic (organic–free) medium.

Strain S-10 was found to have greater capacity to degrade pyrite than M-12 in both organic-containing (final total soluble iron ~ 3.47 and 2.57 g/l, respectively) or organic-free (final total soluble iron ~ 4.3 and 2.95 g/l, respectively) media. By the end of the 56 day leaching period, the total amount of pyrite oxidised by strain M-12 was about 25% less than that by S-10 and 31% less than that by *T. ferrooxidans,* irrespective of yeast extract inclusion.

The bacterial oxidation of pyrite is generally an acid-producing reaction, and a simultaneous decrease of pH was observed in all cases (Fig 6.1b). Lower pH values were found with organic-free medium for isolates S-10 and M-12 as with *T. ferrooxidans*, though isolates RIV-14 and L-15 displayed higher pH in organic-free (final pH 1.68 and 1.52, respectively) than in organic-containing (final pH 1.39 and 1.41, respectively) media during the 56 day leaching period.

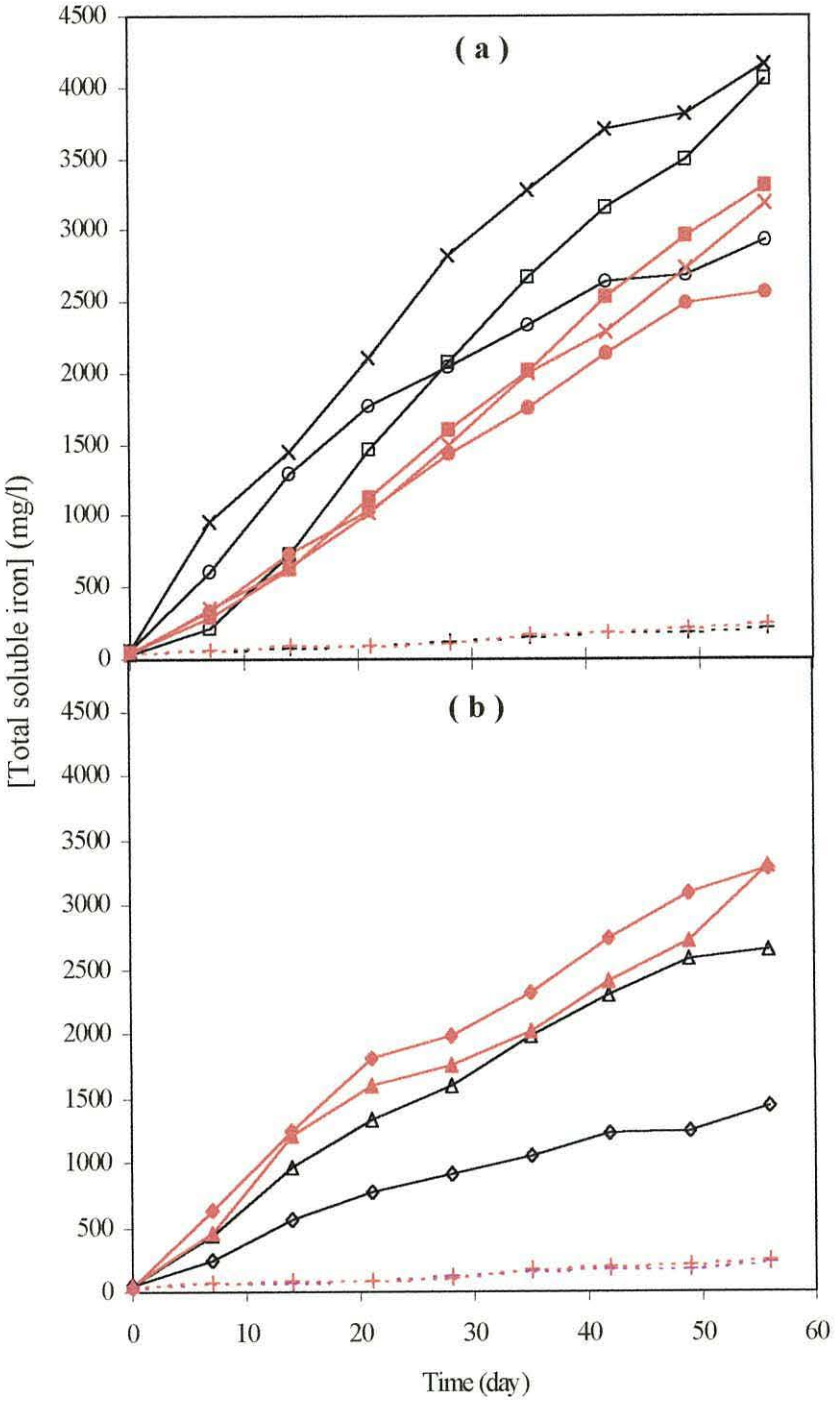


Fig. 6.1a: Effects of yeast extract on pyrite oxidation by iron-oxidising mesophilic acidophiles (a) S-10 (■□), M-12 (●○), and *T. ferrooxidans* (××), (b) RIV-14 (◆◇) and L-15 (▲△). The bacteria were grown in 1% (w/v) pyrite medium; pH 2.0 supplemented (red lines and symbols) or not (black lines and symbols) with 0.02% (w/v) yeast extract. Data for control uninoculated cultures are also shown (+ +; broken lines).

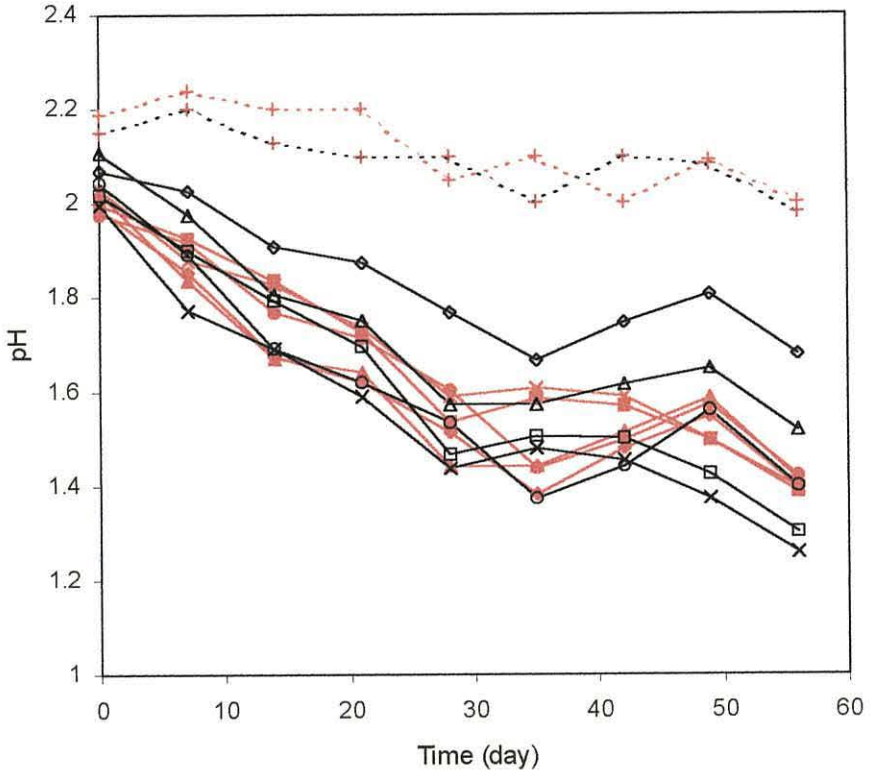


Fig. 6.1b: Changes of pH during pyrite oxidation by S-10 (■□), M-12 (●○), RIV-14 (◆◇), L-15 (▲△) and *T. ferrooxidans* (××) in medium supplemented (solid/red symbols) or not (open symbols) with yeast extract. Data for control uninoculated cultures are also shown (+ + ; broken lines)

6.3 Effect of pH on pyrite leaching by L-15 grown in shake flasks cultures

6.3.1 Methodology

In this experiment, the ability of the Gram-positive Montserrat isolate L-15 to oxidise pyrite was tested in cultures poised initially at different pH values, in shake flasks. Cultures of the type strains of *T. ferrooxidans* (ATCC 23270) and *L. ferrooxidans* (DSM 2705) were also set up. In all cases, pyrite-adapted cultures were used as inocula.

Active cultures were inoculated (5% v/v) into duplicate 250 ml Erlenmeyer flasks containing 100 ml of 2% (w/v) pyrite medium (section 2.2.1.4), initially adjusted to different pH values (1.2, 1.5 and 2.5). All cultures were incubated at 35°C with shaking (150 r.p.m.). Samples were taken regularly for up to 56 days, and pyrite leaching was

monitored by measuring concentrations of total soluble iron using AAS (section 2.5.2.1). Changes in pH were also monitored. Culture purity was tested by streaking the cultures onto ferrous iron overlay for *T. ferrooxidans* and *L. ferrooxidans* (section 2.2.2.1) or ferrous iron/tetrathionate overlay for isolate L-15 (section 2.2.2.2).

6.3.2 Results

The pattern of pyrite oxidation in cultures poised at different initial pH values differed between the three iron-oxidising bacteria (Fig. 6.2). All three were able to oxidise pyrite in cultures poised initially at pH 1.5 and 2.5, but leaching by the type strains of *T. ferrooxidans* and *L. ferrooxidans* was inhibited in pH 1.2 medium. In contrast, the Gram-positive isolate L-15 was able to oxidise pyrite in the pH 1.2 culture (Fig. 6.2a), and continued to do so as the culture pH declined to pH < 1.0.

The effect of pH on the rates of pyrite leaching by the bacteria are summarised in Table 6.1. Pyrite oxidation by L-15 was found to be slower in pH 2.5 medium than at lower pH. At pH 2.5, fastest pyrite oxidation was found with *T. ferrooxidans* (Fig. 6.2c), though there was evidence that pyrite oxidation by L-15 increased after day 44, at which point the pH of the cultures had fallen to around 1.4. *L. ferrooxidans* displayed similar patterns of pyrite oxidation at pH 2.5 and pH 1.5 media. Similar rates of pyrite dissolution were determined by both *T. ferrooxidans* and L-15 in medium initially poised at pH 1.5, though an extended lag period (15 days) was observed with *T. ferrooxidans*.

Table 6.1: Rates of pyrite leaching by isolate L-15 in comparison with *T. ferrooxidans* and *L. ferrooxidans* grown in shake flasks at various initial pH. Values are mean of duplicate cultures.

Bacteria / Medium	Rates of pyrite oxidation (mg Fe solubilised/day)		
	pH 1.2	pH 1.5	pH 2.5
L-15	204	230	68
T. ferrooxidans	n.d	223	240
L. ferrooxidans	n.d	57	136

n.d: not detected

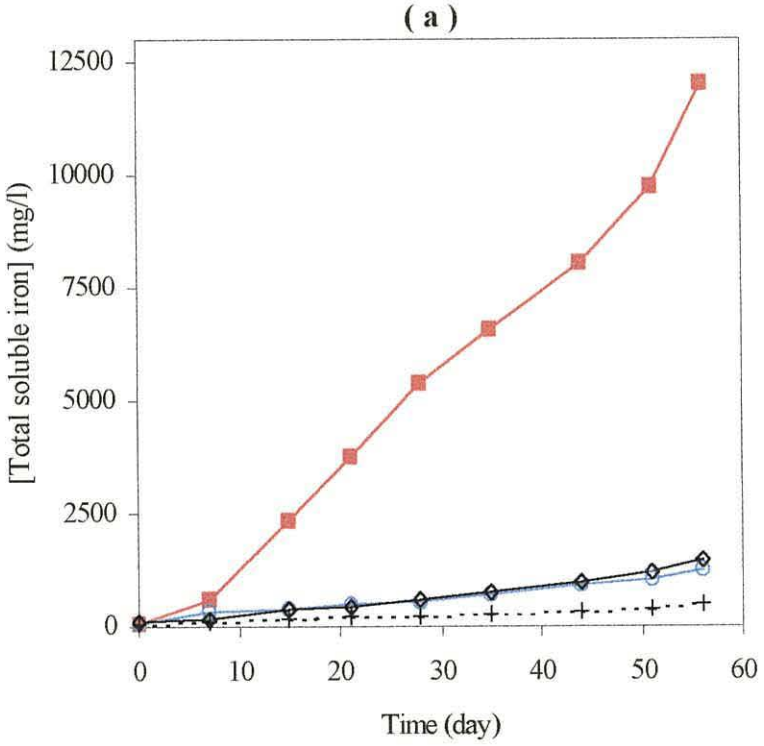


Fig. 6.2a: Oxidation of pyrite at pH 1.2 by the mesophilic Gram-positive Montserrat isolate L-15 (■) in comparison with that of the type strain *T.ferrooxidans* (○) and *L. ferrooxidans* (◇). Data for control uninoculted culture are also shown (+; broken line).

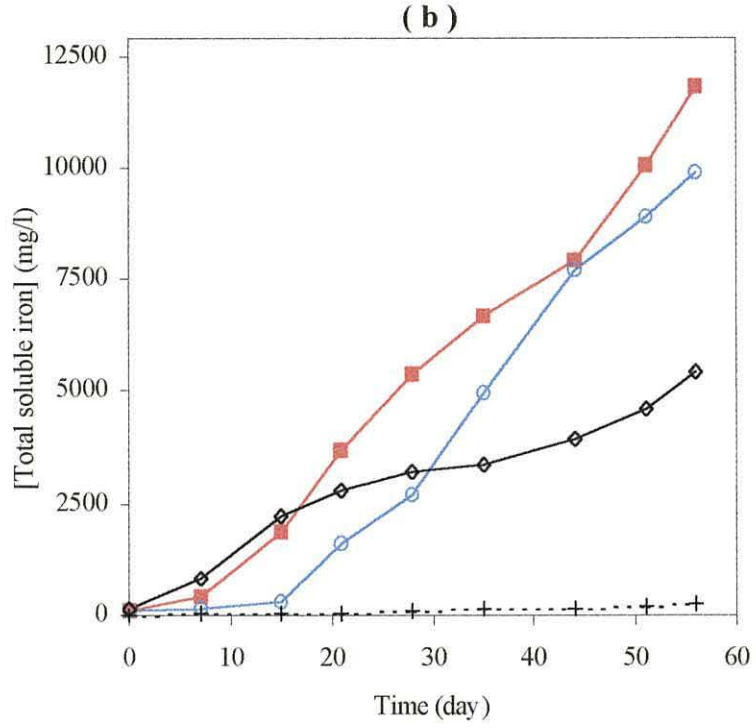


Fig. 6.2b: Oxidation of pyrite at pH 1.5 by the mesophilic Gram-positive Montserrat isolate L-15 (■) in comparison with that of the type strain *T.ferrooxidans* (○) and *L. ferrooxidans* (◇). Data for control uninoculted culture are also shown (+; broken line).

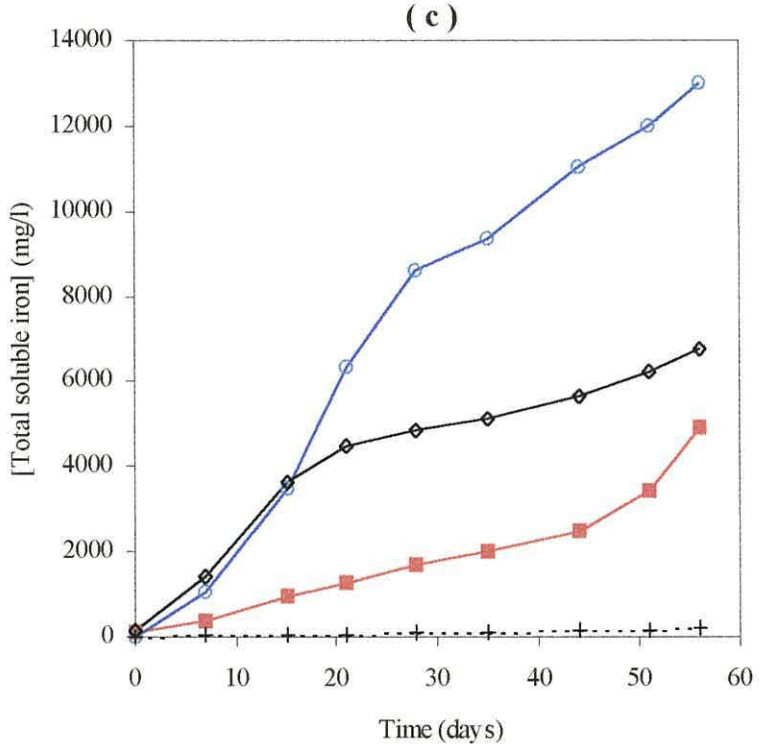


Fig. 6.2c: Oxidation of pyrite at pH 2.5 by the mesophilic Gram-positive Montserrat isolate L-15 (■) in comparison with that of the type strain *T.ferrooxidans* (○) and *L. ferrooxidans* (◇). Data for control uninoculted culture are also shown (+; broken line).

6.4 Pyrite leaching under controlled pH conditions

6.4.1 Methodology

Experiments were carried out in a laboratory bioreactor, fitted with the temperature, pH, agitation and aeration control units (LH Ltd., series 500). The working volume of the batch cultures was 1.5 l. Cultures were stirred at 150 r.p.m, aerated at 0.5 l/min., pH was automatically controlled using H_2SO_4 or NaOH (each 1 M) and the temperature was fixed at 35°C throughout the experiment.

The effect of pH changes on leaching of pyrite by the mesophilic Gram-positive isolate L-15 was studied and compared with that of the type strain of *T. ferrooxidans* (ATCC 23270). Bacteria were first grown in 2% (w/v) pyrite medium (pH 2.25) in shake flask, in a single passage. The bioreactor was equilibrated at 35°C and active cultures were inoculated (10% v/v) into the bioreactor vessel, which contained 2% (w/v) ground pyrite (section 2.2.1.4). The pH of the medium was set at pH 1.5, 1.0 and 0.8 during consecutive experiments. Bacteria were grown in the same conditions for 11 – 17 days at a particular pH value, before set pH points were changed (from pH 1.5 to 1.0 and finally to pH 0.8 for L-15 or from pH 1.5 to 1.0 and finally to pH 1.5 for *T. ferrooxidans*).

Samples were withdrawn daily, and pyrite leaching was monitored by measuring total soluble iron and ferrous iron concentrations. The concentration of ferrous iron was measured colorimetrically using the ferrozine assay (section 2.4.1.3) and total soluble iron was monitored using AAS (section 2.5.2.1). Concentrations of ferric iron were determined by subtracting the concentration of ferrous iron from that of the total soluble iron in the leachate. Redox (E_h) values were also measured offline method using a platinum electrode. Bacteria were enumerated using (i) a Thoma counting chamber (section 2.2.2.2) and (ii) plate counts on ferrous iron overlay (section 2.2.2.1) and ferrous

iron/tetrathionate overlay (section 2.2.2.2) solid media for *T. ferrooxidans* and isolate L-15, respectively.

6.4.2 Results

This experiment was designed to study the abilities of isolates L-15 and *T. ferrooxidans* to leach pyrite under controlled (fixed) conditions of acidity. The patterns of pyrite oxidation obtained are shown in Fig 6.3 and Fig. 6.4.

Both L-15 and *T. ferrooxidans* displayed active degradation of pyrite during the 12 days when the fementers were maintained at pH 1.5. However, the rate of pyrite leaching was found to be higher for isolate L-15 (125 mg Fe solubilised/day) than that of *T. ferrooxidans* (81 mg. Fe solubilised/day). Concentrations of total soluble iron increased in both cases but differed in speciation, with higher Fe^{3+}/Fe^{2+} ratios found for *T. ferrooxidans*. These results were confirmed by the measured redox (E_h)values, which increased from + 780 to + 900 mV for *T. ferrooxidans* in this period, while they fluctuated between + 745 and + 799 mV for isolate L-15. Although Fe^{3+}/Fe^{2+} ratios were lower, pyrite oxidation in cultures of L-15 was greater than by *T. ferrooxidans*, as indicated by the amounts of total soluble iron present in the cultures (1600 and 1200 mg/l respectively) on day 12. Increased numbers of bacteria was also recorded in both cultures during this time (Fig. 6.5).

When the pH was subsequently lowered to 1.0, pyrite leaching was found to continue with *T. ferrooxidans* (after a 4 days lag period) from days 15 – 23, though this subsequently came to a halt. During days 15 - 23, pyrite oxidation was found to correlate with ferric iron reduction and production of ferrous iron. This suggested that pyrite oxidation in the *T. ferrooxidans* culture during this time was entirely abiotic, as; $FeS_2 + 14Fe^{3+} + 8H_2O \rightarrow 15Fe^{2+} + 16H^+ + 2SO_4^{2-}$. A mean rate of 47 mg Fe

solubilised/day occurred between days 15 to 23. The contention that pyrite oxidation was predominantly abiotic was supported by cell counts of *T. ferrooxidans*, which decreased in both total and viable cells 14 days after the start of the experiment (Fig. 6.5).

In contrast, the reduction of pH from 1.5 to 1.0 did not appear to affect pyrite oxidation by isolate L-15 though the rate was found to be slower at pH 1.0 (80 mg Fe solubilised/day) than at pH 1.5 (125 mg Fe solubilised/day). A subsequent further lowering of pH to 0.8 produced an initial response in terms of (i) a temporary halt on pyrite oxidation, and (ii) a decline in Fe^{3+}/Fe^{2+} ratios and a corresponding decrease in redox potential. However, after 10 days the culture appeared to recover and the subsequent rate of pyrite oxidation (238 mg Fe solubilised/day) was actually greater than rates recorded at pH 1.5 and pH 1.0, though this value was based only three analyses of total soluble iron after the 14 days period. In contrast to *T. ferrooxidans*, high numbers of viable cells were found in the culture of L-15 throughout the experiment, indicating its potential for oxidising pyrite in extremely acidic (pH < 1.0) liquors.

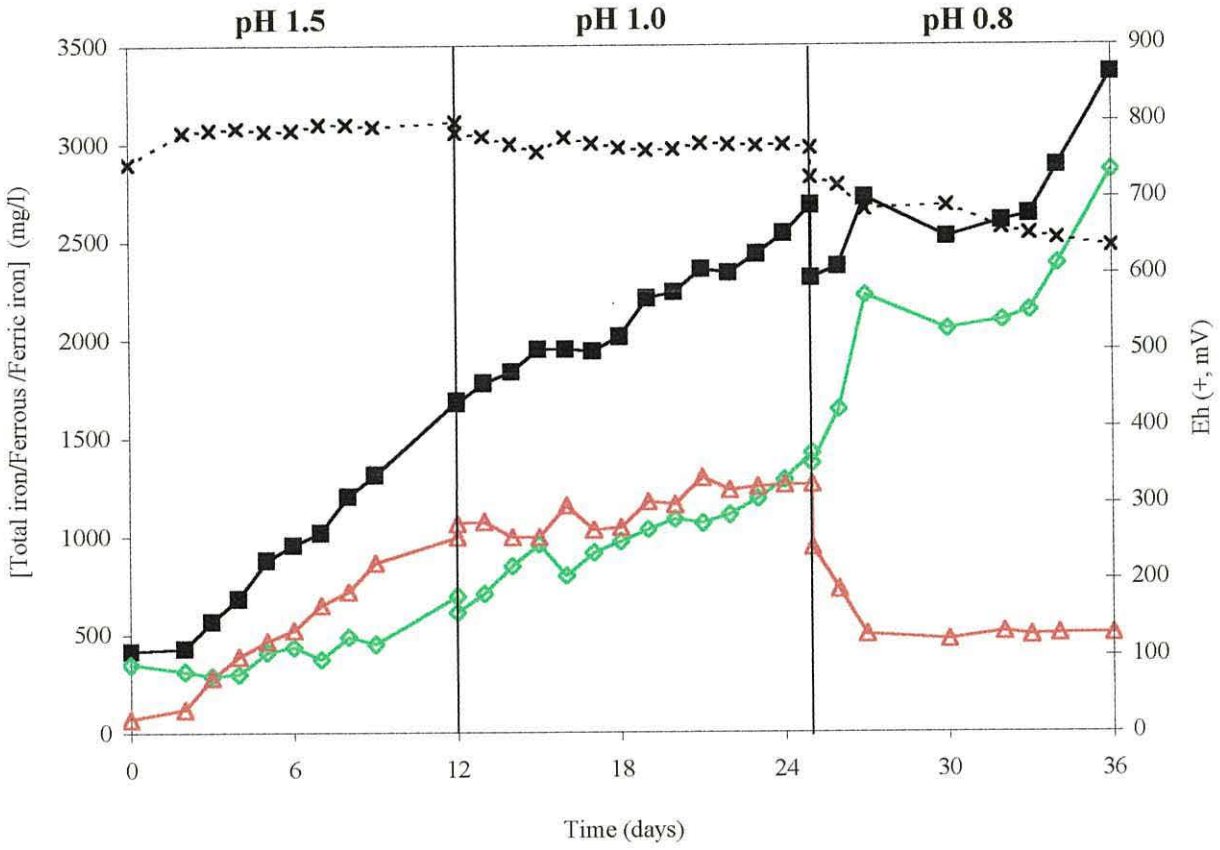


Fig 6.3: Oxidation of pyrite by isolate L-15 under controlled pH. Pyrite oxidation activity was monitored by measuring soluble total soluble iron (■) and changes in iron speciation: ferric iron (△) and ferrous iron (◇). Off line redox (E_h) values (×; broken lines) are also shown.

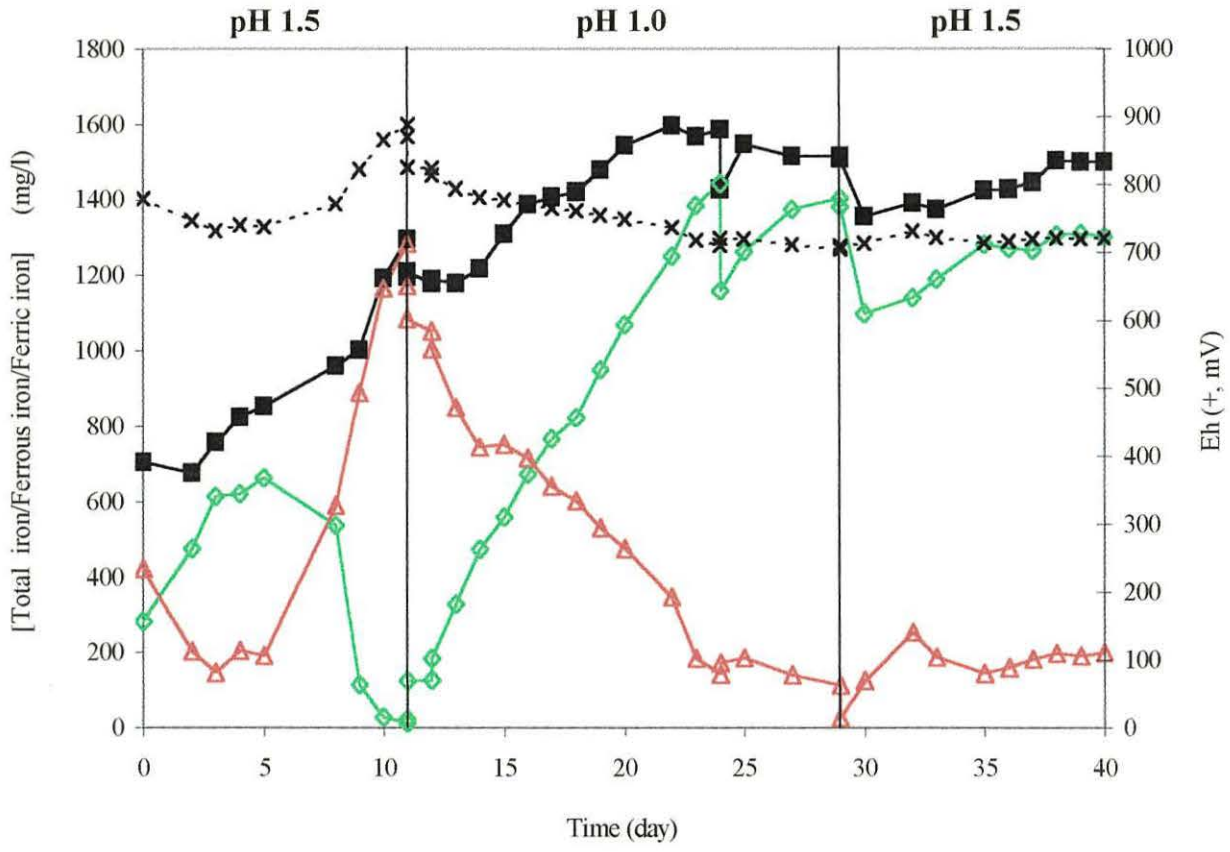


Fig 6.4: Oxidation of pyrite by the type strain *T. ferrooxidans* under controlled pH. Pyrite oxidation activity was monitored by measuring soluble total soluble iron (■) and changes in iron speciation: ferric iron (△) and ferrous iron (◇). Off line redox (E_h) values (×; broken lines) are also shown.

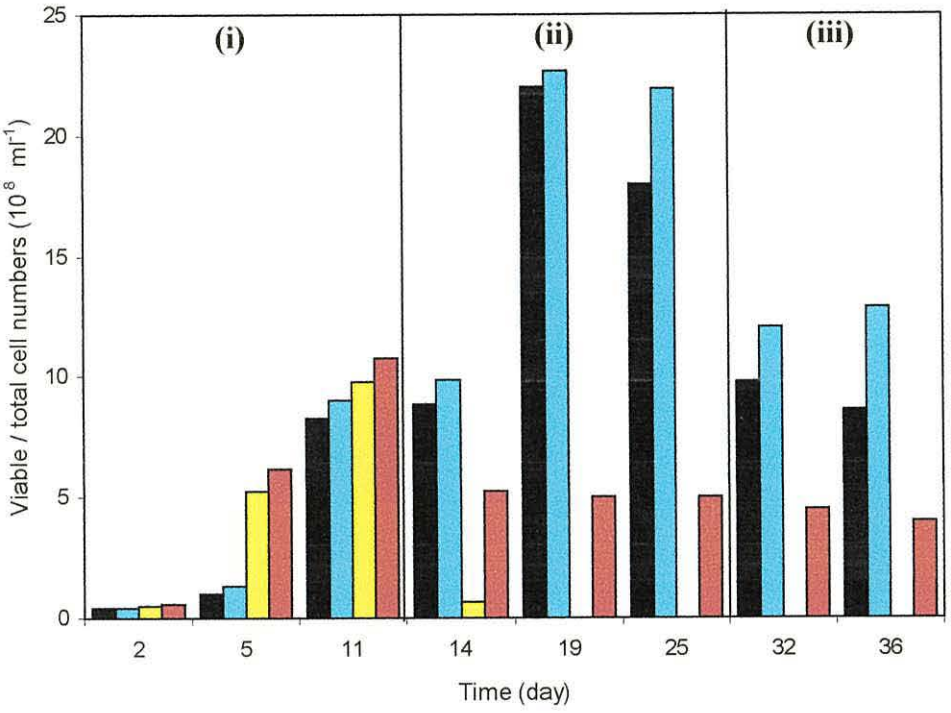


Fig. 6.5: Cell counts of L-15 (■■) and *T. ferrooxidans* (■■) during pyrite oxidation under controlled low pH conditions; (i) pH 1.5, (ii) pH 1.0 and (iii) pH 1.5 (*T. ferrooxidans*) or pH 0.8 (L-15). Values are shown for total (blue/red) and viable (black/yellow) cell counts.

6.5 Pyrite leaching by the moderately thermophilic isolate GSM

6.5.1 Methodology

The novel iron-oxidising moderate thermophilic isolate, GSM, had been studied in detail with respect to its oxidative metabolism of ferrous iron and inorganic sulphur compounds (chapter 5) and thus it was appropriate to investigate its ability, or otherwise, to catalyse the oxidative dissolution of pyrite.

Pyrite leaching by GSM was tested by growing the bacterium in shake flasks containing pyrite medium, with or without added organic compounds. The isolate was adapted to grow in 1% (w/v) pyrite medium, pH 2.0, via several transfers through the same medium. An active culture was inoculated into duplicate 250 ml Erlenmeyer flasks containing sterile 100 ml of pH 2.0 basal salts (section 2.1.1), supplemented with 2%

(w/v) ground pyrite. The medium was supplemented aseptically with either 10 mM ferrous iron or 5 mM fructose plus 0.001% (w/v) yeast extract.

Cultures were incubated at 45°C, shaken at 150 r.p.m, for up to 40 days. A 2 ml sample was removed at regular intervals from each flask and was replaced with the same volume of sterile pH 2.0 distilled water. Pyrite oxidation by GSM was monitored by measuring the changes in concentrations of total soluble iron using AAS (section 2.4.2.1). Ferrous iron was measured colorimetrically using the ferrozine assay (section 2.4.1.3). Changes of pH during pyrite leaching were also recorded throughout the experiment. Fructose utilisation was monitored using the anthrone method (section 2.5.4).

6.5.2 Results

Culture purity was confirmed at the start and at the end of the experiment. Based on the total amounts of soluble iron released, addition of fructose and yeast extract produced no apparent effects on pyrite leaching by isolate GSM (Fig. 6.6). Cell numbers were not monitored on a regular basis, but were analysed at the end of the experiment by plating onto yeast extract plates (section 2.2.2.3). Mean numbers of GSM in the organic-containing medium (8.7×10^8 cell/ml) exceeded those in the organic-free medium (9.2×10^7 cell/ml). Utilisation of organic substrates by GSM was confirmed, as no fructose was detected after the first 5 days of incubation.

A gradual decrease in pH was recorded in all cultures. In the presence of fructose/yeast extract, the initial (0 – 10 day) decline in pH was relatively slow, though it increased subseqently.

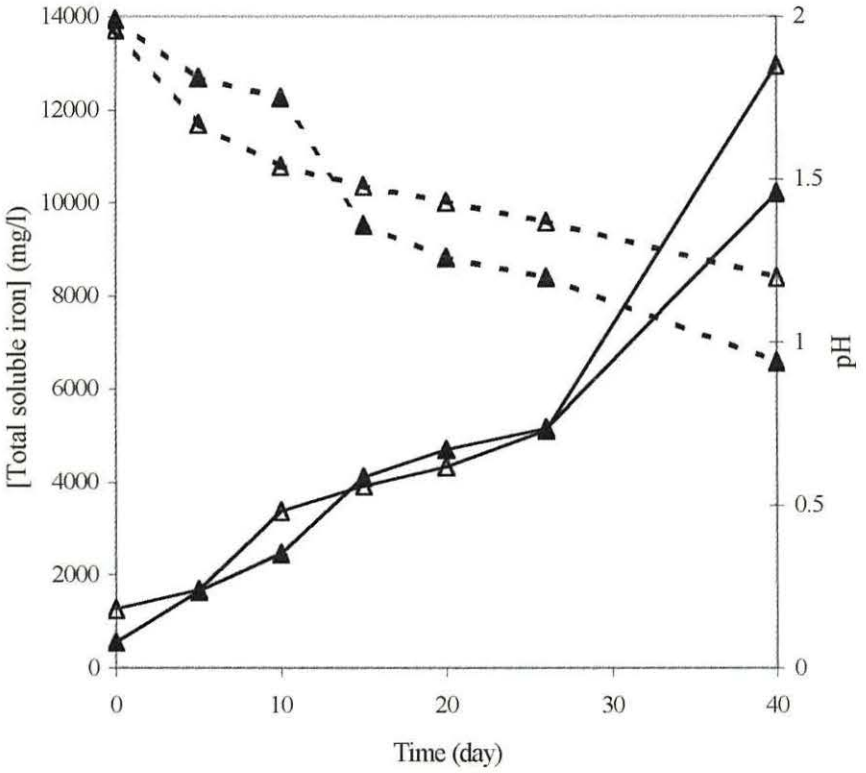


Fig. 6.6: Oxidation of pyrite by the novel iron-oxidising isolate GSM grown in pyrite medium supplemented with either 10 mM ferrous sulphate (open symbols) or 5 mM fructose + 0.001% (w/v) yeast extract (solid symbols). Pyrite oxidation was monitored by measuring concentrations of total soluble iron (▲△) and changes in pH (broken lines)

Distinctive patterns of iron speciation were found in cultures which contained or did not contain added organic compounds (Fig. 6.7). In the organic-containing medium, high Fe^{2+}/Fe^{3+} ratios were maintained throughout the 40 days incubation period, while in the autotrophic (organic-free) cultures, ferric iron concentrations always exceeded those of ferrous iron. Redox values were calculated from measured ferrous and ferric iron concentrations by using the Nernst equation:

$$E = E^{\circ} + \frac{RT}{zF} \log \frac{[Fe^{3+}]}{[Fe^{2+}]}$$

The redox potential of the autotrophic cultures was consistently greater than the organic-amended culture throughout the 40 days incubation period, even though the fructose had apparently been metabolised in the latter within the first five days of incubation.

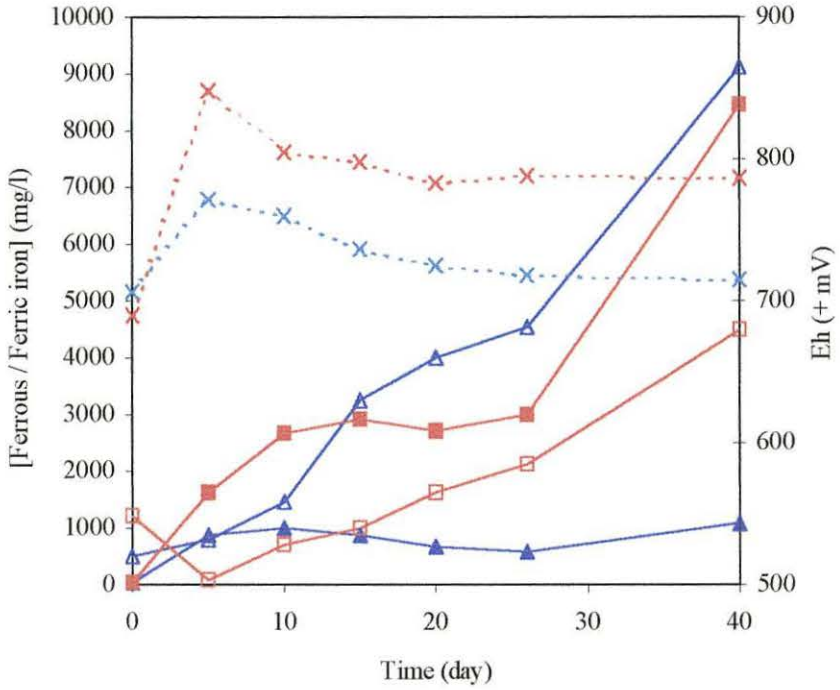


Fig. 6.7: Iron speciation during pyrite oxidation by the moderately thermophilic isolate GSM grown in the presence (blue symbols) or absence (red symbols) of organic compounds. Key: ferric iron (▲■), ferrous iron (△□) and calculated Eh (× ×;broken lines). Values are mean of duplicate cultures.

6.6 Chalcopyrite leaching by the Montserrat isolates

6.6.1 Methodology

All four iron-oxidising mesophilic isolates from Montserrat were able to oxidise pyrite (section 6.2.2). The work was extended to study the abilities of these isolates to oxidise the copper-containing sulphidic mineral, chalcopyrite in acidic solutions. The type strain *T. ferrooxidans* was used in this experiment as a reference bacterium and cell-

free controls were also set up. A chalcopyrite ore (section 2.2.1.4) which contained pyrite in addition to chalcopyrite, was used in these experiments.

Bacterial cultures were set up in 250 ml shake flasks containing 100 ml medium, supplemented with 1% (w/v) chalcopyrite (section 2.2.1.4). The medium was adjusted to pH 1.40 with sulphuric acid prior to autoclaving. All isolates were adapted to grow in 1% (w/v) chalcopyrite via a single passage from pyrite-grown cultures. Active cultures in chalcopyrite were inoculated (2% v/v) into duplicate flasks containing the sterile medium. All cultures were incubated at 35°C, shaken at 120 r.p.m., for up to 49 days. Samples were removed at regular interval to measure the total soluble iron and copper in the leachate, using AAS (Section 2.5.2.1). When assaying for copper, a standard calibration curve was made, over the range of 0 to 100 mg/l of copper sulphate ($CuSO_4.5H_2O$). Ferrous iron was measured using the ferrozine assay (section 2.5.1.3). Ferric iron concentrations were determined by subtracting the concentrations of ferrous iron from those of the total soluble iron. Culture purity was checked at the beginning and the end of the experiment by streaking onto ferrous iron overlay (S-10, M-12 and *T. ferrooxidans*) and ferrous iron/tetrathionate (RIV-14 and L-15) overlay plates (section 2.2).

6.6.2 Results

All four Montserrat isolates were capable of leaching chalcopyrite in pure culture (Fig. 6.8 and 6.9). Isolates S-10 and M-12 displayed similar patterns of chalcopyrite oxidation (Fig 6.8a). High Fe^{3+}/Fe^{2+} ratios were observed for both isolates throughout the experiment (Fig. 6.8b), indicating intensive ferrous iron oxidation by the bacteria. Net decreases in pH were recorded, though there was an initial slight increase (of *ca* 0.3 units) during the first 7 days of incubation. Chalcopyrite oxidation by S-10 and M-12

was superior to that of the type culture of *T. ferrooxidans* at 35°C. This was indicated by the smaller amounts of both copper and total iron solubilised by *T.ferrooxidans* than by the Montserrat isolates. In addition, ferrous iron was found to accumulate in cultures of *T. ferrooxidans*, and the pH gradually increased from 1.4 to 2.3. A decrease in pH was observed in all cultures, though the final pH was slightly lower in cultures of S-10 and M-12 (~pH 1.35) than with RIV-14 and L-15 (~pH 1.6).

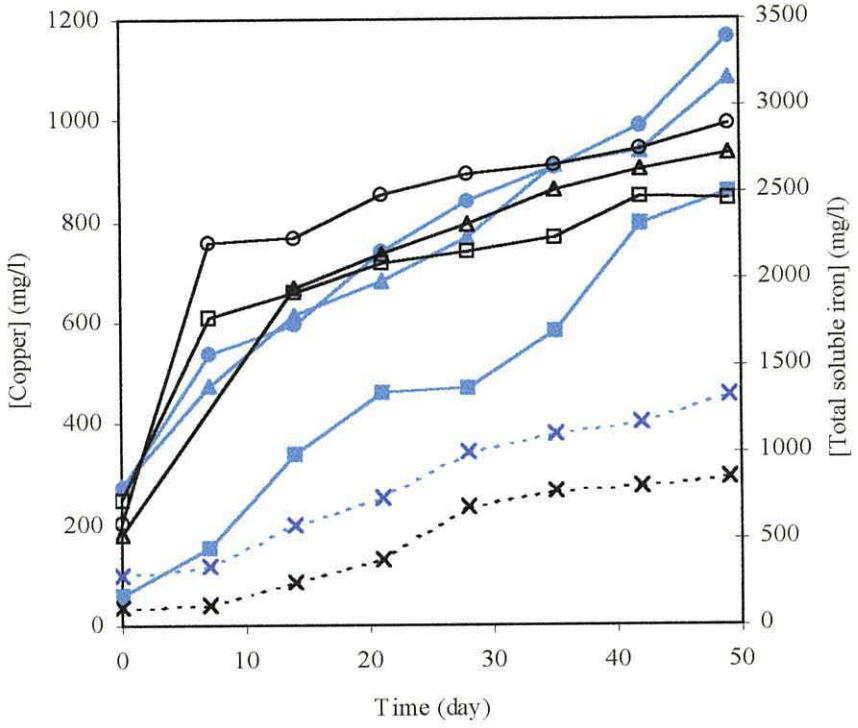


Fig: 6.8a: Chalcopyrite leaching by S-10 (▲△) and M-12 (●○) and comparison with *T. ferrooxidans*^{T} (■□), grown in 1% (w/v) chalcopyrite at 35°C. Bioleaching was determined by measuring concentrations of soluble copper (blue/closed symbols) and total soluble iron (black/open symbols). Data from uninoculated controls are also shown (× ×; broken lines).

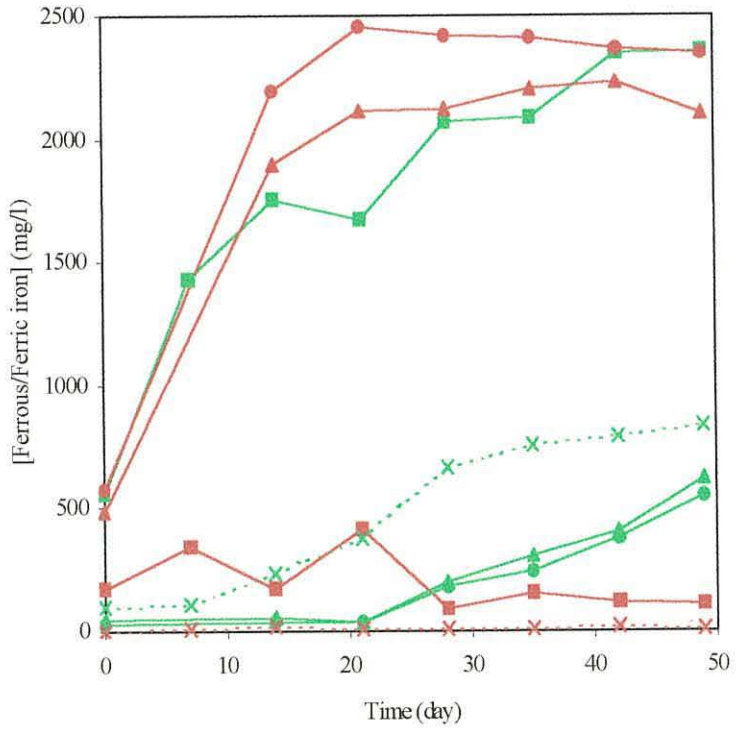


Fig: 6.8b: Iron speciation during chalcopyrite leaching by S-10 (▲▲), M-12 (●●) and *T. ferrooxidans* (■■). Key: ferrous (green symbols) and ferric (red symbols) iron. Data for uninoculated controls are also shown (× ×; broken lines).

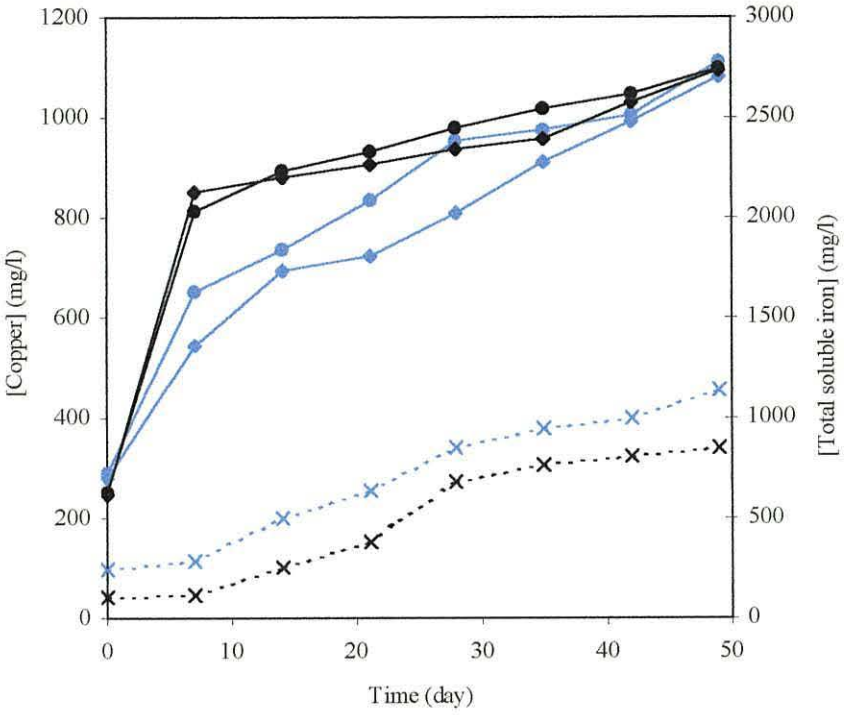


Fig: 6.9a: Chalcopyrite leaching by RIV-14 (◆◆) and L-15 (●●) grown in 1% (w/v) chalcopyrite at 35°C. Bioleaching was determined by measuring concentrations of copper (blue symbols) and total soluble iron (black symbols). .Data from uninoculated controls are also shown (× ×; broken lines).

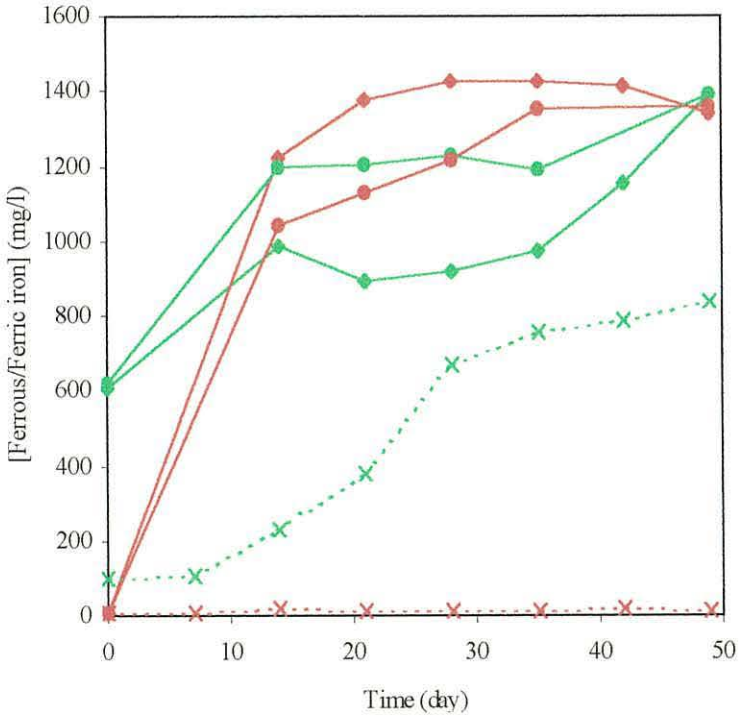


Fig: 6.9b: Iron speciation during chalcopyrite leaching by RIV-14 (◆◆) and L-15 (●●). Key: ferrous (green symbols) and ferric (red symbols) iron. Data for uninoculated controls are also shown (× ×; broken lines).

6.7 Discussion

Leaching of metal sulphides may be accelerated by mesophilic or thermophilic iron-oxidising acidophilic prokayotes such as *T. ferrooxidans*, *L. ferrooxidans*, *Sulfobacillus* spp., *Sulfolobus* spp., etc. via the generation (or regeneration) of ferric iron (Fe^{3+}) and sulphuric acid. In addition, environmental parameters such as pH, temperature, chemical and electrochemical factors are all closely related to the activity of leaching bacteria and consequently to the efficiency of the overall bioleaching process (Donati and Tedesco, 1990; Niemela *et al.*, 1994; Linström *et al.*, 1993; Hansford and Vargas, 1999).

Bacteria used in bioleaching are remarkable in that they have very modest nutritional requirements but in many instances have been shown to be not capable of tolerating more than traces of organic matter when grown in pure culture (Rawling, 1997). All four mesophilic isolates from Montserrat were capable of pyrite oxidation in pure culture, which was not surprising because these bacteria were able to oxidise both iron and reduced sulphur compounds (Chapter 3 and 4). These studies showed that pyrite leaching was slightly suppressed for Gram-negative Montserrat isolates (S-10 and M-12) and *T. ferrooxidans* in yeast extract-containing pyrite medium, compared to autotrophic (organic-free) cultures. On the other hand, oxidative dissolution of pyrite by isolates RIV-14 and L-15 was accelerated by the presence of organic substrates, though some oxidation was observed in organic-free medium. These effects were analogous to the results obtained from iron oxidation experiments in the presence and absence of yeast extract (Chapter 4). Autotrophic growth of RIV-14 and L-15 on pyrite suggests that sulphide may served as a suitable reduced inorganic sulphur compound (RISC) for these bacteria, though a variety of RISCs (including tetrathionate) have been postulated to be

formed during the oxidation of pyrite by ferric iron (Sand *et al.*, 1995; Evangelou, 1995; Schippers and Sand, 1999).

The enhanced rates of pyrite oxidation by the mesophilic isolates RIV-14 and L-15 in organic-supplemented cultures was similar to that reported for moderately thermophilic *Sulfobacillus* spp. (Norris *et al.*, 1996; Ghauri and Johnson, 1991), and also for some mesophilic iron-oxidising heterotrophs (Bacelar-Nicolau and Johnson, 1999). In this study, the response to yeast extract might explain the biphasic pattern of pyrite oxidation displayed by both RIV-14 and L-15 in yeast extract-containing medium. Faster rates were determined during the early phase (21 days) of pyrite oxidation, followed by slower rates in latter phase (21 - 35 days). Despite the more limited abilities of L-15 and RIV-14 displayed in organic-free medium, autotrophic growth on pyrite was found to be enhanced after a period of adaptation in organic-free pyrite medium. Variable responses to yeast extract have also been observed with some strains of moderate thermophiles (Ghauri, 1991).

The oxidation of sulphidic minerals in many cases, results in the production of significant amount of sulphuric acid, which causes culture pH to fall. Due to the poor buffering capacity of the media generally used, acid accumulation causes a continuous pH reduction, which may eventually decline to below pH 1.0. Such extremely acidic conditions may impact the activity and survival of mineral leaching prokaryotes. In general, optimum leaching occurs when the bacteria and archaea in bioleaching systems are capable of growing and remaining active, despite the potentially negative effects of acid and metal accumulation in the leachate solutions.

Bioleaching bacteria are often remarkably adaptable when faced with adverse growth conditions, though there appears to be substantial variation in tolerance to, for

example, pH, temperature and metal ions between species or even strains within a single species. The Gram-positive isolates, RIV-14 and L-15, were extremely acidophilic, as evident by their abilities to grow at pH 1.0 (and below pH 1.0, in the case of isolate L-15) (section 4.5.2). The extremely acidophilic nature of isolate L-15 was also confirmed by its ability to oxidise pyrite in medium poised initially at pH 1.2, and to remain active despite the acidification of the growth medium during pyrite leaching. In contrast, growth of the type strains of *T. ferrooxidans* and *L. ferrooxidans* was completely inhibited at the same conditions (pH 1.2 at 35°C), indicating that these bacteria are more sensitive to extremely low pH. In contrast *T. ferrooxidans* oxidised pyrite more rapidly than L-15 in media poised initially at pH 1.5 and 2.5.

The studies also showed that the type strain of *L. ferrooxidans* was the least effective pyrite oxidiser of the three acidopiles tested. This was possibly due to the fact that it cannot oxidise sulphur, which reduces its efficiency to oxidise sulphide minerals compared to *T. ferrooxidans*, as indicated by a delay in sulphate production (pH reduction) in relation to iron released (Norris *et al.*, 1988). Furthermore, much more energy and therefore greater growth of leaching bacteria, can be gained from oxidation of sulphur compounds compared to oxidation of Fe^{2+}. However, the presence of *L. ferrooxidans* in a mixed culture system with sulphur-oxidising acidophiles such as *T. thiooxidans* has been reported to enhance the overall bioleaching processes (Norris and Kelly, 1982). More recently *Leptospirillum*-like species rather than *T. ferrooxidans* have also been reported to be the dominant bacteria in many commercial processes due to their greater tolerance to low pH and ferric iron. Furthermore *L. ferrooxidans* has also been reported to be more tolerant to slightly elevated temperatures (37° - 45°C; Norris *et al.*, 1988) than *T. ferrooxidans* (< 35°C; Sand *et al.*, 1992, Rawlings *et al.*, 1999).

Measurement of rates of oxidation of pyrite and other sulphide minerals based on the analyses of total iron in solution has been shown to be an accurate indicator of reaction progress in low pH cultures (reviewed in Evangelou, 1995). However, its use at higher pH is precluded due to the low solubility of ferric iron at pH > 2 – 2.5. In the present study, Fe^{3+} precipitates were observed during the early phase of the cultures grown in medium initially adjusted to pH 2.5, which might have given rise to some inaccuracy. Ferric iron precipitates were reported for *T. ferrooxidans* grown at a high ferrous sulphate concentration (120 mM) at 23°C in pH 2.5 medium (Grishin *et al.*, 1988). The production of solid-phase products was also reported during the bacterial oxidation of arsenical pyrite; in this case analyses of the total iron solubilised entailed solubilising the precipitates by acid digestion at high temperature (30 min. at 65°C; Carlson *et al.*, 1992). In highly acidic conditions (pH < 2), the measurement of pyrite oxidation based on changes in oxidation potential of the iron couple (Fe^{3+}: Fe^{2+} ratios or E_h) is also feasible, due to the high solubility of iron (Fe^{2+} and Fe^{3+}). Under acidic conditions, abiotic oxidation of ferrous iron and ferric iron hydrolysis is negligible and therefore, ferrous iron oxidation is totally due to microbial activity. The high solubility of both iron species under extremely acidic condition would also facilitates the accuracy of total iron analyses as a means of monitoring pyrite oxidation. Furthermore, production of solid-phase products during bioleaching have a direct impact on the activity of bioleaching bacteria, as reported for *T. ferrooxidans* (Grishin *et al.*, 1988; Carlson *et al.*, 1992).

The greater tolerance of isolate L-15 to extremely low pH compared to *T. ferrooxidans* was also confirmed in pyrite leaching experiments carried out under controlled pH conditions in a bioreactor. A distinct response was displayed by isolate

L-15, not only by the pattern of pyrite oxidation but also by its ability to grow and remain active at pH as low as 0.8, conditions which were lethal to *T. ferrooxidans*. Tolerance of extreme acidity has also been reported for the sulphur-oxidising mesophile *T. thiooxidans*, which is capable of growth at pH of less than 0.8 (Norris, 1983), though it is far less tolerant of soluble ferric iron (Said, 1990). The ability of leaching microorganisms to tolerate highly acidic conditions is advantageous as these prokaryotes would continue to be active as leaching progresses. Sulphide mineral leaching is often a highly acid producing process, causing the pH of impacted environments to decrease sharply, e.g. during for mesophilic copper bioleaching (Vasquez and Espejo, 1997).

Bacterial leaching technology has primarily focussed on the mesophilic *Thiobacillus* species. However, recent investigations have also revealed the presence of thermophilic bacteria in ore leaching dumps and heaps (Brierley, 1978; Norris and Owen, 1993). In the current study, the novel moderately thermophilic "*Alicyclobacillus*"-like strain (GSM), isolated from a self-heated waste dump rock in Montana and capable of ferrous iron and sulphur oxidation has been characterised (Chapter 5). GSM was also shown to oxidise pyrite. In organic-free (autotrophic) pyrite cultures, growth of GSM was similar to that of chemolithotrophic acidophiles but, unlike the latter, GSM was also able to utilise organic substrates, as evidenced by the rapid utilisation of fructose in pyrite/fructose cultures. There was also evidence of less ferrous iron oxidation by GSM in the organic-containing medium, as indicated by the greater amounts of ferrous iron which accumulated in the leachate. Ferrous iron concentrations were found be *ca.* 50% higher than those in organic-free medium while ferric iron concentrations were corresponding lower. The redox potentials on the organic-free and organic-amended pyrite cultures, calculated from concentrations of ferrous and ferric iron, showed that the

fructose-containing medium maintained a lower redox potential (+711 to +720 mV compared with +794 to +810 mV for the autotrophic cultures) throughout the 40 day incubation period. Interestingly, net leaching of pyrite in these cultures was quite similar, even though it might have been supposed from other studies (e.g. Rawlings *et al.*, 1999) that leaching would have progressed faster in the higher redox cultures. Enhanced leaching of pyrite under relatively low redox values was also reported by Bacelar-Nicolau and Johnson (1999) for "*F. acidophilum*" T23 grown in the presence of glycerol. Mineral leaching under low redox potentials is advantageous with some ores and concentrates (e.g. chalcopyrite) where greater metal extraction and avoidance of passivation layers (secondary minerals; e.g. jarosites) might be expected. The successful oxidation of pyrite by GSM in organic-free medium was somewhat surprising in view of its proclivity for heterotrophic growth. Indeed, other moderately thermophilic mineral-oxidising bacteria (*Ad. ferrooxidans* and *Sulfobacillus* spp.) often display poor pyrite oxidation when grown in organic-free medium in pure culture (Ghauri and Johnson, 1991). Mixed cultures of *Ad. ferrooxidation* with either *S. acidophilus* or *S. thermosulfidooxidans* have been found to be more efficient at pyrite oxidation than pure cultures of these bacteria (Clark and Norris, 1996) Manipulation of the moderately thermophilic acidophiles in commercial bioleaching processes has also been reported to enhanced the dissolution of finely ground mineral sulphide concentrates in stirred tank reactors (Marsh and Norris, 1983; Clark and Norris, 1996) and in bioheap processes (Brierley, 1999).

The ability of isolate GSM to mineralise a diverse range of organic compounds might be very useful in mixed culture leaching systems. A limiting factor for the growth of autotrophic mineral oxidising bacteria is often CO_2, particularly at higher temperatures and concentrated solute solutions (bioleaching liquors), both of which lower the

solubility of CO_2. Commercial bioleaching tanks are generally gassed with CO_2-enriched air (Mintek, South Africa, personal communication). In theory, addition of small quantities of a cheap organic material (e.g. glucose concentrate) to a mineral leachate with a GSM-containing mixed culture would result in the continuous production of CO_2, which could then be fixed by, for example, thermotolerant strains of *L. ferrooxidans*, which may ultimately be more efficient than GSM at oxidising sulphides. There would also be a synergistic effect on mineral oxidation by GSM itself (Fig 6.9).

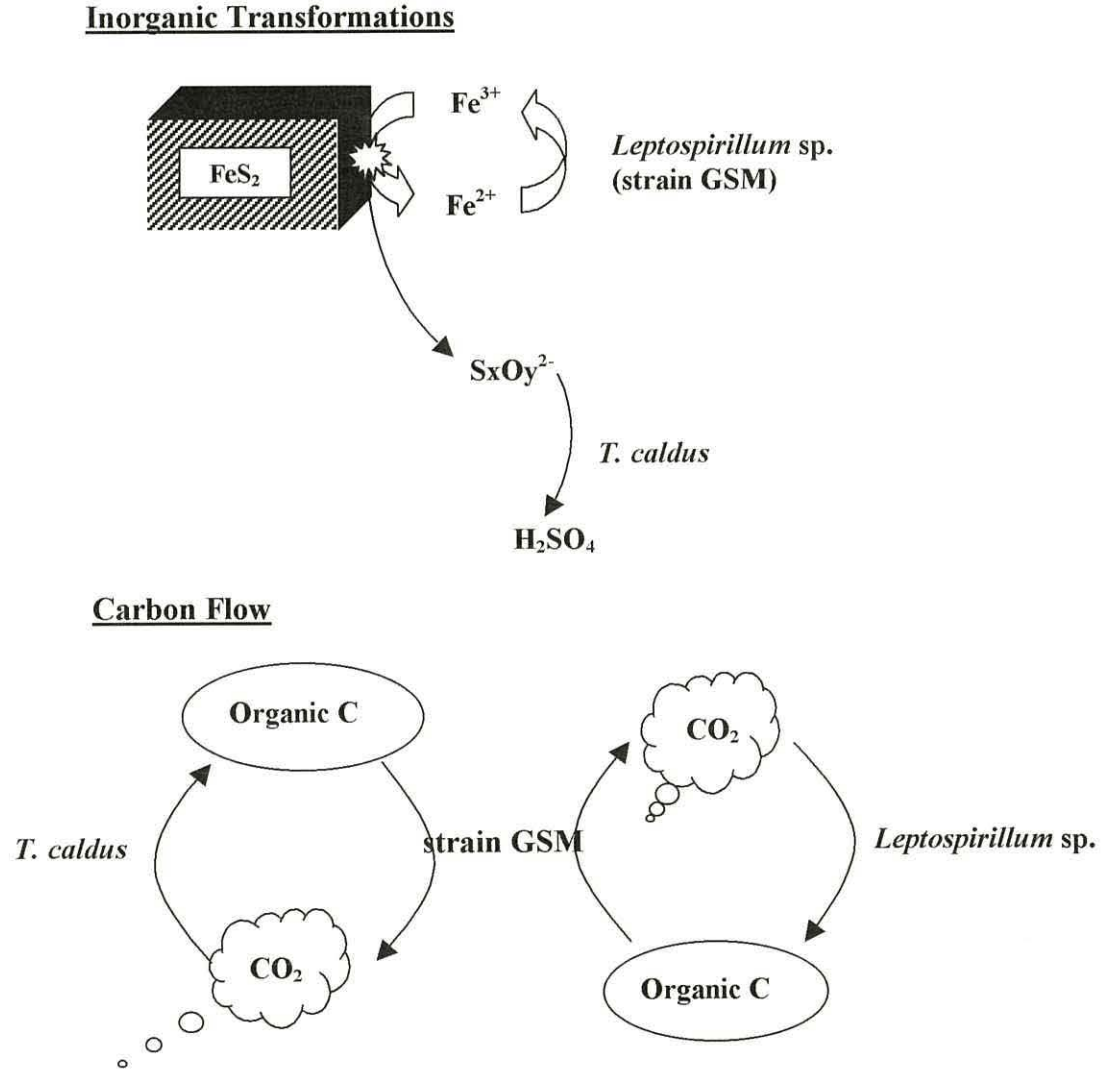


Fig. 6.9: Hypothetical scheme for the leaching of pyrite by mixed cultures of chemolithotrophic iron-oxidising thermotolerant *L. ferrooxidans*, sulphur-oxidising *T. caldus* and chemolithoheterotrophic isolate GSM (proposed by N. Okibe and D.B. Johnson, unpublished).

Chalcopyrite ($CuFeS_2$) is a very important sulphidic mineral being the major copper-containing mineral exploited by mankind. However, it has proved difficult to bioleach chalcopyrite. Problems result from the complex structure of its ores (Hackl *et al.*, 1995) as well as the formation of passivation layers of jarosite, sulphur or polysulfide layers which form on the surface of chalcopyrite during leaching (Hansford and Vargas, 1999; Barrett *et al.*, 1993).It was interesting to note that the amount of copper solubilised from the chalcopyrite ore was similar for all four Montserrat isolates. The ferric : ferrous iron concentrations (and hence redox potential) were, however, much greater with the Gram-negative *Thiobacillus*-like isolates (S-10 and M-12) than the Gram-positive *Sulfobacillus*-like isolates (RIV-14 and L-15). Bioleaching of chalcopyrite at low redox values is perceived to have some major advantages (D.B. Johnson, personal communication). It was also noted that all four Montserrat isolates were more successful than the type strain of *T. ferrooxidans* in liberating copper from this particular ore. One reason for this might be that the low pH (1.4) and slightly elevated (35°C) temperature at which this leaching experiment was set up was less conducive to the growth of *T. ferrooxidans*^{T} than to the Montserrat isolates.

Bacterial leaching (or conventional ore processing) of pyrite to recover iron is not economically feasible though pyrite has, in the past, been used as a source of sulphur. However pyrite was used as a model sulphide mineral in bioleaching experiments, as is frequently the case (e.g. Ghauri and Johnson, 1991; Clark and Norris, 1996; Norris and Owen, 1993). The results have a direct bearing on commercially important gold-bearing arsenopyrite and chalcopyrite ores as well as for the depyritisation of coal. In commercial scale bioleaching processes, the ratio of ferric to ferrous iron (redox potential) is frequently considered to be the main factor affecting the rate of sulphide

mineral oxidation, in which *T. ferooxidans* and *L. ferrooxidans* are thought to dominate the process. In general, a high redox value (~ +790 to +900 mV) is considered to be essential for an effective bioleaching process (Rawlings *et al.*, 1999). In contrast, the present study with the Gram-positive mesophiles (RIV-14 and L-15) and the thermophilic isolate (GSM) showed that successful leaching could be achieved at lower redox values (~ +745 to 780 mV) than have been previously reported. The overall conclusion was that all the bacteria used in this study are possible candidates for bioleaching operations either in the mesophilic temperature range (30° - 40°C) or, in the case of the thermoacidophilic isolate GSM, at elevated temperatures (> 40°C).

(7)

General Discussion and Conclusions

Extremely acidic environments (pH < 3) occur naturally, for example in some geothermal and coastal areas, though more frequently they are associated with human activity, particularly the mining of coal and metal ores. Acidic, metal-rich environments have been considered as complex systems both in terms of variety of microorganisms present and the various interactions between them (Lopez-Archila *et al.*, 1995; Johnson, 1998a). Isolation and characterisation of acidophilic bacteria have also received much attention (e.g. Johnson, 1991; Norris, 1990) though this has tended to focus on acidophiles which have the ability to degrade sulphide minerals. Even in the recent past, cultivation of acidophilic bacteria has sometimes proved to be problematic and has occasionally failed to differentiate mixed populations (Tuovinen and Kelly, 1973; Harrison, 1984). However, successful cultivation techniques have been developed which facilitate cultivation of a wide variety of acidophilic microorganisms in the laboratory (e.g. Johnson and McGinness, 1991; Johnson, 1995). In recent years, the emergence of molecular-based techniques (PCR, DNA hybridisation, RFLP etc.) has increased the overall prospectus and quality of microbial identification and characterisation of acidophilic bacteria (Goebel and Stackebrandt, 1994; Durand *et al.*, 1997; Selenska-Pobell *et al.*, 1998). Therefore, a combination of cultivation and molecular techniques allow characterisation studies to be more accurate and reliable.

In the current project, a group of five obligately acidophilic bacteria were studied which have novel characteristics that distinguish them from known acidophilic bacteria. Characteristics of these isolates are summarised in Table 7.1. They were capable of iron and sulphur oxidation and classed as:

1) mesophilic, Gram-negative, *Thiobacillus*-like eubacteria (S-10 and M-12);

2) mesophilic, Gram-positive *Sulfobacillus*-like eubacteria (RIV-14 and L-15);

3) a moderately thermophilic eubacterium (GSM) which appears to be distinct from other genera of acidophiles.

Table 7.1: Some characteristics of the five mineral-oxidising acidophilic bacteria described in the present study.

Characteristics	Isolates				
	S-10	M-12	RIV-14	L-15	GSM
Morphology:					
• Gram stain	negative	negative	positive	positive	positive
• Endospore formation	-	-	+	+	+
• Shape	rod	rod	rod, filamentous	rod, filamentous	rod, filamentous
• Motility	++	+	±	±	-
• Size (μm)	1.32 x 0.45	1.25 x 0.48	2.25 x 0.55	2.9 x 0.72	2.6 x 0.35
Electron donor:					
• Ferrous iron	+++	+++	+++	+++	++
• Elemental sulphur	+++	+++	-	-	+
• Tetrathionate	+++	+++	+	+	++
• Organic compounds	-	-	+	++	+++
Electron acceptors:					
• Oxygen	+++	+++	+++	+++	+++
• Ferric iron:	+++	+++	++	+	n.d
- autotrophic	-	-	+++	+++	++
- heterotrophic					
Growth on solid media	Feo FeSo	Feo FeSo	FeSo	FeSo	Feo FeSo YE
Growth conditions:					
• Temperature(°C)					
- optimum	30	33	35	37	45
- range	<20 - 35	<25 - 37	25 - 37	<30 - 43	30 - >50
• pH					
- optimum	1.8	1.5	1.5	1.6	1.8
- range	1 – 3	>1 - 3	1 – >2	0.8 - >2	<1.6 – 3
(G + C) mol%					

+++: very good growth; ++: good growth; +: sparce growth; -: no growth

± : not constantly motile; n.d.: not determined; >: indicates that the pH or temperature supporting growth are greater than the values shown but not determined; <: indicates that the pH or temperature supporting growth are less than the values showen but not determined.

The Caribbean island of Montserrat was home to a considerable number of extremely acidic sites of varying temperature, prior to the most recent period of volcanism. A variety of acidophilic bacteria were isolated from various sites in Montserrat (Atkinson *et al.*, 2000), though only four isolates are described in this thesis. Two of these isolates (S-10 and M-12) have some physiological and morphological traits in common with *T. ferrooxidans* (Gram negative, iron oxidising mesophilic rods); however, they differ from *T. ferrooxidans* by their ability to grow mixotrophically in ferrous iron/yeast extract liquid medium and in their higher G + C contents. In contrast, the mesophilic isolates RIV-14 and L-15 showed greater affinity (physiological and phylogenetic) to the genus *Sulfobacillus* (all characterised species of which, with one exception, are thermophilic). The latter two isolates are Gram-positive, endospore-forming rods that display considerable metabolic flexibility. They can, for example, grow chemolithotrophically on ferrous iron (though they have a requirement for reduced sulphur compound, such as tetrathionate), chemolithoheterotrophically (in iron or sulphur medium containing yeast extract) or heterotrophically on a limited range of organic substrates. These bacteria are more sensitive to copper than *T. ferrooxidans* or isolates S-10 and M-12, but somewhat less sensitive to molybdate.

Isolate GSM was readily distinguished from the Montserrat isolates by its thermotolerant nature. This bacterium shared morphological and physiological traits with the mesophilic Gram-positive isolates RIV-14 and L-15, though GSM differed from the Gram-positive Montserrat isolates in displaying greater affinity for heterotrophic growth.

All the Gram-positive isolates were capable of growth on various defined organic substrates, including organic acids. Organic acids have been reported to be toxic to acidophilic bacteria (Schnaitman and Lundgren, 1965; Ingledew, 1982), due to the fact

that they exist predominantly in their non-dissociated (protonated) forms in acidic solutions (pH <3). Since the non-dissociated form is much more membrane-permeable than the dissociated (negatively charged) molecule, the net influx of the non-dissociated molecule into the cytoplasm occurs, followed by the dissociation of the translocated acid leading to a decrease in cytoplasmic pH (Ingledew, 1982) unless the microbes are able to metabolise the organic acid after its entry into the cytoplasm (Booth, 1985).

The metabolic versatility of acidophilic microorganisms is noe recognised to be considerably greater than has been assumed previously. Acidophilic microorganisms exist as mixed populations in both natural and man-made environments and a variety of interactions occur between them, including competition (Johnson, 1998a). The metabolic flexibility of the isolates described in this thesis may confer competitive advantages over obligately chemolithoautotrophic acidophiles. In view of carbon aquisition, the Gram-positive isolates L-15 and GSM displayed an ability to switch from pre-fixed organic carbon to atmospheric CO_2, depending on the availability of organic compounds. As carbon is a major component in all life forms, such a response would confer a better chance for these bacteria to survive and remain active in acidic environments which change with regard to the availability of dissolved organic carbon. In addition, all five isolates are facultative anaerobes. They are capable of anaerobic growth by coupling the reduction of ferric iron and the oxidation of inorganic sulphur (or organic compounds, in the case of three Gram-positive isolates), thus enabling these bacteria to remain active in situations where dissolved oxygen levels fluctuate.

The ability of all five isolates to both oxidise and reduce iron also suggests significant roles in iron cycling in extremely acidic environments. However, their contribution to overall iron cycling might very much depend on specific rates of iron

oxido-reduction. These studies found that the specific rates of iron oxidation by the Montserrat isolates were affected by nutritional conditions and oxygen levels. The iron oxido-reduction systems in RIV-14 and L-15 were inducible under appropriate conditions though iron-reducing activity was generally far smaller than the maximum iron-oxidising activity detected, indicating a higher iron oxidase activity in these isolates. However, specific rates of iron-oxidation declined when organic substrates were present. It has been reported that the contribution of heterotrophs to the overall rate of iron cycling may become quantitatively significant when the ratio of available organic substrates versus available ferrous iron increases (Pronk and Johnson, 1992; Johnson, 1998b). However, detailed knowledge of the fluxes of organic carbon in acidic environments is needed for a quantitative assessment of the role of heterotrophs or mixotrophs in microbial iron cycling.

Iron- and sulphur-oxidising abilities were considered to be the major criteria that equipped these bacteria to grow readily on sulphide minerals. Therefore exploitation of these bacteria for bioleaching processes is possible (Chapter 6), though further investigations would be necessary to further understanding on interactions and synergies with other acidophiles in mixed communities. In natural leaching environments, most of which are characterised by extremely low nutrient concentrations, competition for organic substrates with other obligately heterotrophic and mixotrophic acidophiles would be anticipated. However, being able to conserve metabolic energy from the oxidation of inorganic iron and sulphur compounds, coupled with their flexible carbon fixation mechanisms might allow for more rapid growth, and may contribute to their ecological success in acidic environments.

The phylogeny of the five isolates was determined from the comparative analyses of their 16S rRNA gene sequences with those of other bacteria found in the database. Their relationship with other characterised acidophilic iron-oxidising bacteria is shown in Fig 7.1. Based on the comparative analysis of 16S rRNA gene sequences, isolate S-10 exhibits a very close relationship to isolate M-12 (99% sequence identity). Both isolates are more closely related (99% sequence identity) to *T. ferrooxidans* DSM 9465 than to the type strain of *T. ferrooxidans* ATCC 23270 (98% sequence identity). However, both S-10 and M-12 have a G + C content of 65%, which is significantly greater than the type strain *T. ferrooxidans* ATCC 23270 (58% – 59%). Based on the physiological and phylogenetic characteristics, both Gram-negative Montserrat isolates S-10 and M-12 are currently considered to be strains of *T. ferrooxidans*, though their mixotrophic nature and differences in G + C contents suggest that they may represent a novel species.

The two Gram-positive Montserrat isolates RIV-14 and L-15 have a close phylogenetic relationship to the genus *Sulfobacillus*, with *S. thermosulfidooxidans* as the closest neighbour (94% sequence identity). RIV-14 and L-15 are also closely related to each other, though the binary level comparison of 16S rRNA between them is low enough (97% sequence similarity) to consider them as two novel and distinct species of the genus *Sulfobacillus*. In addition there were also several physiological traits that distinguished these isolates from each other (Chapter 4). It is proposed that isolate L-15 will be named (subject to official acceptance and confirmation) as "*Sulfobacillus montseratensis*", to reflect its island of origin. The current proposed name for isolate RIV-14 is "*Sulfobacillus ambivalens*" referring to its apparent ability to both oxidise and reduce sulphur.

The Gram-positive moderate thermophilic isolate GSM is most closely related (16S rRNA gene sequence) to the genus *Alicyclobacillus*, with *Al. cycloheptanicus* as the

closest neighbour (93% sequence identity). However, many of the physiological characteristics of GSM are very different to those of the four classified *Alicyclobacillus* spp., particularly with regard to its chemolithotrophy and metabolism of iron and sulphur. In addition, GSM does not contain ω-alicyclic fatty acids therefore cannot be considered as a member of the genus *Alicyclobacillus* as currently defined. Isolate GSM appears, therefore, to be a novel (and third) genus of moderately thermophilic, acidophilic Gram-positive eubacteria. A possible genus name is "*Caldibacillus*", indicating that it is a moderately thermophilic *Bacillus*-like bacterium, with the species name "*Caldibacillus ferroxidans*" to indicate its propensity for iron oxidation.

It is likely that further experimental data are needed to firm the novel species/genus identities of the bacteria described in this thesis, most notably DNA-DNA hybridisation studies with nearest-neighbour known organisms.

This study has shown further that acidophilic, mineral-oxidising bacteria are a diverse group of microorganisms. Future developments in the biotechnology of microbially-catalysed mineral leaching may well involve the use of different microorganisms to the *Thiobacillus* / *Leptospirillum* spp., which have traditionally been considered to be the major organisms involved in the process. Defined mixed cultures, possibly including one or more of the bacteria described in this thesis, are likely to become of greater significance as the range and complexity of sulphide ores subjected to bioleaching increases in the 21st century.

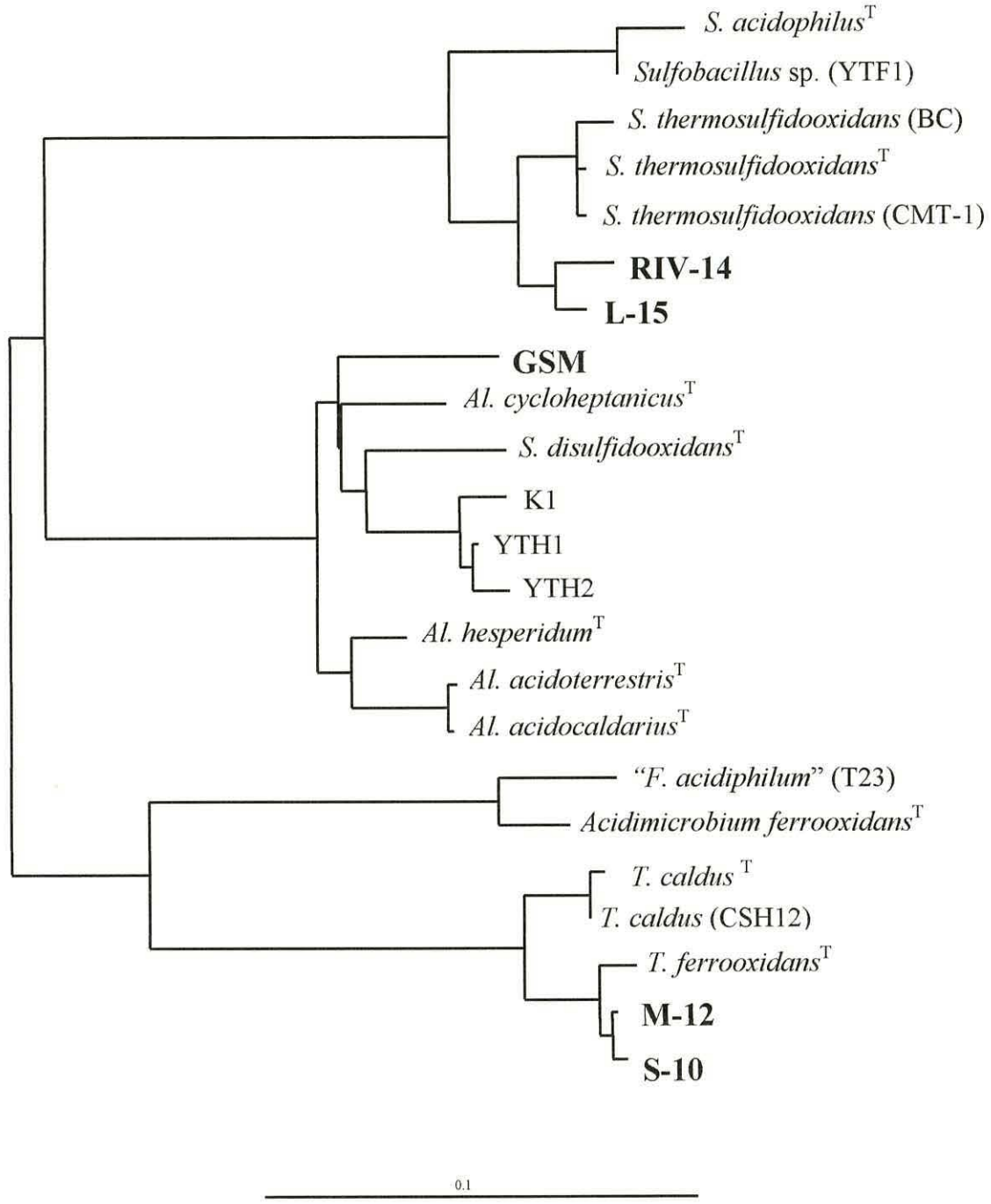


Fig. 7.1: Phylogenetic relationship of four Montserrat isolates and a moderately thermophilic acidophilic iron oxidiser, base on the 16S rRNA gene sequences analysis. The tree branches were proportional to the calculated distances. Tree was rooted with *Korarchaeote* as an outgroup. The bar indicates 0.1 nucleotide substitutions per site.

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