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Series on Harmonisation of Regulatory Oversight in Biotechnology No. 37

CONSENSUS DOCUMENT ON INFORMATION USED IN THE ASSESSMENT OF ENVIRONMENTAL APPLICATIONS INVOLVING *Acidithiobacillus*

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Series on Harmonisation of Regulatory Oversight in Biotechnology

No. 37

Consensus Document on Information Used in the Assessment of Environmental Application Involving *Acidithiobacillus*

Environment Directorate

Organisation for Economic Co-operation and Development

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FOREWORD

Consensus documents contain information for use during the regulatory assessment of a particular product. This document addresses information of environmental applications involving the genus *Acidithiobacillus*. Canada served as the lead country in the preparation of this document. The draft has been revised on a number of occasions based on the inputs from other member countries. At the 17th meeting of the Working Group (24-26 October 2005), it was agreed that the document be forwarded to the joint Meeting of OECD's Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology, which then agreed that this document be declassified.

TABLE OF CONTENTS

Tables

PREAMBLE

The environmental safety/risks of transgenic organisms are normally based on the information on the characteristics of the host organism, the introduced traits, the environment into which the organism is introduced, the interaction between these, and the intended application. The OECD's Working Group on Harmonisation of Regulatory Oversight in Biotechnology decided at its first session, in June 1995, to focus its work on identifying parts of this information, which could be commonly used in countries for environmental safety/risk assessment to encourage information sharing and prevent duplication of effort among countries. Biosafety Consensus Documents are one of the major outputs of its work.

Biosafety Consensus Documents are intended to be a "snapshot" of current information on a specific host organism or trait, for use during regulatory assessments. They are not intended to be a comprehensive source of information on everything that is known about a specific host or trait; but they do address the key or core set of issues that member countries believe are relevant to risk/safety assessment. This information is said to be mutually acceptable among member countries. To date, 25 Biosafety Consensus Documents have been published. They include documents which address the biology of crops, trees and micro-organisms as well as those which address specific traits which are used in transgenic crops.

In reading this Consensus Document, it is useful to consult another document: *An Introduction to the Biosafety Consensus Document of OECD's Working Group for Harmonisation in Biotechnology.* This text explains the purpose of the consensus documents and how they are relevant to risk/safety assessment. It also describes the process by which the documents are drafted using a "lead country" approach.

The Consensus Documents are of value to applicants for commercial uses of transgenic organisms, regulators in national authorities as well as the wider scientific community. As each of the documents may be updated in the future as new knowledge becomes available, users of Consensus Documents are encouraged to provide any information or opinions regarding the contents of this document or indeed, OECD's other harmonisation activities. If needed, a short pre-addressed **questionnaire** is attached at the end of this document that can be used to provide such comments.

The published Consensus Documents are also available individually from OECD's website (http://www.oecd.org/biotrack) at no cost.

GENERAL INTRODUCTION

1. This document presents information that is accepted in the literature about the known characteristics of bacteria in the genus *Acidithiobacillus*. Regulatory officials may find the technical information useful in evaluating properties of micro-organisms that have been derived for various environmental applications. Consequently, this document provides a wide range of information without prescribing when the information would or would not be relevant to a specific risk assessment. The document represents a snapshot of current information (end-2002) that may be potentially relevant to such assessments.

2. In considering information that should be presented on this taxonomic grouping, the Task Group on Micro-organisms has discussed the list of topics presented in the "Blue Book" (*i.e.* Recombinant DNA Safety Considerations (OECD, 1986)) and attempted to pare down that list to eliminate duplications as well as those topics whose meaning is unclear, and to rearrange the presentation of the topics covered to be more easily understood (the Task Group met in Vienna, 15-16 June, 2000). This document is a first draft of a proposed Consensus Document for environmental applications involving organisms from the genus *Acidithiobacillus*.

A. GENERAL CONSIDERATIONS

1. Subject of document: species included and taxonomic considerations

3. The four species of *Acidithiobacillus* covered in this document were formerly placed in the genus *Thiobacillus* Beijerinck. In recent years several members of *Thiobacillus* were transferred to other genera while the remainder became part of three newly created genera, *Acidithiobacillus*, *Halothiobacillus*, *Thermithiobacillus,* and to the revised genus *Thiobacillus sensu stricto* (Kelly and Harrison, 1989; Kelly and Wood, 2000). The reassignment to these three newly designated genera was based on physiological characteristics and 16S rRNA gene sequence comparisons (Kelly and Wood, 2000). *Acidithiobacillus* contains two species (*A. ferrooxidans* and *A. thiooxidans*) of the original genus *Thiobacillus* that have the potential to cause significant ecological damage. Two other species have been reassigned to this new genus, *A. caldus* and *A. albertensis*. All these species have been, or are likely to be, employed in various biotechnological applications in the environment.

2. Characteristics of the organism: identification and the methods used to identify the organism

2.1 Characterisation of the genus Acidithiobacillus

4. The genus was established by Kelly and Wood (2000), with *A. thiooxidans* (formerly *Thiobacillus thiooxidans)* as the type species. The four species included in the genus are Gram-negative, rod-shaped (0.4 x 2.0 μm), motile with one or two flagella, and possess the ability to use reduced sulphur compounds as electron donor for autotrophic growth, in common with various other unrelated "sulphur bacteria" (Kelly and Harrison, 1989; Kuenen *et al*., 1992). As with other *Thiobacillus* species now redistributed, members of this genus are distinguished morphologically from other colourless sulphur bacteria by forming external rather than internal sulphur particles (Kuenen, 1989). They are strictly aerobic and obligatory acidophilic (optimum pH < 4.0). Some species oxidise ferrous iron and hydrogen (Table 1) or use natural and synthetic metal sulphides to generate energy, while one species (*A. ferrooxidans*) can oxidise iron. The optimum temperature ranges from 30-35 ^{o}C for mesophilic species to 45 ^{O}C for moderately thermophilic species. All of the species contain ubiquinone Q-8, and the G+C content of the DNA is 52-64 mol %. *Thiobacillus sensu stricto* now contains only species belonging to the β-subclass of the Proteobacteria, but *Acidithiobacillus*, together with *Halothiobacillus* and *Thermithiobacillus*, have been assigned to the γ-subclass (Kelly and Wood, 2000). A full account of the genus is given in the section contributed by Kelly and Wood (2005) in the 2nd edition of Bergey's Manual of Systematic Bacteriology.

2.2 Differentiation of Acidithiobacillus from related taxa

5. Members of the genus are distinguished by their obligate acidophilic nature (pH < 4.0) and possession of ubiquinone Q-8.

2.3 Characters used in classification

2.3.1 Phenotypic characters

6. Many of the phenotypic characters of *Acidithiobacillus* such as the rod-like shape, motility,

Gram-negative reaction and utilisation of sulphur compounds are shared in common with species formerly placed in *Thiobacillus.* These characters are useful for broad recognition but no longer for critical identification.

2.3.2 Mol% G+C content

7. The determination of the mol % G+C content of the DNA of bacterial isolates has been used for a long time to determine whether strains could be related to each other. It is to some extent, a negative test. While widely differing G+C values can suggest that two isolates are not related, matching G+C values do not guarantee that they are the same. G+C values for the four species of *Acidithiobacillus* are, however, often sufficiently far apart to serve as useful species characteristics.

2.3.3 Ubiquinones and cellular fatty acid analysis

8. Lane *et al*. (1985) determined that there was a correlation between ubiquinone type and physiological behaviour. Katayama-Fujimura *et al*. (1982) used types of ubiquinones and the DNA base composition to differentiate 11 species of the former genus *Thiobacillus*. The association between species and ubiquinone type was constant except for *T. perometabolis*, one strain having 8 and the other 10-isoprene units. Species presently assigned to *Acidithiobacillus* all possessed eight units. The strain of *A. ferrooxidans* examined by the later authors was unique in that it had ubiquinones with 9 as well as 8 isoprene units.

2.3.4 Nucleotide structure

9. 5S rRNA sequences were obtained for thirteen species of the original genus *Thiobacillus* (Lane *et al*., 1985, 1992) and these sequences were shown to be distinct for each species. Similarities within the sequences also enabled the species also to be assigned to the α, β or γ groups of the four groups of Proteobacteria, the last group including species of *Acidithiobacillus.*

2.3.5 DNA homologies

10. DNA hybridisation studies on *A. ferrooxidans* and *A. thiooxidans,* together with *Thiobacillus thioparus* and five bacteria formerly placed in *Thiobacillus* (Huber and Stetter, 1989, 1990) established the value of these tests because they showed that there was usually a high degree of homology (>70%) between strains of the same species.

Table 1. *Acidithiobacillus* : Characters used in classification

Species	Optimum pH[a]	pH range	Optimum temperature	Temperature range	Ubiquinone	Mol % G+C	Subclass of Proteobacteria	References
Strictly chemolithotrophic and autotrophic								
Acidithiobacillus albertensis	3.5-4.0	2.0-4.5	28-30	ND	Q-8	61-62	ND	b, c
Acidithiobacillus ferrooxidans	2.0-2.5	1.3-4.5	30-35	2-37	Q-8	58-59	γ	b, d, e
Acidithiobacillus thiooxidans	2.0-3.0	0.5-5.5	28-30	10-37	Q-8	52	γ	b, f
Facultatively chemolithotrophic or mixotrophic with tetrathionate								
Acidithiobacillus caldus[1]	2-2.5	1-3.5	45	32-52	Q-8	63-64	γ	g

ND: not determined
[1]Moderately thermophilic
[a]Katayama-Fujimura *et al*. (1982); [b]Kelly and Harrison (1989)); [c]Bryant *et al*. (1983); [d]Leduc and Ferroni (1994); [e]McCready (1988); [f]Fliermans and Brock (1972); [g]Hallberg and Lindstrom (1994).

2.4 Comments on the species

2.4.1 Acidithiobacillus albertensis

11. Syn. *Thiobacillus albertensis* (Bryant *et al*., 1983; Kelly and Harrison, 1989; Kelly and Wood, 2000). This species is distinguished morphologically by a tuft of polar flagella and a glycocalyx extending outwards from the outer membrane of the bacterial cell envelope and which is used to attach itself to elemental sulphur (Bryant *et al*., 1983). These features together with the relatively high G+C content of the DNA, differentiate this species from the other three (Kelly and Wood, 2000). It has been tentatively assigned to *Acidithiobacillus* because a 16S rRNA sequence for the species is not yet available (Kelly and Wood, 2005).

2.4.2 Acidithiobacillus caldus

12. Syn. *Thiobacillus caldus* (Hallberg and Lindstrom, 1994; Kelly and Harrison, 1989; Kelly and Wood, 2000). This species is distinguished by extremely short rod-shaped cells, each with a single polar flagellum (Hallberg and Lindstrom, 1994), and by its moderately thermophilic nature. It cannot oxidise sulphidic ores, but it may be found associated with others involved in leaching. It is facultatively rather than obligatory chemolithotrophic. A specific, fast and sensitive non-radioactive immuno-binding assay had been used for the detection and enumeration of this species (Amaro *et al*., 1994). Chemiluminescence or peroxidase-conjugated immunoglobulins are employed in a dot or slot blotting system. This method is very convenient for monitoring bioleaching micro-organisms in effluents from industrial bioleaching processes.

2.4.3 Acidithiobacillus ferrooxidans

13. Syn. *Thiobacillus ferrooxidans* (Temple and Colmer, 1951; Kelly and Harrison, 1989; Kelly and Wood, 2000). Morphologically this species appears to be distinguished by a single coiled flagellum in mature cells (Gonzalez and Cotoras, 1987). Also, this is the only species in the genus so far to be able to utilise iron as well as sulphur.

14. Serological and electrophoretic methods have been employed for the rapid detection of isolates of *A. ferrooxidans* and the differentiation of strains (Jerez *et al*., 1986). Different serotypes, characterised by specific lipopolysaccharide banding patterns in polyacrylamide gels, have been described (Koppe and Harms, 1994). A specific and very sensitive dot-immuno-binding assay for the detection and enumeration of *A. ferrooxidans* has been developed by Arredondo and Jerez (1989). Samples were spotted onto nitrocellulose membranes and first incubated with polyclonal antisera, derived from a rabbit inoculated with whole cells of *A. ferrooxidans*, and in ^{125}I-labeled protein A or ^{125}I-labelled goat anti-rabbit immunoglobulin G. The membranes were then dried, autoradiographed on Fuji Rx X-ray film and scanned at 550 nm. The antisera reacted with every strain of *A. ferrooxidans* tested but not with *A. thiooxidans* and *H. neapolitanus* and three species formerly placed in *Thiobacillus*.

15. A specific, fast and very sensitive immuno-electron microscopy method was also developed to identify *A. ferrooxidans* present with other iron oxidising bacteria in acidic mine waters (Coto *et al*., 1992). Polyclonal antisera, produced against whole cells of *A. ferrooxidans*, *A. thiooxidans* and *Leptospirillum ferrooxidans* gave highly specific reactions when cross-reacted with 23 strains of acidophilic bacteria using an immuno-fluorescence staining technique (Koppe and Harms, 1994). These methods have been criticised, however, because of the inability of the antisera to distinguish between dead and living cells (Khalid *et al*., 1993).

16. A systematic study of a large collection of strains ascribed to *A. ferrooxidans* revealed considerable diversity among them (Harrison, 1982). The members of seven DNA homology groups recognised by Harrison (1982) were largely homologous with strains inside each group, but to a lesser degree with strains in other groups. Although all strains grew between 25 and 30 ^{O}C, some were able to grow at 5 ^{O}C and others at 40 ^{O}C, and two genomic groups could be distinguished by different temperature optima. The strains in two other genomic groups (1 and 7) were apparently unable to use elemental sulphur, and their high mol% G+C values (53 and 65 respectively) were well outside the range normally accepted (see Table 1), giving rise to the suspicion that they might represent different species. Although strain m-1, comprising the seventh genomic group, was later shown to be able to oxidise elemental sulphur on prolonged incubation (Johnson, 1995b), it has been further distinguished from a more typical strain of *A. ferrooxidans* by its classification in the 5S rRNA sequence group III rather than in group II (Lane *et al*., 1985).

2.4.4 Acidithiobacillus thiooxidans

17. Syn. *Thiobacillus concretivorus* Parker *Thiobacillus thiooxidans* (Waksman and Joffe, 1922; Kelly and Harrison, 1989; Kelly and Wood, 2000). This species is motile by means of a single polar flagellum, and as with *A. caldus*, it cannot oxidise iron or pyrite, although it can grow on sulphur from pyrite in conjunction with *Leptospirillum ferrooxidans* (Kelly and Wood, 2000). In a study by Harrison (1982), four strains of *A. thiooxidans* were found to be largely homologous with a fifth strain but not with representatives of the seven homology groups of *A. ferrooxidans*. However, a sixth strain ascribed to *A. thiooxidans* showed no similarity to any of them. This indicates that there may be atypical representatives of *A. thiooxidans* as well as *A. ferrooxidans*.

3. Information on the organisms' reproductive cycle (sexual/asexual)

18. *Thiobacillus sensu lato*, in which *Acidithiobacillus* was included, was found to reproduce by binary cell division (Sokolova and Karavaiko, 1968). No spores were observed.

4. Biological features and environmental conditions which affect survival, reproduction, growth, multiplication or dissemination

4.1 Growth requirements

19. Most strains are able to produce colonies on appropriate media solidified with agar or agarose. The use of elemental sulphur is avoided because of its insolubility. Formation of hydrogen sulphide is potentially toxic in moderate concentrations, and the most widely used sulphur compound is thiosulphate (Smith and Strohl, 1991).

20. Some strains, especially those of *A. thiooxidans*, grow poorly on agar media, possibly due to the toxicity of agar hydrolysis products (Kelly and Harrison, 1989). The problem is generally solved by the use of a minimal concentration of agar, screening for suitable brands of purified agars, use of agarose, and, in the case of *A. thiooxidans*, a combination of low agar concentration with pH 2.2-2.5 and ferrous sulfate at only about 20 mM (Johnson, 1995a). The growth of strains of *A. ferrooxidans* on solid media is also difficult. However, the double-layered plates such as FeTSBo and described by Johnson (1995a) allow also the growth of most strains.

21. Species of *Acidithiobacillus*, in common with other former species of *Thiobacillus*, are able to use carbon dioxide as the sole source of carbon for synthesis of cell material (Kuenen, 1975). Ribulose diphosphate carboxylase, the enzyme responsible for the fixation of carbon dioxide, appears to be located in polyhedral inclusions in the cell.

22. In contrast to species of *Halothiobacillus* which have been recorded as either halotolerant or having a strict NaCl requirement (Sievert *et al*., 2000), *A. ferrooxidans* has been recorded as unable to grow at salt concentrations above 1% (Lazaroff, 1963; McCready, 1987; Razzell and Trussell, 1963) whereas specific information for other species of *Acidithiobacillus* for their tolerance to salt is not available.

23. *Acidithiobacillus ferrooxidans* has a remarkable physiology that allows it to thrive in an inorganic mining environment. Its minimum growth requirements can be satisfied by water, air, an oxidisable iron or sulphur source and trace minerals. The trace elements required are usually present as impurities in the water or ore (Rawlings and Woods, 1995). This statement is also true for *A. thiooxidans*, however elucidation of these abilities for other members of *Acidithiobacillus* is still not available. *A. caldus* was found to be relatively insensitive to a number of xanthate and dithiocarbamate-based flotation reagents but sensitive to a number of mercapto-benzthiazole-based reagents (Okibe and Johnson, 2002).

4.2 Oxidation of hydrogen

24. In contrast to strains of *A. thiooxidans*, *H. neapolitanus*, *T. prosperus*, *Leptospirillum ferrooxidans* and four species formerly placed in *Thiobacillus*, three strains of *A. ferrooxidans* were found to be facultative hydrogen oxidisers, being able to use molecular hydrogen as a sole source of energy (Drobner *et al*., 1990). The ability to oxidise hydrogen was repressed by ferrous iron or sulphur and occurred only in the presence of oxygen.

4.3 Nitrogen

25. In addition to their ability to fix carbon dioxide all strains of *A. ferrooxidans* examined so far are also able to fix atmospheric nitrogen (*i.e.* they are diazotrophic)(Rawlings and Kusano, 1994).

4.4 Aerobic/Anaerobic growth

26. *Acidithiobacillus* species are strict aerobes with the exception of *A. ferrooxidans*, which is a facultative aerobe. In the absence of oxygen, *A. ferrooxidans* is able to grow on reduced inorganic sulphur compounds using ferric iron as an alternative electron acceptor (Pronk *et al*., 1992; Sugio *et al*., 1985).

4.5 Resistance to metals

27. Resistance to metal ions is a function of those thiobacilli tested to date. *Acidithiobacillus ferrooxidans* is resistant to a variety of metal ions such as chromium (Baillet *et al*., 1998), copper, zinc, nickel, thorium and uranium (Leduc *et al*., 1997; Tuovinen *et al*., 1971) and mercury (Takeuchi *et al*., 1999, 2001; Sugio *et al*., 2001). According to Iwahori *et al*. (2000), the resistance of *A. ferrooxidans* to mercury is ferrous iron dependent.

4.6 Role of Rusticyanin

28. Rusticyanin is a blue copper protein present in the periplasmic space of *A. ferrooxidans*. Consisting of a single polypeptide chain with one copper atom as a cofactor (Hazra *et al*., 1992), rusticyanin reportedly serves as the initial electron acceptor upon oxidation of ferrous iron (Hazra *et al*., 1992; Hutchins *et al*., 1986). An acid-stable cytochrome c was found to catalyse the reduction of rusticyanin (Blake *et al*., 1988).

4.7 Survival

29. No information on factors influencing survival of *Acidithiobacillus* in the natural environment appears to be available. According to Kelly and Harrison (1989), *A. ferrooxidans* survives in culture on pyrite (FeS_2) for very long periods when stored at 5-15 ^oC. Many strains have been successfully freeze-dried or have survived storage in liquid nitrogen or in glycerol suspension at -20 ^oC (Kelly and Harrison, 1989). Hubert *et al*. (1994) have shown that survival rates of *A. ferrooxidans* decreased rapidly under laboratory conditions above and below the individual temperature ranges of psychrotrophic and mesophilic strains.

4.8 Adhesion

30. The ability to adhere to surfaces seems to be a peculiar feature of *Acidithiobacillus*. Myerson and Kline (1983) observed the physical adsorption of cells of *A. ferrooxidans* to the surface of different non-porous solid particles (glass, pyrite, sulphur). Selective adherence to iron containing minerals appears to occur naturally (Ohmura *et al*., 1993), and the ferrous ion, but not the ferric ion, inhibited such selective adhesion. A model of the biofilm structure has been proposed by Karamanev (1991). Intimate contact and adhesion are required for enzymatic attack by *A. ferrooxidans* on insoluble substrates such as sulphur, pyrite (FeS_2), and chalcopyrite ($CuFeS_2$), and this is brought about by either a proteinaceous surface appendage (Devasia *et al*., 1993) or by extra-cellular polymeric substances (specifically lipopolysaccharides) combined with iron (III) (Gehrke *et al*. 1998, 2001; Blais *et al*., 1994). *Acidithiobacillus ferrooxidans* does not randomly adsorb onto pyrite or other surfaces, but congregates selectively at sites where dislocations, grain boundaries and other non-uniformities in the crystal structure emerge to the surface (Andrews, 1988; Bagdigian and Myerson, 1986; Gehrke *et al*., 1998). It is possible that diffusion of sulphur atoms along dislocations in the substrate is an important part of the mechanism of microbial decomposition. This pattern of diffusion provides a great advantage to bacteria because sulphur oxidation has a much higher yield of free energy than iron oxidation. No corresponding advantage would be gained by adsorption onto pure pyrite sites because the diffusion through pyrite crystals is several orders of magnitude lower.

31. Growth of bacteria adhering to the mineral surface initiates the oxidation process in arsenopyrite bioleaching (Fernandez *et al*., 1995). Corrosion patterns appear, with the liberation of ferrous ions and formation of elemental sulphur. With the increase in number of the bacteria, the ferrous ions are oxidised to ferric ions with the ultimate production of ferric arsenate (Fernandez *et al*., 1995).

32. Adhesion structures, consisting of a filamentous matrix, have been observed in *A. thiooxidans* (Blais *et al*., 1994), linking the cells to the surface of sulphur particles. They were not observed however in *T. thioparus*, where the cells fixed directly onto the sulphur (Sokolova and Karavaiko, 1968). The latter process is the usual type of fixation observed in the species placed formerly in *Thiobacillus.*

33. Adhesion can be estimated using the technique of Dziurla *et al*. (1998), who developed an immuno-filtration assay (ELIFA) for this purpose. ELIFA is a modified ELISA using micro-titer plates with 0.2-µm pore-size filters in place at the bottom. Particles, either previously inoculated with bacteria or to be reacted with added bacteria are incubated in the wells and then successively filtered and washed by applying a vacuum to the bottom of the plate. The inoculated particles are retained by the filter. Polyclonal antiserum raised against a strain of *A. ferrooxidans* is added and the plates incubated at 35 °C for one hour. The polyclonal rabbit antibody used was shown to react with different *A. ferrooxidans* and *A. thiooxidans* but not with other bacterial genera. Following washing the bound antibody is detected with goat-anti-rabbit globulin conjugated to alkaline phosphatase and ρ-nitrophenylphosphate was used as the detection substrate.

4.9 pH and nutrition preference

34. Species of *Acidithiobacillus* are acidophilic as well as obligate or facultative chemolithotrophs according to the nutritional table presented by Kuenen *et al*. (1992). Species of *Acidithiobacillus* are abundant in acid mine drainage water where they oxidise and gain energy from the oxidation of metals such as iron. The optimum pH for *A. ferrooxidans* is between 2-3, but when the substrate is in large part pyritic, the pH can reach extremely low values, (less than 1). This is due to the availability of abundant sulphur and the precipitation of ferric hydroxide when the solution reaches saturation (Morin, 1995). *Acidithiobacillus caldus* is the single mixotrophic species which can utilise sulphur or tetrathionate and yeast extract or glucose (Hallberg and Lindstrom, 1994).

35. Blais *et al*. (1993) have demonstrated that less acidophilic bacteria in sludge such as *Thermithiobacillus tepidarius, T. aquaesulis*, *T. denitrificans*, *T. thioparus* and other species formerly placed in *Thiobacillus*, may initiate the acidification to the point where the acidophilic species can take over. Acidophilic bacteria decreased the pH of a sulphur-containing synthetic salts medium to the level of 1.4-1.6 in 10 days. Evangelou (1995) mentioned pH 3.5 as the upper limit below which Fe^{2+} is oxidised by *A. ferrooxidans*. For the less acidophilic bacterial species, the limit of acidification was more variable, between pH 2.2 and 6.9. This has important implications for the removal of heavy metals from sludge (presented later in this document).

4.10 Temperature relations

36. *Acidithiobacillus* contains one thermophilic species, *A. caldus*, but little is known about its actions. *Acidithiobacillus ferrooxidans* has been traditionally regarded as mesophilic. Recently, however, psychrotrophic strains have been isolated with a growth range on iron of 2-37 °C (Leduc and Ferroni, 1994). Some Canadian isolates have a greater cold tolerance than most strains with a temperature optimum of only 20 ^{o}C (McCready, 1988). The occurrence of broad temperature range for psychrotrophic isolates of *A. ferrooxidans* has been reported by Leduc *et al*. (1993) and by Berthelot *et al*. (1993). Temperature ranges of 2-35 °C and 4-21 °C, respectively, were observed. However, psychrophiles have not been isolated from cold tailing effluents where they would be expected (Berthelot *et al*., 1994). The temperature used for bioleaching in most studies is 35 ^{o}C. Although *A. ferrooxidans* was reported to grow most rapidly at 30 °C, it oxidised iron faster at 35 °C (Holmes, 1988). This has important implications for industrial bioleaching since the oxidation of sulfides is exothermic, and therefore cooling may be necessary to maintain a satisfactory industrial process (Morin, 1995). Industrial applications are further described in section 8.

4.11 Metabolic pathways: involvement of Acidithiobacillus in bioleaching

37. Bioleaching is a biochemical oxidation process, catalysed by a living organism, whereby an insoluble mineral is oxidised to a soluble form and recovered in a pure form. A large number of acidophilic bacteria capable of attacking mineral sulphides have been isolated from industrial leaching operations or from sites of natural leaching. *Acidithiobacillus ferrooxidans* and *A. thiooxidans* are prominent among the species isolated from leaching sites, although the redox potential and concentration of ferric iron will influence which species dominates (Rawlings *et al*., 1999).

4.11.1 Acidithiobacillus ferrooxidans

38. This bacterium derives its energy from oxidation-reduction reactions using insoluble sulphidic minerals as growth substrates, including pyrite (FeS_2), chalcopyrite ($CuFeS_2$), chalcocite (Cu_2S) and sphalerite (ZnS). This, coupled with its resistance to high concentrations of normally toxic metal ions in solution, accounts for the ubiquity of this organism in leaching systems (Cripps, 1980). Combinations of *A.*

ferrooxidans and either *A. thiooxidans* or *Acidiphilium acidophilum* and *Leptospirillum ferrooxidans* have been associated with degradation of pyrite and chalcopyrite. Metals can be released from sulphidic ores by direct or indirect leaching or by galvanic conversion (Hutchins *et al*., 1986).

4.11.1.1 Direct leaching

39. This involves oxidation of the substrate by the bacterium and may require physical attachment of the bacteria to particles of the mineral sulphide (see section 4.8). The process can be described in general by a simplified reaction:

$MS + 2O_2 \Rightarrow MSO_4$ where M represents a bivalent metal [1]
(Hutchins *et al*., 1986)

40. Several different metal sulphides can be acted on directly by cells of *A. thiooxidans* (Leduc and Ferroni, 1994). These include sulphides of copper, nickel, lead, iron, gallium, cobalt and zinc. Examples of direct leaching include the following:

$CuS + 2O_2 \Rightarrow CuSO_4$ (Woods and Rawlings, 1989) [2]
Covellite

$2FeAsS + 7O_2 + 2H_2O \Rightarrow 2FeAsO_4 + 2H_2SO_4$ (Brierley, 1995) [3]
Arsenopyrite

4.11.1.2 Indirect leaching

41. This occurs through the production of an oxidative reagent or lixiviant that causes solubilisation to occur. The use of iron pyrites by *A. thiooxidans* as an energy source is a good example of how both direct and indirect leaching processes work together. The overall reaction describing pyrite oxidation is usually written as:

$4FeS_2 + 15O_2 + 2H_2O \Rightarrow 4Fe_2(SO_4)_3 + 2H_2SO_4$ (Rawlings and Woods, 1995) [4]

42. However, the actual pathway for the oxidation of pyrite is not a simple one-step reaction, but a series of reactions, passing through a number of intermediates. Under natural conditions, the two main oxidising agents that act on the pyrite are oxygen and the ferric ion. The reaction can be catalysed by *A. ferrooxidans*, which increases the rate of reaction by more than 10^6 (Singer and Stumm, 1970).

$2FeS_2 + 7O_2 + 2H_2O \Rightarrow 2FeSO_4 + 2H_2SO_4$ [5]
pyrite

$4FeSO_4 + O_2 + 2H_2SO_4 \Rightarrow 2Fe_2(SO4)_3 + 2H_2O$ [6]
(bacteria)

$Fe_2(SO_4)_3 + FeS_2 \Rightarrow 3FeSO_4 + 2\ S$ [7]

$2S + 3O_2 + 2H_2O \Rightarrow 2H_2SO_4$ [8]
(bacteria)

(Leduc and Ferroni, 1994; Monticello and Finnerty, 1985)

43. Reactions [5], [6] and [8] are part of the direct leaching mechanism, which may require the physical attachment of the bacteria to pyrite particles (section 4.8). Reaction [7] comprises the indirect mechanism that can take place independently of the bacteria. The role of the bacteria is to reoxidise the ferrous ions to ferric ions and sulphur to sulphuric acid (Monticello and Finnerty, 1985). A cyclical system develops in which ferrous ions released from pyrite are oxidised by the bacteria to ferric ion, which can then oxidise pyrite again, generating more ferrous ions. The biomass specific oxygen consumption rate is dependent on the ratio of ferric to ferrous irons in the culture (Boon *et al*., 1999). These ferric ions are known as the lixiviant because they carry electrons from the mineral to the bacterium's cell membrane (Leduc and Ferroni, 1994). The electrons are subsequently transported via an electron-transport chain to molecular oxygen in reaction [4]. Since iron is nearly always available in natural leaching environments, both the direct and indirect leaching mechanisms probably operate simultaneously in nature (Leduc and Ferroni, 1994; Monticello and Finnerty, 1985).

44. In the indirect reaction, bacterial activity is limited to the oxidation of pyrite (FeS_2) and ferrous iron. *Acidithiobacillus ferrooxidans* does not directly interact with the metal in the minerals. The role of the bacterium is to continuously provide a powerful oxidation agent, ferric sulphate [$Fe_2(SO_4)_3$] which is capable of dissolving a wide variety of metal sulphide minerals (Torma, 1991).

45. The following are examples of indirect leaching of minerals other than pyrites (Hutchins *et al*., 1986)

$CuFeS_2 + 2\ Fe_2(SO_4)_3^- \Rightarrow CuSO_4 + 5FeSO_4 + 2S^0$ [9]
Chalcopyrite

$Cu_2S + 2Fe_2(SO_4)_3^- \Rightarrow 2CuSO_4 + FeSO_4 + S^0$ [10]
Chalcocite

$CuS + 2\ Fe_2(SO_4)_3^- \Rightarrow CuSO_4 + 2FeSO_4 + S^0$ [11]
Covellite

$Cu_5FeS_4 + 6Fe_2(SO_4)_3^- \Rightarrow 5CuSO_4 + 13FeSO_4 + 4S^0$ [12]
Bornite

46. Ultimately, the indirect leaching mechanism depends on biological regeneration of ferric sulphate. Elemental sulphur (S^0) generated by the reactions above can be converted to sulphuric acid by *A. ferrooxidans*:

$2S^0 + 3O_2 + 2H_2O \Rightarrow 2H_2SO_4$ [13]

47. The sulphuric acid maintains the pH at levels favourable to the bacteria and effectively leaches a variety of copper oxide minerals, giving copper sulphate as an end product (Hutchins *et al*., 1986).

48. Uranium leaching proceeds in a similar fashion, again without the bacterium metabolising the uranium ion:

$$UO_2 + Fe_2(SO_4)_3 + H_2SO_4 \Rightarrow UO_2(SO_4)_3^{4-} + 2FeSO_4 + 4H^{4+} \quad [14]$$

or:

$$UO_3 + 3H_2SO_4 \Rightarrow UO_2(SO_4)_3^{4-} + H_2O + 4H^{+} \quad [15]$$

49. *Acidithiobacillus ferrooxidans* can be used to extract uranium from sulfidic ore bodies because the bacterium uses the iron and the sulfur in the ores, as energy sources (See reaction [5]). The oxidation of reduced sulfur results in the production of sulfuric acid, whereas the oxidation of reduced iron produces the oxidant Fe^{3+}. Under such acidic conditions, the insoluble tetravalent uranium is oxidised by ferric ions to the soluble hexavalent state as follows (Berthelot *et al*., 1993):

$$UO_2 + 2Fe^{3+} \Rightarrow UO_2\ 2+ + 2Fe^{2+} \quad [16]$$

50. Thus, the soluble, oxidised uranium is released from the mineral into the leaching liquid and can be easily recovered. For other minerals where the sulphate formed from the mineral is insoluble, as is the case with lead, gold and silver, the metal may be concentrated by leaching away other soluble metal sulphates, leaving a concentrated metallic product (Hutchins *et al*., 1986). Gold ores are often recalcitrant. The gold may be encased in a pyrite/arsenopyrite matrix that has to be decomposed before the gold is accessible to cyanide extraction (Rawlings and Woods, 1995).

4.11.1.3 Galvanic conversion

51. This is a lesser-known process in bioleaching where physical contact between two dissimilar metal sulphide phases immersed in an electrolyte such as dilute sulphuric acid or ferric sulphate solution creates a galvanic cell. In a mixture of pyrite and chalcopyrite, the former acts as a cathode while the chalcopyrite behaves as an anode and undergoes rapid dissolution. *Acidithiobacillus ferrooxidans* may accelerate the reaction by continuously oxidising the film of elemental sulphur that would obstruct the diffusion of copper and iron salts.

4.11.2 Acidithiobacillus thiooxidans

52. This species differs from *A. ferrooxidans* in its inability to oxidise iron (Fe^{2+}). Sulphur appears to provide the main source of energy for this bacterium (Sokolova and Karavaiko, 1968), and its ability to oxidise elemental sulphur allows it to take part in the indirect leaching of some minerals, particularly sulphides of cobalt, nickel and zinc (Hutchins *et al*., 1986; Norris, 1990). The sulphuric acid generated by *A. thiooxidans* through oxidation of sulphur (see reaction [8]), results in acid solubilisation of the metal. In mixed culture with iron-oxidising bacteria, *A. thiooxidans* oxidises the protective sulphur covering on the surface of minerals such as chalcopyrite. This allows bacteria such as *Leptospirillum ferrooxidans*, which cannot remove the sulphur, to attack the iron component beneath.

53. *Acidithiobacillus thiooxidans* can operate synergistically with *A. ferrooxidans*, or with *Leptospirillum ferrooxidans* which is able to use only ferrous iron. Either combination of bacteria can efficiently attack mineral sulphides and rapidly degrade a variety of ores (Paiment *et al*., 2001; Rawlings and Woods, 1995). *Acidithiobacillus thiooxidans* can augment the oxidation of pyrite in coal by *A. ferrooxidans* by oxidising elemental sulphur produced by the latter organism to sulphuric acid (see reaction [3]), which is then converted by *A. ferrooxidans* firstly to ferrous sulphate and then to ferric sulphate (Ford *et al*., 1977). Khalid and Naeveke (1991) have also observed that *A. thiooxidans* had the ability to solubilise heavy metals from carbonate-bearing complex sulphidic ore more efficiently than *A. ferrooxidans*. The reason may be that *A. ferrooxidans* alone could not produce sufficient acid to neutralise the carbonate contents and lower the pH, so that suitable conditions for growth of the bacterium could not

be attained. A combination of both bacteria works efficiently since the sulphuric acid produced from sulphur by *A. thiooxidans* fulfils this condition.

4.12 Inhibition of bacterial oxidation and growth

54. A number of simple organic compounds have been found to inhibit bacterial oxidation of ferrous ion at low concentrations under laboratory conditions, including benzoate, sorbate and sodium dodecyl sulphate (SDS) (Onysko *et al*., 1984) and sodium tungsten (Sugio *et al*., 2001). The inhibitory effect of glucose, cellobiose, galacturonic acid and citric acid compared favourably with that of SDS (Frattini *et al*., 2000). Ferric and arsenite ions have a most detrimental effect on the growth of *A. ferrooxidans* and *A. thiooxidans* (Collinet and Morin, 1990). These compounds have been suggested for use in control of these species.

5. Characterisation of the genomes (*e.g.* presence of large plasmids, insertion sequences) and stability of these characteristics

5.1 Chromosome

55. Recently, Rawlings (2001) provided sequencing data of the chromosome of *A. ferrooxidans* and of other iron- or sulfur-oxidising bacteria. The *A. ferrooxidans* chromosome consists of 2.9 Mb and contains 61ats larger than 500bp (Tettelin *et al*., 2002). Harrison (1986) determined that *A. ferrooxidans* showed a wide range of genetic diversity encompassing seven DNA homology groups. This, with the discovery of high frequency mutations (Holmes *et al*., 1988; Schrader and Holmes, 1988; Yates and Holmes, 1987), may explain the frequently observed ability of *A. ferrooxidans* to adapt to specific laboratory culture conditions such as pH and resistance to metals. High frequency mutants possess special insertion sequences that replicate and migrate along the bacterial chromosome, so that the mutants can frequently revert back to the original phenotype (Holmes and Yates, 1990). The mobility of the sequences is believed to cause a dramatic increase in the frequency of spontaneous phenotypic variations (Holmes *et al*., 1988). Recently, this phenomenon was correlated to the high frequency of the insertion and excision of ISAfe1 (1.3 kb), an ISL3 family insertion sequence in a gene *ResB* that encodes for a cytochrome c-type maturation protein (Cabrejos *et al*., 1999). In addition, Holmes *et al*., 2001 demonstrated recently that ISAfe1 or similar ISAfe-1 sequences exist in diverse strains of *A. ferrooxidans* and *A. thiooxidans.*

5.2 Genes

56. Several genes and their functions have been elucidated in the thiobacilli (Rawlings and Kusano, 1994; Rawlings and Woods, 1995; Tuovinen and Fry, 1993; Heinhorst *et al*., 2002). A list of some of the genes in *A. ferrooxidans* have been identified and cloned as shown in Table 2.

Table 2. Genes in Acidithiobacillus ferrooxidans

Gene	Gene Product	Function
glnA	Glutamine synthetase	Ammonia assimilation
nifH	Nitrogenase	Reduces N_2 to NH_3
ntrA	ntrA sigma factor	Promotes transcription
rbcL, rbcS,	RuBPCase	Fixes CO_2
recA	alanyl-tRNA synthetase	Recombination/ DNA repair
merA	Mercury reductase	Resistance to Hg
merC	unnamed	Mercury transport
merR	unnamed	merA regulator
iro	Fe(II) oxidase	Iron oxidation
Rusticyanin gene	Rusticyanin	Respiratory Electron Transport Chain[a]
ATP synthase genes	ATP-synthase	ATP synthesis
cysC, cysD	unnamed	Sulphur assimilation

[a] Exact function of the gene is unknown

5.3 Plasmids

57. Plasmids are found in a large number of *A. ferrooxidans* strains. With the exception of the arsenic and antibiotic resistance plasmids described in next section, most of the plasmids evaluated to date, are cryptic in that no phenotypic characteristic has been linked to their presence (Valenti *et al*., 1990). Rawlings *et al*., 1984 reported on the isolation of a 12,190 bp IncQ plasmid (pTF-FC2) from A. *ferooxidans* present in 12-15 copies per chromosome. Recently, another IncQ plasmid pTC-F14 was isolated from *Acidithiobacillus caldus* (previously *Thiobacillus caldus*) consisting of 14 000 bp, 12-16 copies per chromosome (Gardner *et al*., 2001). A large review of these plasmids characteristics can be found in (Rawlings and Tietze, 2001).

58. Dominy *et al*. (1998) isolated and characterised a 19.8 kb plasmid from *A. ferrooxidans* ATCC33020. Fourteen complete open reading frames (ORFs) were identified, most of which were proteins involved in maintenance although three of the ORFs appeared to correspond to redox-active proteins and thus could constitute part or all of an electron transport chain.

59. None of the plasmids describe to date, have been correlated with metal resistance (Hutchins *et al*., 1986; Chisholm *et al*., 1998; Leduc and Ferroni, 1993), although circumstantial evidence suggests that genes for mercury and silver resistance may be located on an unnamed 19 x 10^6 daltons plasmid isolated from *A. ferrooxidans* (Visca *et al*., 1986). One isolate of *A. ferrooxidans* was found to possess mercuric reductase activity similar to that in heterotrophic bacteria containing the mer operon, but this could not be associated with a plasmid.

60. Valenti *et al*. (1990) discovered a 20-kb plasmid (pTFO) in eight out of twelve strains of *A. ferrooxidans* from Italy and Mexico, which was stably maintained for many bacterial generations. All of the strains resisted similar concentrations of metal ions in spite of overall differences in their plasmid pattern.

5.4 Genetic variation among leaching bacteria

61. Suzuki *et al*. (1989) demonstrated that naturally occurring strains of *A. ferrooxidans* vary widely in their ability to utilise iron and sulphur compounds, resistance to metal and mineral leaching activities. The abilities to adsorb onto solid surfaces and to oxidise sulphur with ferric ion are other variable

properties. Natural variation and /or selection of mutants can be inferred from the ease with which new isolates are selected to enhance process efficiency in various industrial settings (see section 8).

5.5 Mutation

62. The occurrence of high-frequency mutation strains of *A. ferrooxidans* was previously mentioned (section 5.1). It was noted that high frequency mutants possess special insertion sequences that replicate and migrate along the bacterial chromosome, so that the mutants can frequently revert back to the original phenotype (Holmes and Yates, 1990). The mobility of the sequences is believed to cause a dramatic increase in the frequency of spontaneous phenotypic variations (Holmes *et al*., 1988; Holmes *et al*., 2001). In addition, Holmes and Debus (1991) ascribed the high mutation rate of *A. ferrooxidans* to repetitive DNA sequences in the chromosomes and plasmids whose mobility around the genome could cause mutations to arise.

63. The high frequency of spontaneous mutations overshadows mutations derived by chemical mutagenesis and makes the latter difficult to detect.

64. An important implication for environmental regulation is that the inherent instability of these 'naturally' engineered strains makes it difficult to predict ecological behaviour thus complicating assessment of risk.

6. Genetic transfer capability

65. Classical genetic engineering techniques for the development of new microbial strains include mutation, conjugation, transformation, transduction and electroporation. Very little work has been done in these areas with bioleaching micro-organisms (Holmes and Debus, 1991). However, Young (1993) has presented a good review of attempts at genetic engineering of these micro-organisms, particularly *A. ferrooxidans*.

6.1 Transduction

66. The high mutation rate of *A. ferrooxidans* was ascribed by Holmes and Debus (1991) to repetitive DNA sequences in the chromosomes and plasmids whose mobility around the genome could cause mutations to arise (see also sections 5.1 and 5.5). One of these DNA sequences has been shown to be an insertion sequence. However, bacteriophages for transduction have not been reported for *Acidithiobacillus*, although these authors cited a report of bacteriophage-like particles in acidophilic heterotrophs, which may prove valuable for developing transduction systems.

6.2 Conjugation

67. Transfer of plasmid DNA from heterotrophic bacteria to chemolithotrophic colourless sulphur bacteria by conjugation was first achieved experimentally by Kulpa *et al*. (1983). *Halothiobacillus neapolitanus* was used in the study because of its ability to grow at the near neutral pH required by the heterotrophic donor, *Pseudomonas aeruginosa*. It was discovered that plasmid RP1, which governed resistance to three common antibiotics was accepted, replicated and expressed in the chemolithotrophic bacterium. Transfer from *Escherichia coli* into *A. thiooxidans* and back of four broad-range IncP plasmids with antibiotic resistance markers has also been achieved (Jin *et al*., 1992), and two of the three antibiotic resistance markers have been expressed in *A. thiooxidans*.

68. However, although plasmids have been demonstrated in many strains of *A. ferrooxidans*, most of them are cryptic in that no identifiable phenotype has been linked to their presence (Leduc and Ferroni, 1994), and there are few cases where plasmid transfer has been successful. In spite of being cryptic in *E. coli*, however, the plasmid pTF-FC2 has been found to have a broad host-range of replication, and the discovery of such plasmids does suggest that a conjugation system exists in *A. ferrooxidans* (Rawlings and Woods, 1995; Rawlings and Tietze, 2001). This plasmid has been deemed particularly suitable for use as a cloning vector for genetic manipulation of *A. ferrooxidans* (Rawlings *et al*., 1986). Four plasmids (pTF35, pTF-FC2, pTF3320-1, pTF3302-2) from three different strains of *A. ferrooxidans* have been successfully cloned into the plasmid pBR325 and two into the related plasmid pBR322 (Rawlings and Woods, 1995). However, the desirable properties of increased uranium and arsenic resistance, present in the *A. ferrooxidans* parents, were not expressed by the transformed *E. coli* mutants.

69. Two arsenic-resistant plasmids, pSDRA1 and pSDRA21 have been constructed and introduced into *A. ferrooxidans* by conjugation with *E. coli*, using Solid 2:2 Medium as a mating medium (Peng *et al*., 1994b, 1994c). Arsenic resistance was demonstrated in the progeny. Unfortunately most attempts at returning functioning genes from *E. coli* to *A. ferrooxidans* have been unsuccessful. This means that, in principle, the statement by Holmes and Yates (1990) that genes can be extracted from *A. ferrooxidans* and be genetically modified, yet cannot be returned to *A. ferrooxidans* to create an improved organism is still largely true. However, this position may change as techniques improve for introducing foreign genetic information into *A. ferrooxidans* and other thiobacilli.

6.3 Transformation

70. Transformation does not appear to take place naturally in *Acidithiobacillus* and other genera of colourless sulphur bacteria, but transformation has been accomplished experimentally through electroporation. Transport of naked DNA into bacterial cells by application of a high voltage electrical discharge (electroporation) has been successful in two cases. *Thiomonas intermedia* was transformed by this method using the plasmid pRK415Km (Jin *et al*., 1994), conferring kanamycin resistance. The transformation efficiency ranged from 10^3 to 10^4 transformants μg^{-1} plasmid DNA under optimal conditions. Kusano *et al*. (1992) transformed *A. ferrooxidans* with natural plasmids and an artificially constructed one, but the efficiency was much less. The reason why only one strain out of the thirty tested was amenable to transformation by electroporation is still uncertain. The plasmids in the transformed cells were stable for at least 110 generations.

7. Behaviour in simulated natural environments such as microcosms

71. Microcosms have been used to investigate the role of *A. thiooxidans* and other thiobacilli in the degradation of concrete (Sand, 1987; Sand and Bock, 1988). Samples of concrete were inoculated in a simulation apparatus with bacteria originally isolated from concrete and incubated for nine months at an relative humidity of 95% and a temperature of 30 ^oC. Three compounds were tested as sources of energy: hydrogen sulphide, thiosulphate, and methyl mercaptan. Hydrogen sulphide at a concentration of 15 mg/m^3 resulted in severe corrosion after nine months, and *A. thiooxidans* was the dominant species in the microflora. At the lesser concentration of 2 mg/m^3 moderate corrosion resulted and the dominant species were *Thiomonas intermedia*, *Starkeya novella* and *Halothiobacillus neapolitanus*. Similar results were obtained with thiosulphate but methyl mercaptan at concentrations of 22 and 2 mg/m^3 caused negligible corrosion and only heterotrophic bacteria and fungi thrived on the concrete blocks.

8. History of use (including selection of mutants and examples of environmental applications of the organism, and information derived from these):

8.1 Selection of industrially useful mutants

72. The rate of growth and mineral oxidation by a population of leaching bacteria can be improved simply by cultivating a population of bacteria in a continuous flow apparatus. If the flow rate through the apparatus is slowly increased, those bacteria that are capable of the most rapid growth will replace the others. Spontaneous mutants will be selected by their growth potential on the available substrate. The advantage of this method is that it does not require sophisticated procedures, but it may take a long time to improve the bacterial strain to any economically significant extent. This approach has nevertheless been used to improve the leaching rates of *A. ferrooxidans* to several fold over that of the original isolates. Vian *et al*. (1986) were able to progressively select mutants of *A. ferrooxidans* with high oxidative efficiency at low pH values (down to pH 1.5) and with high resistance to ferric ions, thus improving the leaching of metals from low-grade ore deposits and avoiding precipitation of oxidised iron compounds.

73. The improvement of biomining bacteria by mutation and selection has had a dramatic effect on the economics of biooxidation of gold-bearing arsenopyrite ores in particular (Rawlings and Woods, 1995). Natural selection in the laboratory and in pilot and full-scale plants over several years has produced *A. ferrooxidans* strains that are highly resistant to arsenic and are capable of rapid oxidation of gold-bearing arsenopyrite ores in a continuous industrial bioleaching process. Highly adapted strains decompose arsenopyrite ores to an extent that allows more than 95% gold recovery in 3 days compared to the more than 12 days required by the original isolates. Further improvement however, is likely to require the application of DNA recombinant technology to amplify genes or to enable the introduction of new genetic material. Similarly, resistance to nickel ions was enhanced in *A. ferrooxidans* by repeated culturing in a medium containing nickel and gradually increasing the nickel concentration (Kai *et al*., 1995) and the use of the modified strain, in turn, significantly increased the rate of industrial nickel extraction. No literature data was found on the selection of mutants of the three other species of *Acidithiobacillus*.

8.2 Main Industrial Uses

74. Historically there are nine main uses to which species of *Acidithiobacillus* have been applied. This is not meant to imply that their use should be restricted to these categories, but rather to illustrate the potential and diversity of organisms within this genus. The main uses of *Acidithiobacillus* are directly related to their bioleaching ability.

8.2.1 Removal of sulphides from industrial wastes

75. The toxicity, corrosive properties, and unpleasant odour dictate stringent control of release of sulphides into the environment. Reduced sulphur compounds can occur in industrial wastes of the oil and gas industries as a result of several processes. Whether directly as an end product of sulphate reduction (Buisman *et al*., 1989), or indirectly as a result of methanogenesis, the effluent from which the methane is generated may contain significant quantities of sulphate. The release of large amounts of sulphide into natural waters can result in oxygen depletion due to direct or biological oxidation (Kuenen, 1975) as well as corrosion of the concrete walls of reactors, sewer systems, and steel pipelines.

8.2.1.1 Acidithiobacillus ferrooxidans

76. *Acidithiobacillus ferrooxidans* has often been preferred for sour gas removal because the costs of neutralising sulphuric acid produced by the other bacteria can be avoided, since the sulphur is converted to ferric sulphate, and the bacterium is not inhibited by H_2S (Jensen and Webb, 1995; Shiratori and Sonta, 1993). The Bio-SR Process (Satoh *et al*., 1988) comprises the following steps. Sour gas is introduced into

an absorber containing ferric sulphate solution where it is oxidised to elemental sulphur and the ferric sulphate is reduced to ferrous sulphate:

$$H_2S + Fe_2(SO_4)_3 \Rightarrow S^0 + 2FeSO_4 + H_2SO_4 \quad [17]$$

77. Elemental sulphur is removed from the solution by a separator and the bacteria in the bioreactor then oxidise the ferrous sulphate in the solution back to ferric sulphate:

$$4FeSO_4 + 2H_2SO_4 + O_2 \Rightarrow 2Fe_2(SO_4)_3 + 2H_2O \quad [18]$$

78. The oxidised solution is then recycled to the absorber to repeat the cycle. This procedure is environmentally sensible in that it is easy to operate, there are no waste products, no special chemicals are needed and the operating cost is low.

79. In earlier gas treatment processes (Onken *et al*., 1984; Sumitomo Jukai Envirotech, 1983) the hydrogen sulphide is first precipitated as CuS or FeS. These sulphides are then oxidised by the *A. ferrooxidans* to regenerate the precipitating agent. Cadenhead and Sublette (1990) have commented that the requirement of a low pH for these processes may induce corrosion, and these authors prefer *Thiobacillus denitrificans* with its higher pH tolerance as the microbiological conversion agent.

8.2.1.2 Acidithiobacillus thiooxidans

80. Berzaczy *et al*. (1990) have patented a microbiological conversion process for degradation of sulphur-containing pollutants such as H_2S, CS_2, COS, thioalcohols, thioethers and thiophenes in waste gas, especially from cellulose fibre manufacture. The gas makes contact with cells of the bacterium immobilised on packing material in a packed-bed reactor. The metabolic products (mainly H_2SO_4) draining from the reactor are neutralised by addition of lime and lime-water.

81. It has been suggested that *A. thiooxidans* could be used to convert hydrogen sulphide to sulphur or sulphate in industrial plants. This process has already been demonstrated experimentally, using a continuous column contacter (Lizama and Sankey, 1993). *Acidithiobacillus thiooxidans* was also shown experimentally to act as a bacterial deodorant in removing hydrogen sulphide and trimethylamine simultaneously from a mixture of these two compounds (Hirano *et al*., 1996). It has also been used as a deodoriser in a carrier-packed biological deodorisation reactor used in a sewage treatment plant (Shinabe *et al*., 1995). More than 99% of the hydrogen sulphide and 70-80% of the methanethiol were removed from the raw gas in the early section of the packed bed. Lizama and Sankey (1993) pointed out that *A. thiooxidans* might have some advantages over *T. denitrificans*, since the energy requirement of *A. thiooxidans* for fixing carbon dioxide from the atmosphere is high. It must oxidise large quantities of sulphide for the production of relatively little biomass, and its tolerance to low pH makes it resistant to the sulphuric acid produced.

8.2.2 Removal of heavy metals from sludge and mine wastes

8.2.2.1 Extraction of heavy metals from sewage sludge

82. Application of sewage sludge to agricultural land is one of the most economical methods for final sludge disposal (Bruce and Davis, 1989), since it is a very good soil conditioner and sources of plant nutrients. However, the levels of toxic metals in sewage sludge make them unsuitable for agricultural land

application because food plants for humans and animals take up these metals, causing them to accumulate in the food chain (Tyagi *et al*., 1993b).

83. Couillard and Mercier (1994) determined that bacterial leaching of metals from sludge using *A. ferrooxidans* was more economical than traditional methods of sludge management except in the case of a processing plant treating only 20,000 m^3 of wastewater per day. Furthermore, the use of biological leaching had less of an environmental impact and the product was acceptable for use on agricultural lands (Wong and Henry, 1984). Bacterial leaching was also more economical because acid consumption is reduced by more than 80% (Couillard and Mercier, 1991).

84. The presence of sulphur-oxidising microflora in sewage sludge is potentially useful for the removal of toxic metals found there (Blais *et al*., 1993). An acid medium is useful in inactivating many bacteria and viruses, though not all organisms are affected. The process is enhanced through the addition of elemental sulphur as an energy substrate, preferably in solid rather than powdered form since the sulphur is then easier to recover at the end of the operation (Ravishankar *et al*., 1994). Rapid decrease of sludge pH by a mixed culture through sulphur oxidation into sulphuric acid solubilised toxic metals to levels recommended for intensive use of residual sludge in agriculture (Tyagi *et al*., 1993b). The solubilised metals in the leachate could be separated from decontaminated sludge solids by centrifugation or filtration, precipitated by neutralising the leachate with lime and then safely disposed of. There exists the potential to recycle these metals for the metal industry. Decontaminated sludge solids must also be treated with lime to reduce acidity before application to agricultural land (Tyagi *et al*., 1993b).

85. Evidence seems to indicate that a combination of thiobacilli is more effective in the treatment of sewage sludge than the use of a single species. *Acidithiobacillus ferrooxidans* on its own, was not as capable of efficient solubilisation of metals as when compared to a mixture of indigenous sulphur-oxidising bacilli or with other *Acidithiobacillus* species (Couillard and Zhu, 1992; Tyagi and Couillard, 1987; Tyagi *et al*., 1993b). In Table 3, the efficiency of thiobacilli species to solubilise heavy metals is illustrated.

Table 3. Comparative % solubilisation of heavy metals in sludge

Heavy Metals	Indigenous bacterial microflora Wong and Henry (1984)	*A. ferro-oxidans* Tyagi *et al*. (1993a)	*A. ferro-oxidans* + *A. thiooxidans* Tyagi and Couillard (1987)	Mixture of thiobacilli* Tyagi *et al*. (1993b)	*T. thioparus* + *A. thio-oxidans* Blais *et al*. (1992)	*T. thioparus* + *A. thio-oxidans* Blais *et al*. (1993)
Cd	80-85%	55-98%	50%	51-93%	83-96%	83-90%
Cr	-------	0-32	-------	16-58	16-54	19-41
Cu	66-80	39-94	75	47-95	85-87	69-92
Mn	70-78	71-98	-------	------	91-94	88-99
Ni	37-98	37-98	-------	48-97	78-79	77-88
Pb	0	0-31	55	7-63	28-46	10-54
Zn	84-90	66-98	96	65-98	82-96	88-97

*Named species were *A. ferrooxidans* and *A. thiooxidans*. Other sulphur oxidising organisms were also present (*e.g. Sulfolobus acidocaldarius*)

86. Blais *et al*. (1992, 1993) showed that the bioleaching of metals from sewage sludge could be carried out by successive growth of moderately acidophilic bacteria (*H. neapolitanus, T. denitrificans, T. thioparus*) and the acidophilic *A. thiooxidans*. *Thiobacillus thioparus* VA-7 and *A. thiooxidans* VA-4 possess distinctive physiological characteristics that allow them to easily grow and solubilise heavy metals

in municipal sludge (Blais *et al*., 1992). Strain VA-7 decreased the pH of the sludge initially from pH 7-8.5 to a value between pH 4.0 and 4.5. Strain VA-4 began to grow and further reduced the pH to values below 2.0.

8.2.2.2 Extraction of heavy metals from industrial wastes

87. Bosecker (1987) found that some products such as copper, chromium, zinc and vanadium were completely extracted from a variety of industrial waste products by the sulphuric acid produced by *A. thiooxidans*. In some cases bacterial leaching was as effective as chemical leaching with sulphuric acid. Heavy metals such as Cu, Pb, Zn, Fe, As and Cd could also be recovered from flue dust from a flash-smelting furnace (Shiratori and Sonta, 1993) using *A. ferrooxidans*. There were several advantages to bacterial oxidation, including low cost and clear separation of metals.

88. Aluminium could be recovered from red mud, a chemical waste produced by alkaline extraction of aluminium from bauxite (Bayer process). The mud usually contains about 25% Al_2O_3 (Vachon *et al*., 1994) and is still highly alkaline (pH 12-13). Traditionally, red mud has either been disposed of in the sea or allowed to settle in 'red lakes' for further processing; neither process is environmentally innocuous. Bioleaching, after the addition of sewage sludge to a concentration of 30% V/V to red mud, solubilised up to 47% of the aluminium and brought down the pH to 3.5-2.2. The bacteria responsible were not identified but were probably *T. thioparus* and *A. thiooxidans*.

89. Silver recovery from waste photographic processing solutions has been accomplished using *A. ferrooxidans*, *A. thiooxidans, Starkeya novella, T. denitrificans* and *T. thioparus* (Kitajima and Abe, 1979).

8.2.3 Biomining and acid production

90. Bacterial leaching is used in the recovery of metals from ores that are often too poor for conventional metallurgical extraction methods (Robertson and Kuenen, 1992; Paiment *et al*., 2001). The main metals that have been recovered on a commercial scale by microbial leaching are copper and uranium. In the U.S.A., 10-15% of all copper is obtained in this way (Cripps, 1980). The potential of this technique, however, is not limited to copper and uranium, since it can, in principle, be extended to all sulphide and some oxide ores. Other metals that have been extracted using processes that involve bacteria include zinc, cobalt, lead, gold and molybdenum (Robertson and Kuenen, 1992).

91. Biological copper leaching is practised in many countries, including the U.S.A., Russia, Chile, Peru, Australia, Spain, Canada and Mexico. Typically, copper ore mined from open pits is segregated, higher-grade material being concentrated for smelting and the lower-grade ore subjected to leaching. The latter is piled to form a 'dump' up to 40 m high and several hectares wide. After the top is levelled, a leaching solution containing ferric sulphate and *A. thiooxidans* is flooded or sprayed onto the dump (Merson, 1992). Bacterial colonisation occurs mainly in the top metre. Leachates enriched with copper exit at the base of the dump and are conveyed to a central recovery facility. The copper in the solution is recovered by mixing it with iron scraps in large container units according to the following reaction:

$$CuSO_4 + Fe^0 \Rightarrow FeSO_4 + Cu^0 \qquad [19]$$

92. The finely divided "cement copper" is periodically recovered and refined for sale, while the barren solution is recycled to the leachate dumps. A typical large dump may have an operating life of over ten years (Hutchins *et al*., 1986). Total copper recoveries of 80% were attained by the Chilean company *Sociedad Minera Pudahuel* (SMP) after leaching times ranging from 150 to 230 days, and about 90% recovery was attained after 7 to 11 months, more than double the quantities that would have been obtained without bacteria (Acevedo and Gentina, 1993).

93. *Acidithiobacillus thiooxidans* has also been used to obtain a high degree of copper extraction from covellite (Curutchet *et al*., 1995) by oxidising the layer of sulphur covering the sulphide surface and allowing sulphide oxidation by ferric ion. Both *A. thiooxidans* and *A. ferrooxidans* are effective in leaching covellite, although at different rates (Donati *et al*., 1996).

94. *Acidithiobacillus ferrooxidans* was used to extract uranium from low-grade ores, as for example in the Denison Mine project in Elliott Lake, Ontario, Canada (Brierley, 1990; McCready, 1988). The operation is similar to copper leaching in that it employs a bacterially assisted, flood-leaching process, but it is performed underground on mine waste rubble. About 12-13% of the uranium production is attributed to bioleaching at present, which could increase to 25% after refinement. After the uranium-bearing ore is leached, a uranium-bearing solution drains to lower portions of the mine and accumulates in sumps, after which it can be pumped to the surface for uranium recovery (Hutchins *et al*., 1986). The addition of elemental sulphur or sulphur slag as an external energy source enhanced the leaching process (Bhatti *et al*., 1991).

95. *Acidithiobacillus ferrooxidans* and *A. thiooxidans* were used together to extract cobalt from an ore containing 40.3% iron and 1.4% cobalt (Battaglia *et al*., 1994). It was found that for the system to operate at the highest efficiency, the acidity had to be maintained at a pH of between 1.1 and 2. Furthermore, the dissolution of pyrite was depressed when the concentration of ferric ions reached a level of 35 g/L.

96. According to Brierley (1995), an estimated one-third of the world's total gold production is now from refractory deposits such as gold-arsenic concentrates. In these concentrates, gold and silver are finely disseminated in sulphide minerals, mainly arsenopyrite as well as pyrite, and partly antimonite (Karavaiko *et al*., 1986). Bio-oxidation, in which chemolithotrophic bacteria such as *A. ferrooxidans* have been used to decompose the ore, is a low-energy alternative to conventional methods which involve roasting and pressure leaching.

97. Lindstrom *et al*. (1992) and Morin (1995) have reviewed the process of microbial leaching for recovery of gold. *Acidithiobacillus ferrooxidans* was found to rapidly and selectively oxidise arsenopyrite and other sulphide minerals in the concentrates. Bio-oxidation increased gold recovery from low levels up to 95-99% (Maturana *et al*., 1993; Morin, 1995) in comparison with traditional acid leaching. Compared to roasting, bio-oxidation with thiobacilli and their relatives could generally reduce capital costs by 12-20%, operating costs by 10% in some cases and construction time by 25% (Brierley, 1995). At the same time, the process was less polluting to the environment and had lower energy requirements since it operated at relatively low temperatures (Cripps, 1980). Highly adapted strains decompose arsenopyrite ores to an extent that allows more than 95% gold recovery in 3 days compared to the more than 12 days required by the original isolates.

98. *Acidithiobacillus ferrooxidans* accelerated the leaching of silver and other metals present in a mixed sulphide ore from Idaho (Ehrlich, 1986; 1988). Continuous leaching where iron in the solution was supplied to the reactor from a reservoir resulted in selective leaching of the silver.

99. Zinc has been effectively recovered from a zinc sulphide concentrate by continuous microbiological leaching with *A. ferrooxidans* using a two-stage reactor sequence (Sanmugasundaram et. al., 1986). Direct leaching by *A. ferrooxidans* together with *A. thiooxidans* has been found to be effective (Pistorio *et al*., 1994).

100. Sub-marginal mercury/antimony sulphidic ores were separated under experimental conditions into their components using a culture of *A. ferrooxidans* isolated from a coal field in the Moscow region (Lyalikova and Lyubavina, 1986).

8.2.4 Oil recovery and purification of oil shale

101. The position with oil reserves is similar to that of metallic ores outlined above: some 30,000 billion barrels of oil are present in shales, of which only about 2% are available because the recovery of the rest is uneconomical. Conventional extraction methods involve crushing and heating the shale to high temperatures to release the oil from the inorganic matrix. In this way, vast quantities of energy are consumed, only 75% of the organic material is liberated and large quantities of expended shale must be disposed of (Dalton, 1979). A biological process has been invented (Yen *et al*., 1976) to extract oil at ambient temperatures, giving good yields and avoiding the production of vast quantities of insoluble residue. Organisms mentioned in the patent included *A. ferrooxidans, A. thiooxidans, H. neapolitanus, T. thioparus,* other species of *Thiobacillus* since relocated, and various species of *Desulfovibrio.* These bacteria remove most of the organically bonded disulphides and polysulphides in the inorganic matrix of the shale oil, leaving an organic structure that can be used as fuel or can be converted into other materials such as petroleum or synthetic natural gas.

102. Bioleaching of pyrite from the Aleksinac oil shale in Yugoslavia was successfully carried out by using *A. ferrooxidans* as an alternative to chemical removal; chemical removal formerly led to undesirable changes in the oil substrate (Vrvic *et al*., 1988).

8.2.5 Desulphurisation of coal

103. To some extent coal desulphurisation is similar to the process above, in that in both cases sulphidic ores are oxidised; however both the aim and the end products are different. Coal, being of fossil origin, is not a homogeneous substance, containing a variable quantity of fixed carbon, hydrogen, oxygen, sulphur, nitrogen and trace minerals (Mannivannan *et al*., 1994). The aim of coal desulphurisation is to produce coal which is as free of sulphur and its derivatives as possible and it is necessary therefore to convert reduced sulphur compounds to soluble forms (Robertson and Kuenen, 1992).

104. Microbial desulphurisation by *A. ferrooxidans, A. thiooxidans* and *Sulfolobus brierleyi* can remove 90% or more of the inorganic sulphur from coal within a few days (Khalid and Aleem, 1991; Kilbane, 1989). The pyrite-oxidising capacity of *A. ferrooxidans* and related organisms has also been successfully exploited in the desulphurisation of coal (Bos *et al*., 1988; Bos and Kuenen, 1990; Tuovinen and Fry, 1993) with the production of sulphuric acid instead of sulphur dioxide.

105. Sulphur is bound in inorganic and organic form in coal. Sulphur dioxide emissions arising by coal burning represent an important ecological problem, which can be solved by the conversion of the sulphur, compounds in the coal into different end products (Beck *et al*., 1988). These workers found that *A. ferrooxidans* was the most useful species to use, but other species such as *A. acidophilum, (T. acidiphilus*), *Thiomonas perometabolis* (*T. perometabolis)* and *T. plumbophilus* could also be used. Beyer *et al*. (1988) and Bos *et al*. (1988) found that it was possible to remove 90% of the pyrite from coal within 8 to 10 days, using a mesophilic pyrite-oxidising microbial system for which a plant design involving a cascade of Pachuca tank reactors was devised.

106. Mixed cultures of *A. ferrooxidans* and *A. thiooxidans* have also been used to remove sulphur from lignite, the lowest rank of coal intermediate between peat and anthracite (Raman *et al*., 1994).

8.2.6 Desulphurisation of rubber

107. Mixed cultures of *A. ferrooxidans* and *A. thiooxidans* satisfactorily removed sulphur inclusions in rubber materials that could be recycled from urban wastes (Torma and Raghaven, 1990). The two bacteria together were more efficient than the individual bacteria alone.

8.2.7 Detection of sulphur impurities in wine

108. A rapid and accurate sensor system was developed to determine free sulphite in wine (Nakamura *et al*., 1989), using immobilised cells of *A. thiooxidans* S3. The concentration of free sulphite could then be controlled so as to protect wine from oxidation processes and microbial spoilage. The same strain was used to detect sulphur dioxide in wine and various foodstuffs. The bacterium is converted into a microbial sensor by setting a piece of microbial membrane onto an O_2 electrode soaked in 0.1 M citrate buffer and covered with a gas-permeable Teflon membrane (Kawamura *et al*., 1992; Kurosawa *et al*., 1990, 1994). A similar biosensor using strain JCM7814 was developed to detect concentrations of sulphur dioxide in wine up to 50 mg/l, with a limit of detection of 5 mg/l and a response time of 20 minutes (Nakamura *et al*., 1993).

8.2.8 Agricultural fertilisation

109. Since thiobacilli are involved in the sulphur cycle, the presence in the soil can be used to assess fertility. In Australia, for example, thiobacilli are scarce in sulphur-deficient areas (Kuenen *et al*., 1992).

110. Under warm climatic conditions rock phosphate pelleted with sulphur and seeded with thiobacilli has been shown to be a useful slow release source of phosphate and sulphate for soil fertilisation (Swaby, 1975). However, the addition of *A. thiooxidans* to a mixture of rock phosphate and sulphur granules, called 'Biosuper', has given variable results; in some cases the mixture increased the level of phosphorus in the soil and gave plant yields equivalent to those produced by the more expensive super-phosphate (Muchovej *et al*., 1989; Schofield *et al*., 1981), whereas in others, addition of *A. thiooxidans* did not improve performance of the fertiliser (Alvarez *et al*., 1981; Rajan, 1982). In pot experiments with guavas and ryegrass, the presence of *A. thiooxidans* in the soil enhanced the uptake of Fe, Zn and Mn (Azzazy *et al*., 1994; Schnug and Eckardt, 1981).

111. *Acidithiobacillus thiooxidans* is perhaps more useful in low-cost production of a supply of sulphuric acid for the dissolution of apatite in the production of phosphate fertilisers (Donati and Curutchet, 1995).

8.2.9 Soil Reclamation

112. Incorporating certain species of *Thiobacillus* with sulphur enrichment can reclaim alkali soils, since these soils are naturally poor in sulphur-oxidising bacteria. *Thiobacillus thioparus* has been inoculated with crushed sulphur into a calcareous solonetzic soil in virgin Alberta prairie land in order to promote acidification. This, combined with ripping the soil at a 60 cm depth and weekly irrigation, released several soluble salts, particularly those of sodium, calcium and magnesium (Bole, 1986). *Acidithiobacillus thiooxidans* was used to reclaim a saline alkaline soil by inoculating it together with elemental sulphur, thus lowering the pH and increasing the quantity of soluble salts (Bardiya *et al*., 1972).

B. HUMAN HEALTH CONSIDERATIONS

1. Diseases caused and mechanism of pathogenicity, including invasiveness and virulence

113. No species of *Acidithiobacillus* are known to be pathogenic based on the results of literature search conducted in various databases such as PubMed, Biosis, CAB Health and Current Contents.

2. Toxigenicity

114. There is no evidence to indicate that any species of *Acidithiobacillus* are toxigenic based on the results of literature search conducted in various databases such as PubMed, Biosis, CAB Health,Toxnet and Current Contents.

3. Allergenicity

115. No literature was found on the allergenicity of thiobacilli. However, the organisms are Gram negative and would therefore be expected to exhibit some of the characteristics associated with endotoxin. Nevertheless, no allergens of significance to humans have as yet been traced to this group of bacteria.

C. ENVIRONMENTAL AND AGRICULTURALCONSIDERATIONS

1. Natural habitat and geographic distribution: Climatic characteristics of original habitats

1.1 General overview

116. Although *Acidithiobacillus* is probably widely distributed, this distribution is usually related to the presence of sulphur as for example, coastal ecosystems such as salt marshes, sediments, mine dumps and sulphur-rich products of human industrial activity such as metal pipelines and concrete (Kelly and Harrison, 1989; Smith and Strohl, 1991).

117. As well, some of the products of human industrial activity such as metal pipelines and concrete have become a new ecological niche for some species. This has potentially destructive consequences (see below).

1.2 Correlation of natural incidence with usage and environmental impacts

118. While it can be assumed that *Acidithiobacillus* plays a role in the sulphur cycle, the extent of their involvement is largely unknown. Vitolins and Swaby (1969) showed that most thiobacilli in Australian soils rich in sulphur were autotrophic. *Acidithiobacillus* species' natural habitats, usage and environmental impacts are presented in Table 4.

Table 4. Usage and environmental impacts of Acidithiobacillus

Species	Physiological status	Natural habitats	Usage	Impacts
Acidithiobacillus albertensis	AA	Acid sulphurised soil (Bryant *et al*., 1983)	None at present	Not known
Acidithiobacillus caldus	AF	Coal spoil (Hallberg and Lindstrom, 1994)	None at present	Not known
Acidithiobacillus ferrooxidans	AA	Sulphurised soil and rock, in nature, *e.g.* pyrite (FeS_2). Iron and sulphur springs, sulphur iron-rich acidic waters, mines with various ores (Berthelot *et al*., 1993; Blowes *et al*., 1995; De Kimpe and Miles, 1992*;* Harrison, 1982; Johnson, 1995b; Valenti *et al*., 1990; Vitolins and Swaby, 1969; Zagury *et al*., 1994).	• Removal of heavy metals, • Bioleaching of ores, • Desulphurisation of coal and rubber.	Pyrite-oxidising bacterium involved in Acid Mine Drainage (AMD) (Evangelou, 1995)
Acidithiobacillus thiooxidans	AA	Sulphurised soil and deposits fresh water, mines + various ores, corroded concrete (Cho and Mori, 1995; Emde *et al*., 1992; Evangelou and Zang, 1995; Fliermans and Brock, 1972; Harrison, 1982; Parker, 1945; Robertson and Kuene, 1992; Sokolova and Karavaiko, 1968).	• Oxidation and removal of sulphidic pollutants in gas • Recovery of heavy metals • Recovery of silver in photoprocessing, • Recovery of certain ores • Sulphur transformation • Desulphurisation of coal and rubber • Detection of sulphur impurities in wines • Enhancement of phosphorus fertiliser • Soil Amelioration	• Potential threat to buildings, drains, • Deterioration of rubber, • Pyrite-oxidising bacterium involved in AMD (Evangelou, 1995), • Development of acid soils (Arkesteyn, 1980).

AA = Acidophiles, strictly chemolithotrophic and autotrophic
AF = Acidophiles, facultatively chemolithotrophic or mixotrophic
It is assumed that all thiobacilli play some role in the sulphur cycle.

2. Significant involvement in environmental processes, including biogeochemical cycles and potential for production of toxic metabolites

2.1 Utilisation of sulphur

2.1.1 The sulphur cycle

119. Colourless sulphur bacteria, which include the thiobacilli, play an important role in the sulphur cycle by oxidising sulphur and sulphides to sulphates so that they can be utilised by plants (Weir, 1975). Sulphide, which originates from anaerobic sulphate reduction and from decaying organic matter, is oxidised to sulphate under both aerobic and anaerobic conditions and by both chemical and biological means. Sulphate is assimilated by plants and micro-organisms and reduced to sulphides by other micro-organisms when these die.

120. In nature, a variety of reduced inorganic sulphur compounds occur as intermediates between sulphide and sulphate, which normally react very slowly with oxygen. Biological oxidation by the colourless sulphur bacteria plays an important role in the recycling of reduced sulphur compounds under aerobic conditions (Kuenen, 1975). The thiobacilli have received more attention than the other main groups of sulphur-oxidising micro-organisms. These comprise several genera of heterotrophic and facultative autotrophic bacteria and yeasts and are far more numerous than the thiobacilli. The thiobacilli, however, were deemed to be more efficient when conditions suited them (Vitolins and Swaby, 1969).

2.1.2 Role of thiobacilli in geologic sulphur deposits

121. Evidence shows that thiobacilli play a fundamental role in the development and weathering of sulphur deposits. In a review of sulphur deposits and waters with high sulphidic content in the former USSR, Sokolova and Karavaiko (1968) found that *T. thioparus* and *A. thiooxidans* were often associated. However, slight differences in distribution, according to the redox potential and the acidity of the environment, roughly correlating to the pH ranges cited in Table I were observed. Two examples show that these bacteria may play a role in the build-up and breakdown of sulphur deposits:

2.1.2.1 Formation of sulphur deposits

122. In the Shor-Su sulphur mines, *A. thiooxidans* occurred in the upper horizons of the deposit where an oxidative environment prevailed and the rocks were highly acidic (Sokolova and Karavaiko, 1968). In the aquifers throughout the lower horizon where the pH was neutral or weakly alkaline due to the proximity of limestone, *T. thioparus* was widespread and *A. thiooxidans* was absent. The presence of hydrogen sulphide prevented oxidation of the ore bed in the main deposit. Hydrogen sulphide was produced daily up to a rate of 0.2 mg/l by numerous sulphur-reducing bacteria in the groundwater and in the rocks, and ascended towards the surface waters where it was oxidised by *T. thioparus* to sulphur and water.

$$2H_2S + O_2 \Rightarrow 2H_2O + 2S^0 \qquad [20]$$

123. In several parts of the sulphur mines, sedimentation of molecular sulphur still continued by oxidation of the hydrogen sulphide of the underground waters, as shown by the presence of bacterial cells and small, freshly-deposited sulphur crystals retrieved on culture slides.

2.1.2.2 Degradation of sulphur deposits in the soil

124. In the early 1970s, millions of tonnes of elemental sulphur were extracted from sour natural gas

and stored in blocks in Alberta, Canada (Maynard *et al*., 1986). Since 1979, large quantities of sulphur have been deposited in adjacent forest systems due to mechanical break-up and weathering of these blocks, causing considerable damage to the understory vegetation. Three sites at distances of 50, 250 and 750 m from a sulphur block were studied. The pH was 2.6, 3.7 and 4.4 respectively for each site. This increasing acidification with the declining proximity to the sulphur block, was attributed to *A. thiooxidans*, which was the main soil micro-organism responsible for elemental sulphur oxidation at all three sites. *Thiobacillus thioparus* was also present, but at a significantly lower population level at the first site (that with the lowest pH) than at the other two. The nutrient concentration of the soil as measured by recoverable calcium, magnesium and potassium also decreased sharply towards the sulphur block.

2.1.3 Reaction on pyrite (FeS_2) in nature

125. Pyrite is the most prevalent form of iron disulphide and is usually associated with coalfields in the U.S. and elsewhere in the world. It is associated with many ores, including zinc, copper, uranium, gold and silver. Pyrite is formed in a reducing environment with a continuous supply of sulphates and iron in the presence of easily decomposable organic matter (Evangelou and Zhang, 1995).

126. Oxidation of pyrite deposits due to the combined action of *A. ferrooxidans* and *A. thiooxidans* in empoldered or flooded land has caused a pronounced acidification of the soil (Arkesteyn, 1980; Kuenen, 1975). When soils rich in pyrite are brought into agricultural production, "cat clay" is often formed where clay particles are cemented together by jarosite formed during the oxidation of pyrite, reducing agricultural production (Kuenen, 1975; Pronk and Johnson, 1992). Jarosite [$KFe_3(SO_4)_2(OH)_6$] is a basic ferric sulphate also found in deposits associated with pyrite (Ivarson, 1973). *Acidithiobacillus ferrooxidans* has been shown to play a part in the weathering of sulphide minerals with jarosite formation under humid conditions in metamorphic and igneous rocks in Ontario (De Kimpe and Miles, 1992). Other acidophilic species, such as *T. prosperus,* are able to perform the same reactions (Johnson, 1995b) but strains of *A. ferrooxidans* tend to grow more rapidly on ferrous iron than do other iron-oxidising acidophiles, thus causing them to dominate mixed populations (Pronk and Johnson, 1992).

2.1.4 Acid mine drainage (AMD)

127. Acid and metal pollution can be the result of the activities of thiobacilli in mine wastes (Tuovinen and Kelly, 1972). The natural oxidation of sulphide to sulphates, including sulphuric acid, as part of the sulphur cycle has become greatly enhanced by the world's increasing demand for metals and fossil fuels. Acid mine drainage (AMD) result from land disturbances due to mining and ore processing, and has become an economic and environmental burden (Evangelou and Zhang, 1995). Acid mine drainage may be enriched with soluble iron, manganese, aluminium, sulphate and heavy metals, and the pH may be as low as 2. One of the effects of AMD is to kill established vegetation associated with mine sites and spoil tips. The typical situation found on a reclaimed spoil tip is a slow fall in pH over most of the site. Severe acid generation tends to neutralise any added lime or buffering systems in the soil quickly, and further accelerates the rate of oxidation of pyrite (Backes *et al*., 1986). The large quantities of sulphuric acid that are produced make the environment in which *A. ferrooxidans* grows, and to which it is well adapted, inhospitable to most other organisms (Rawlings and Woods, 1995; Léveillé *et al*., 2001; Leduc *et al*., 2002).

128. Mine spoils that were alkaline in nature (pH 9), with low sulphur content and a high concentration of chlorides tended to be free of *A. ferrooxidans* (Twardowska, 1986). The limiting pH value for growth of *A. ferrooxidans* in rock material and drainage was found to be about 7.2 (Twardowska, 1987).

2.2 Corrosion

129. Because many of the colourless sulphur bacteria produce sulphuric acid or ferric ion, they are often associated with the oxidative corrosion of concrete and pipes, and have been implicated in the corrosion of buildings and ancient monuments.

2.2.1 Corrosion of concrete by Acidithiobacillus thiooxidans (Thiobacillus concretivorus)

130. Bacterial involvement in concrete corrosion has been known since the pioneering work of Parker (1945). *Acidithiobacillus thiooxidans* has been found mainly responsible for the deterioration of concrete in sewage pipelines (Cho and Mori, 1995; Jozsa *et al*., 1995; Mori *et al*., 1991, 1992; Parker, 1945; Sand and Bock, 1988, 1991). The bacterium is able to use hydrogen sulphide released from the sewage and oxidise it to sulphuric acid which then attacks the concrete. It was found to coexist with an acid-resistant fungus which could oxidise H_2S to thiosulphate, and it is thought that the bacterium used the latter as an energy source, producing sulphuric acid that was responsible for the corrosion.

$CaCO_3 + H_2SO_4 \Rightarrow CaSO_4 + H_2O + CO_2$ (Sand and Bock, 1988) [21]

131. The calcium loss results in a reduction in the stability of the structure.

132. The pH of fresh concrete sewer pipes is about 13, so that little microbial activity would normally occur in them. The pH would be lowered by carbonation of the concrete due to exposure to air, as well as by exposure to H_2S and then by the activities of the bacterium itself. Other bacteria, including *H. neapolitanus, T. intermedia* and *S. novella,* have been found to accompany *A. thiooxidans* but played a minor role in corrosion (Sand and Bock, 1991).

133. *Acidithiobacillus thiooxidans* was shown experimentally to be able to degrade Sulphlex, a mixture of elemental sulphur and plasticisers used as a paving material and as a substitute for asphalt in road construction with a simultaneous production of sulphuric acid (Ferenbaugh *et al*., 1992). In concurrent studies, plants grown in soils amended with Sulphlex exhibited higher sulphur content and reduced growth consistent with poisoning. Indications are, therefore, that *A. thiooxidans* has the potential for adverse effects on sulphur-containing construction materials as well as on the local environment.

2.2.2 Corrosion of steel

134. Transportation of low-grade coal in railway carriages has been linked with accelerated corrosion of the steel framework (Brozel *et al*., 1995), involving scaling, pitting and cracking. Corrosion of 3CR12 steel coupons embedded in coal occurred under experimental conditions after the pH of the coal had been lowered to 2.5. The extent of the damage increased when the fungus *Hormoconis resinae,* another dominant member of the natural flora, was present in the substrate.

2.3 Deterioration of rubber

135. Thaysen *et al*. (1945) reported that the deterioration of rubber hoses was the result of the microbial oxidation of elemental sulphur present in the rubber by *A. thiooxidans*. This was confirmed by Raghaven *et al*. (1990) who also note similar effects on polyethylene.

3. Interactions with and effects on other organisms in the environment

136. *Acidithiobacillus* plays an important role in making sulphur available to plants. *Acidithiobacillus thiooxidans* may also make phosphorus, iron, zinc and manganese more available for plant growth.

However, plants can be adversely affected by high concentrations of sulphate and increased acidity in the soil as a result of activity due to *A. thiooxidans*. Very little is known about antagonists of these bacteria.

4. Routes of dissemination: biological or physical

4.1 Biological

137. All species of *Acidithiobacillus* are motile (Kelly and Wood, 2000) so that they are able to disseminate within their immediate environment.

4.2 Physical

138. Water appears to be the major means of passive dissemination, and some dispersal must also be due to the spread of particles of soil or rock to which the bacteria have become attached.

D. APPLICATION OF THE ORGANISM IN INDUSTRY

1. Containment and decontamination

1.1 Chemical Methods

139. The traditional method for controlling acidity in coal spoil and the deposition of pyrite in field drains and soils was to add high levels of lime in order to maintain the pH well above 4 to limit the activity of ferric ions and of *A. ferrooxidans*, so restricting the oxidation of pyrite to a process involving oxygen alone (Backes *et al*., 1986; Poissant, 1986; Trafford *et al*., 1973). Alkaline chemicals, such as limestone, sodium carbonate or sodium hydroxide, have been applied or pumped into active mines to neutralise acid soils. Limestone could hydrolyse most heavy metals, precipitating them as metal hydroxides (Evangelou and Zhang, 1995). This method depended on maintenance of the pH at a consistent value, and very often reacidification occurred as the lime was neutralised or washed out of the surface layers. Limestone is usually readily available but massive over liming of the site may result (Pulford *et al*, 1986) and its effectiveness is reduced because a coating of ferric hydroxide precipitates develops to shield it from further dissolution (Evangelou and Zhang, 1995). The use of soluble neutralising agents such as sodium hydroxide avoids this problem but can be costly and not very practical.

140. McCready (1987) successfully controlled *A. ferrooxidans* in pyritic shale in the laboratory by adding sodium chloride to reach a concentration of 1.5%. He has suggested that the incorporation of a salt layer would prevent AMD in pyritic sites. Another promising method of controlling pyrite oxidation is considered to be the application of phosphate, which can precipitate Fe^{3+} in an insoluble form as $FePO_4$ or $FePO_4.2H_2O$ (strengite). Coating of the pyrite with iron is prevented by leaching the pyrite with low but critical concentrations of H_2O_2 and a pH buffer, with or without KH_2PO_4. In the first case, iron phosphate precipitates as a coating on the pyrite surface; in the second, it precipitates as an iron oxide (Evangelou and Zhang, 1995). Treatments with phosphate, silicate, citrate and EDDDHA (ethylenediamine di-orthohydroxyphenylacetic acid) inhibited the release of acid and iron from pyritic mine waste (Pulford *et al*., 1986).

141. Growth of *A. thiooxidans* strain NB1-3 was also greatly reduced in the laboratory by 5 mM of $NiSO_4$, the nickel binding to the cells and inhibiting the enzymes involved in sulphur oxidation (Maeda *et al*., 1996).

142. Inhibition of iron-oxidising bacteria may be achieved through the use of anionic surfactants (including common cleaning detergents), organic acids and food preservatives (Kleinman, 1989). Acid production may be reduced by 60 to 95%. However, wide use of surfactants is limited by the necessity for frequent treatments, since they are very soluble and motile, and they may also be adsorbed onto the surfaces of minerals without reaching the pyrite-bacterial interface (Evangelou and Zhang, 1995). The Witco product Microwet II^{TM} incorporating various surfactants, when continuously applied to refuse leaving a coal mine satisfactorily controlled *A. ferrooxidans* and reduced acid production (Stancel, 1982). Sodium lauryl sulphate was shown to be inhibitory at a concentration of about 10^{-6} M in culture media and in mine spoils at higher concentration (Fox and Rastogi, 1983). It also has the additional benefit of being low in mammalian toxicity and quite biodegradable, so subsequent environmental problems are unlikely. For restoration of a good ground cover over reclaimed overburden, these authors proposed that a controlled release system be set up to promote a good ground cover, which, over a period of years, would eventually

deprive the mine spoil of oxygen and water needed to generate acidity. 0.25% Sodium dodecyl sulphate at about 5,000 litre ha^{-1} resulted in dramatic reductions in acidity, sulphate, and dissolved-iron concentrations of discharge water for 3-6 months (Monticello and Finnerty, 1985).

143. Application of ProMac, a bactericide that can be applied both as a spray and in controlled-release monolithic pellets has been successful in controlling *A. ferrooxidans* by destroying its protective outer coating, making it susceptible to the acid it produces (Sanda, 1989). Controlled release bactericides including ProMac inhibited *A. ferrooxidans* and promoted regeneration of a mining site (Sobek, 1987; Sobek *et al*., 1990).

1.2 Biological Control

144. Inundation of pyritic material and acid sulphate soils or of disused mine shafts has been suggested (Backes *et al*., 1986); Evangelou and Zhang, 1995), since no significant growth of *A. ferrooxidans* has been demonstrated to occur in water-saturated environments (Kleinman and Crerar, 1979). A similar concept has been applied to the construction of wetlands to receive AMD in an anaerobic environment (Evangelou and Zhang, 1995). This environment encourages the activity of sulphate-reducing bacteria and so reduces acidity. Most of the hydrogen sulphide produced by these bacteria react with heavy metals to yield insoluble precipitates. Typical wetlands however, may not have sufficient permeability to take full advantage of this process.

145. Canada's Natural Resources Department 'Centre for Mineral and Energy Technology' (CANMET) proposed to develop an anaerobic "sulphuretum" for mitigation of acidic mine drainage by envisioning construction of a system of drainage ditches to control the flow of effluent through a bed of straw (McCready, 1991). As aerobic degradation of the straw proceeds, the sugars released will be fermented to organic acids by acidophilic heterotrophs. The organic acids will then be utilised by anaerobic sulphate reducers to reduce the sulphate in the effluent to hydrogen sulphide. Hydrogen sulphide percolating through a water column will precipitate dissolved metal ions as metal sulphides. Microbially produced CO_2 assists in buffering the system and provides a carbon source that may be combined with excess hydrogen by the methanogens to produce methane. In a laboratory study, the pH of the incoming liquid was 3.5 and after passage through the treatment zone it rose to pH 8.1. This process achieved a 65% reduction in the sulphate concentration and metals were not detected in the effluent.

146. Christison *et al*. (1985) reported that an unidentified zooflagellate was an effective predator, reducing the population of *A. ferrooxidans* from 10^8 cells/ml to 10^2 cells/ml within 18 hours at pH 2.3. Rotifers, as well as flagellated and ciliated protozoa were recorded as significant predators of *A. ferrooxidans* (McCready, 1987) but were incapable of eradicating them in liquid culture; moreover their large size makes it difficult for them to pursue their prey in interstitial spaces of mine tailings.

147. Padival *et al*., (1995) found that an unidentified strain of yeast introduced into continuously stirred tank reactors with *H. neapolitanus* or *A. thiooxidans* resulted in a 99% decrease in the population of the latter. The effect on these thiobacilli was enhanced by limitation of nitrogen. The results suggest that strategies based on the competitive displacement of thiobacilli to inhibit corrosion of concrete sewers may be feasible.

2. Description of detection and monitoring techniques, including specificity, sensitivity and reliability

2.1 Thallous sulphide test

148. The ability to oxidise thiosulphates to sulphates with the production of elemental sulphur can be utilised to distinguish the sulphur-producing bacteria including *Acidithiobacillus*, from natural samples (Galizzi and Ferrari, 1976). Thallous sulphide paper moistened with pyridine is pressed onto agar plates

with the colonies to be tested and then placed in dilute (0.12 N) nitric acid. The black thallous sulphide paper is bleached except in the presence of free sulphur, due to the presence of thallous polysulphides. If sulphur is present, a brown spot is left at the site of the replicated colony. This test specifically enabled quantification of *T. thioparus* and *A. thiooxidans* in natural samples.

2.2 Molecular probes

149. There are some specific problems associated with the identification and quantitation of micro-organisms in biohydrometallurgical operations (Yates and Holmes, 1986). The numerous species of autotrophic and heterotrophic bacteria may be morphologically similar and are often difficult to purify since they grow poorly or not at all on solid media. Analysis may be frequently aggravated by the presence of small rock particles and by the production of ferric precipitates. Molecular probes using cloned DNA sequences in Southern Blots and Dot blots, could distinguish between *A. ferrooxidans* and other species (*Acidiphilium acidophilum*, *Starkeya novella* and *T. thioparus*) as well as recognising several strains of *A. ferrooxidans*. This technique could, moreover, detect as few as 10^5 bacterial cells of a given species. The reverse sample genome probing (RSGP) technique was used by Léveillé *et al*. (2001) to monitor the presence of *Acidithiobacillus* species in AMD environments. Another genomic tool such as fluorescent *in situ* hybridisation (FISH) was successfully used in the laboratory to detect strains of *A. ferrooxidans* in an ADM environment (Leduc, personal communication).

2.3 Polymerase-chain-reaction (PCR) and related methods

150. Recently PCR has been used in the detection and identification of *Acidithiobacillus* and other sulphur bacteria. Strains of *A. ferrooxidans* were differentiated by use of RAPD (random primer amplified polymorphic DNA) (Novo *et al*. 1996). Extending this observation, Selenska-Pobell *et al*. (1998) used genomic fingerprinting in the form of RAPD, rep-APD (repetitive primer amplified polymorphic DNA) and ARDREA (amplified ribosomal DNA restriction enzyme analysis) to distinguish four strains of *A. ferrooxidans,* and one strain each of *A. thiooxidans, E. coli, Burkholderia cepacia, T. thioparus* and *Thiomonas cuprinus*. The procedures not only discriminated between the different species but also suggested that one of the strains of *A. ferrooxidans* was only distantly related to the three others. This variable sequence homology was attributed by the authors to the greater ability of the variant strain to accumulate uranium, although all strains were isolated for some ability to do this. Such variability suggests that more than one method should be used to identify or distinguish different strains or species of thiobacilli.

151. The differentiation of one strain from the others tested may not be surprising since the strains were isolated from different strata and Novo *et al*. (1996) suggested that strains of *A. ferrooxidans* isolated from different micro-environmental sources could give varying patterns on RAPD.

2.4 Isolation Media

152. Most sulphur bacteria can be isolated from natural habitats by the use of mineral media containing elemental sulphur or thiosulphate as an energy substrate. Use of media of different pH will assist differential selection of the neutrophilic and acidophilic species, whereas use of acid ferrous sulphate medium will frequently select for *A. ferrooxidans* (Kelly and Harrison, 1989).

153. A procedure has been described for the enrichment of facultatively autotrophic, mixotrophic bacteria, using a continuous flow chemostat provided with both organic and inorganic substrates (Gottschal and Kuenen, 1980). This provides a means of avoiding the predomination of heterotrophs in standard batch enrichment media containing supplements such as thiosulfate and glucose or acetate. In the latter, a mixture of obligatory chemolithotrophic thiobacilli and chemo-organotrophs normally develops. Harrison

(1984) has described a general medium for cultivation of acidophilic bacteria comprising a basal mineral salts solution (MS) with the following (%w/v):

Media composition (per litre)	
$(NH_4)_2SO_4$	0.200
KCl	0.010
K_2HPO_4	0.025
$MgSO_4.7H_2O$	0.025
$Ca(NO_3)_2$	0.001

154. The pH is adjusted to pH 2-4 with 1N H_2SO_4.

155. Johnson (1995a) has since reviewed various solid media formulations for acidophilic bacteria published in the literature. The use of agarose, a purified derivative of agar, was recommended to overcome the fastidiousness of thiobacilli in this and other media, as well as the use of a double-layered medium, the underlayer incorporating an acidophilic heterotroph. The latter significantly lowered the proportion of monosaccharides and resulted in a dramatic increase in plating efficiency of most strains of *A. ferrooxidans*.

156. The basic medium recommended comprised ferrous sulphate and tryptone soya broth and potassium tetrathionate, and enabled differentiation and identification of isolates of several iron-oxidising bacteria based on colony characteristics.

2.4.1 Acidithiobacillus albertensis

157. This species can be grown on the media suitable for *A. thiooxidans* (Kuenen *et al*., 1992) (see below). The pH should not be lower than pH 2.0.

2.4.2 Acidithiobacillus caldus

158. Hallberg and Lindstrom (1994) used tetrathionate as a main growth substrate adjusted to pH 2 and held at 32 ^{O}C or 52 ^{O}C according to the strain.

2.4.3 Acidithiobacillus ferrooxidans

159. This species does not readily form colonies on standard agar media because it is inhibited by some of the organic compounds found in unpurified agar (Holmes and Yates, 1990). The ability to form colonies on a solid medium is a necessary precondition for growth studies and for strain development. This problem has been overcome by substituting pure agarose for the routine agar medium or by growing the bacterium on membrane filters placed on the solid agar medium (Tuovinen and Kelly, 1973).

160. Growth of this species on solid media can be enhanced by the addition of small quantities of a surfactant such as Tween 80 (Garcia *et al*., 1992). Use of ferrous sulphate ($FeSO_4.7H_2O$) with other basal salts has also been successful in enhancing the growth of the bacterium (Kuenen *et al*., 1992; Harrison, 1984; Visca *et al*., 1989). One of the *Thiobacillus* solid media, TSM-1, developed by Visca *et al*., (1989), produced discrete and easily countable colonies and could be used for the isolation of single clones.

161. The ferrous sulphate medium is given by Kuenen *et al*. (1992) as follows:

Solution I (per litre)
K_2HPO_4 0.5 g.
$(NH4)_2SO_4$ 0.5 g
$MgSO_4.7H_2O$ 0.5 g
H_2SO_4 5 ml of a 15N solution

Solution II (per litre)
$FeSO_4.7H_2O$ 0.5 g
H_2SO_4 150 ml of a 15N solution

162. Four parts of solution I are mixed with 1 part of solution II to give a medium containing 120 $mMFe^{2+}$. Formation of iron precipitates can be avoided by lowering the pH through successive subcultures to a value of 1.3 with H_2SO_4.

Solid 2:2 medium for genetic manipulation of *A. ferrooxidans*

163. This medium was developed by Peng *et al*. (1994a) for the isolation of mutants. The medium, prepared in four parts, contains ferrous sulphate and sodium thiosulphate as energy sources for the growth of the bacterium at pH 4.6-4.8. Strains resistant to kanamycin and streptomycin could be obtained by incorporating increasing concentrations of these antibiotics in the medium and selecting out those colonies which developed. The medium encouraged the growth of a wide morphological range of colonies. It is also possible to introduce plasmids into bacterial cells using this medium and to develop strains resistant to heavy metals.

2.4.4 Acidithiobacillus thiooxidans

164. Kuenen *et al*. (1992) list the isolation and growth media for this species as follows:

Media (per Litre)
K_2HPO_4 3.5 g
$(NH_4)_2SO_4$ 0.3 g
$MgSO_4.7H_2O$ 0.5 g
$FeSO_4.7H_2O$ 0.018 g
CaCl 0.25 g
Flowers of Sulphur 5.0 g
Adjust to a pH of 4.5

165. Both *A. ferrooxidans* and *A. thiooxidans* may be cultivated in minimal salts (MS) containing 1% powdered sulphur. Sulphur melts at ~113 ^{O}C, so the MS-sulphur slurry is sterilised by heating at 105 ^{O}C for one half-hour on two successive days (Harrison, 1984). These acidophilic thiobacilli have also been isolated on a mineral basal salts medium supplemented with ferrous sulphate and substituting 0.4% Gelrite, a bacterial polysaccharide, for agar (Khalid *et al*., 1993). Dark brown circular colonies have been observed to develop on this medium within 72-96 hours.

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