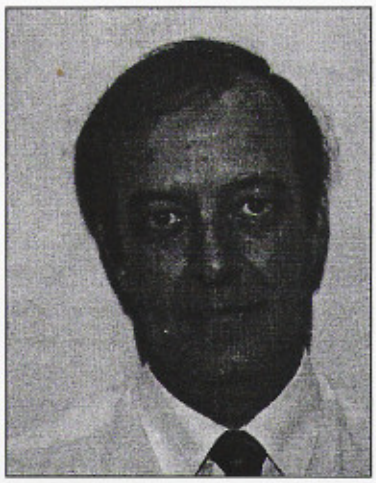
INTRODUCTION



Douglas Eric Rawlings was born in East London on 11 November 1950. After matriculating from Selborne College (1968), he studied at Rhodes University where he completed a BSc degree in Chemistry and Microbiology, a BSc (Hons) in Microbiology, and in 1976 a PhD degree in Microbiology under the supervision of Professor David Woods. He spent two years as a research officer at the Leather Industries Research Institute (Grahamstown) and four years as a lecturer at the University of the Witwatersrand. In 1982 he was appointed Senior Lecturer in the Department of Microbiology at the University of Cape Town and reestablished his research collaboration with Professor David Woods, who had moved to UCT. He was promoted to Associate Professor in 1987 and a year later to an *ad hominem* chair as Professor of Microbiology. In July 1998 he took up his present appointment as Professor of Microbiology at the University of Stellenbosch.

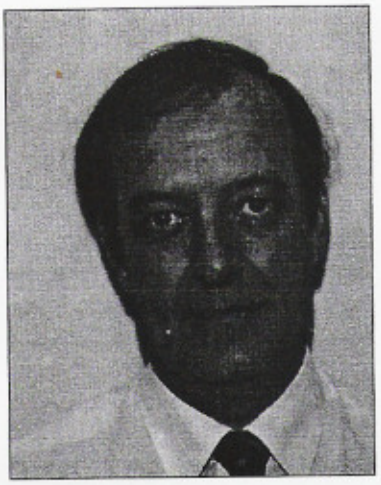
Professor Rawlings's initial research was in the field of waste water treatment. However, over the past 18 years his research has mostly concerned the bacteria involved in the extraction of minerals from ores. He was involved in the initial conceptual development of the process for the biooxidation of difficult-to-treat gold-bearing arsenopyrite ores. The engineering scale-up work and application of this technology was carried out by Genmin Process Research Laboratories (now Billiton, Randburg, South Africa). Most of Prof Rawlings's research has concerned the molecular biology of the biomining bacterium *T. ferrooxidans* and more recently this work has been extended to include other biomining bacteria.

Professor Rawlings has supervised eight completed MSc and thirteen PhD students. The findings of this research have been published in over seventy peer-reviewed journal articles and book chapters. He has been inventor or co-inventor of two international and two local patents. In 1997 he edited a book entitled *Biomining: Microbes, Theory and Industrial Processes*, which was sold out within the first year of publication. He has delivered many plenary and keynote lectures at local and international conferences. In recognition of the output of the research group he leads, he received an A-rating from the FRD in 1992 and again in 1996.

He currently serves on the editorial board of the journals *Applied and Environmental Microbiology* and *International Deterioration and Biodegradation* and as an occasional reviewer for several other journals. Professor Rawlings has acted as external examiner for undergraduate and postgraduate degrees at several universities in South Africa and abroad. He is a member of several learned bodies and is currently General Secretary of the Royal Society of South Africa and is on the council of the SA Society for Microbiology. He is a life-fellow of the University of Cape Town (1990), received the UCT distinguished teachers award (1992) and in 1998 was appointed for five years as an Honorary Professor of Microbiology at the same university. In 1992 he was awarded a silver medal from the SA Society for Microbiology and in 1997 the PanLab's award from the Society for Industrial Microbiology. He was elected a fellow of the Royal Society of South Africa (1993) and a founder member of the South African Academy of Science (1995).

Professor Rawlings is a keen squash player and an occasional cyclist (completed the last five Argus-Pick 'n Pay cycle tours). Much of his spare time is devoted to his duties as an elder in the Assembly of God church in which capacity he has served for the past 24 years. He and his wife, Janet, recently celebrated their twenty-fifth wedding anniversary and have three children, Barry (21), Kim (19) and Sarah-Jane (18).

INTRODUCTION



Douglas Eric Rawlings was born in East London on 11 November 1950. After matriculating from Selborne College (1968), he studied at Rhodes University where he completed a BSc degree in Chemistry and Microbiology, a BSc (Hons) in Microbiology, and in 1976 a PhD degree in Microbiology under the supervision of Professor David Woods. He spent two years as a research officer at the Leather Industries Research Institute (Grahamstown) and four years as a lecturer at the University of the Witwatersrand. In 1982 he was appointed Senior Lecturer in the Department of Microbiology at the University of Cape Town and reestablished his research collaboration with Professor David Woods, who had moved to UCT. He was promoted to Associate Professor in 1987 and a year later to an *ad hominem* chair as Professor of Microbiology. In July 1998 he took up his present appointment as Professor of Microbiology at the University of Stellenbosch.

Professor Rawlings's initial research was in the field of waste water treatment. However, over the past 18 years his research has mostly concerned the bacteria involved in the extraction of minerals from ores. He was involved in the initial conceptual development of the process for the biooxidation of difficult-to-treat gold-bearing arsenopyrite ores. The engineering scale-up work and application of this technology was carried out by Genmin Process Research Laboratories (now Billiton, Randburg, South Africa). Most of Prof Rawlings's research has concerned the molecular biology of the biomining bacterium *T. ferrooxidans* and more recently this work has been extended to include other biomining bacteria.

Professor Rawlings has supervised eight completed MSc and thirteen PhD students. The findings of this research have been published in over seventy peer-reviewed journal articles and book chapters. He has been inventor or co-inventor of two international and two local patents. In 1997 he edited a book entitled *Biomining: Microbes, Theory and Industrial Processes*, which was sold out within the first year of publication. He has delivered many plenary and keynote lectures at local and international conferences. In recognition of the output of the research group he leads, he received an A-rating from the FRD in 1992 and again in 1996.

He currently serves on the editorial board of the journals *Applied and Environmental Microbiology* and *International Deterioration and Biodegradation* and as an occasional reviewer for several other journals. Professor Rawlings has acted as external examiner for undergraduate and postgraduate degrees at several universities in South Africa and abroad. He is a member of several learned bodies and is currently General Secretary of the Royal Society of South Africa and is on the council of the SA Society for Microbiology. He is a life-fellow of the University of Cape Town (1990), received the UCT distinguished teachers award (1992) and in 1998 was appointed for five years as an Honorary Professor of Microbiology at the same university. In 1992 he was awarded a silver medal from the SA Society for Microbiology and in 1997 the PanLab's award from the Society for Industrial Microbiology. He was elected a fellow of the Royal Society of South Africa (1993) and a founder member of the South African Academy of Science (1995).

Professor Rawlings is a keen squash player and an occasional cyclist (completed the last five Argus-Pick 'n Pay cycle tours). Much of his spare time is devoted to his duties as an elder in the Assembly of God church in which capacity he has served for the past 24 years. He and his wife, Janet, recently celebrated their twenty-fifth wedding anniversary and have three children, Barry (21), Kim (19) and Sarah-Jane (18).

THE BACTERIA, BIOLOGY AND BIOTECHNOLOGY OF BIOMINING

Microbes have been involved in the formation and decomposition of minerals in the earth's crust since geologically ancient times. Mining operations have benefited from the activities of such microbes, especially from the ability of some bacteria to leach metals from insoluble ores. As early as 1000 BC mine workers in Mediterranean countries recovered copper that was leached into mine drainage waters by bacteria, although they would not have been aware that microbes were involved. Romans in the first century, Welsh in the 16th century (Figure 1) and the Spanish who worked the Rio Tinto mine in the 18th century almost certainly used microbial leaching for extracting metals.

Figure 1. Site of the now disused Parys copper mine, where bioleaching was used for centuries. The mine is located on the island of Angelsey, North Wales. Dr Barrie Johnson is in the foreground.

Research on biomining and the microbes involved in biomining is a field in which I have been involved for the past 19 years. During this time the use of microbes in the recovery of metals from ores has been extended to the point where biomining has become a very significant part of industrial microbiology. Large-scale copper bioleaching takes place by using what may be considered to be a low-technology process, the irrigation of dumps (Brierley, 1982). Since the early 1960s the copper recovery process has been made more efficient by the construction and irrigation of specially designed heaps (Brierley 1982, Schnell, 1997). Also in the 1960s it was discovered that uranium could be recovered by bioleaching and industrial-scale uranium bioleaching was carried out by spraying stope walls with acid mine drainage and by the *in situ* irrigation of fractured underground ore deposits (Mc Cready and Gould, 1990). Then in the 1980s a process for the biooxidation of gold-bearing arsenopyrite ores in highly aerated stirred-tank reactors was developed by Gencor, South Africa (now sold to Billiton) (Livesey-Goldblatt *et al.*, 1983). With that process, biomining became a significant part of the fermentation industry. An illustration of this is that the biooxidation plant built at the Ashanti goldfields in Ghana (Dew *et al.*, 1997) is currently the largest fermentation plant in the world (with the possible exception of some sewage treatment facilities). Today at least six commercial stirred-tank gold-ore biooxidation plants operate in five countries using highly controlled fermentation technology. Biomining has come of age as an important part of industrial microbiology.

An aspect of biomining that has interested me is the fascinating physiology of the microorganisms involved (Kelly *et al.*, 1979; Lundgren and Silver, 1980; Rawlings, 1997). The most important microbes are characterised by their ability to grow chemolithotrophically using ferrous iron or reduced sulfur compounds as an energy source. Microorganisms with this ability are found in a large number of environments ranging from the mesophilic to the hyperthermophilic and in water which varies from neutral pH to highly acidic (Norris, 1990; Norris, 1997). All current industrial bioleaching or biooxidation processes are carried out at below 50°C, using a consortium of mesophilic or moderately thermophilic iron and sulfur-oxidising bacteria, at a pH of 1.6 or less. With the exception of a single biooxidation plant (Miller, 1997), currently operating commercial bioleaching plants function at 40°C or below.

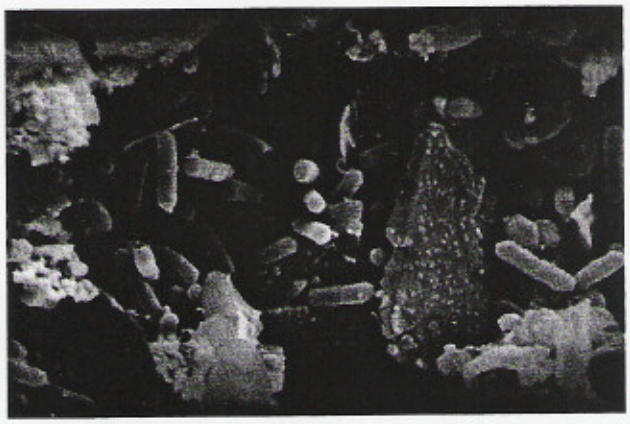


Figure 2. An electron micrograph of rod-shaped bacteria belonging to the genus *Thiobacillus* among particles of crushed ore. The iron- and sulfur-oxidising bacteria *Thiobacillus ferrooxidans* and the strictly sulfur-oxidising bacteria *Thiobacillus thiooxidans* look very similar to each other and it is not possible to tell them apart from this photograph.

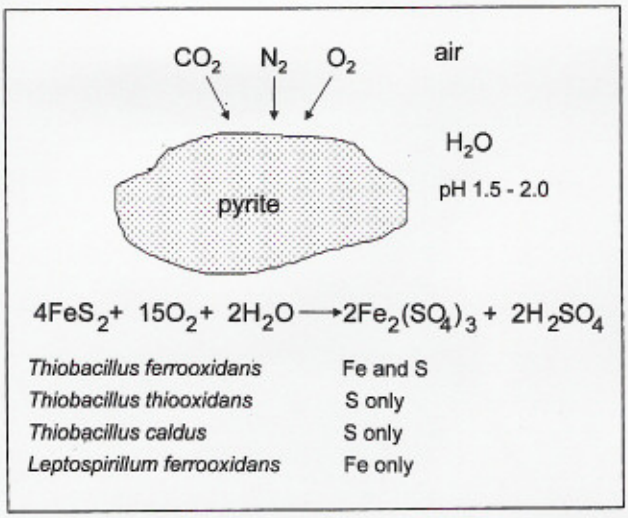


Figure 3. Biomining bacteria require remarkably few nutrients for growth. Air, water, trace elements and an iron- or sulphide-containing ore are sufficient.

BIOMINING BACTERIA

Mesophiles

The most important "players" in biomining operations that take place at less than 40°C are the bacteria, *Thiobacillus ferrooxidans*, *Leptospirillum ferrooxidans* and *Thiobacillus thiooxidans* (Figure 2). These Gram-negative bacteria have a physiology which is ideally suited for growth in an inorganic mining environment (Lundgren, 1980; Rawlings, 1997). They are all obligately chemoautolithotrophic bacteria which obtain their energy through the oxidation of ferrous to ferric iron (*T. ferrooxidans* and *L. ferrooxidans*) or reduced inorganic sulfur compounds to sulfate (*T. ferrooxidans* and *T. thiooxidans*) (Kelly and Harrison, 1989). The production of sulfate results in the accumulation of sulfuric acid so that the pH of their environment is typically pH 1.5 -2.0 (or lower) and all of the important biomining bacteria are obligately acidophilic. A typical source of energy is the oxidation of a mineral such as pyrite.

$$4\ FeS_2 + 15\ O_2 + 2\ H_2O \longrightarrow 2\ Fe_2(SO_4)_3 + 2\ H_2SO_4$$

Biomining bacteria are considered to be aerobic, although in the absence of oxygen *T. ferrooxidans* is able to grow on reduced inorganic sulfur using ferric iron as an alternate electron acceptor (Sugio *et al.*, 1985). *T. ferrooxidans* has been shown to fix atmospheric nitrogen and all *T. ferrooxidans* and *L. ferrooxidans* tested have *nif* genes (Norris *et al.*, 1995; Pretorius *et al.*, 1986). Their unique physiology means that biomining bacteria are able to grow in what would appear to be very nutrient-poor solutions. Aeration of a suitable ore in water is usually sufficient to satisfy their essential growth requirements. Air provides the carbon (CO_2), nitrogen (N_2) and electron acceptor (O_2) source, ore is the source of energy and water the growth medium. Essential trace elements are provided as impurities in the ore or water (Figure 3). The organisms are probably not capable of fixing nitrogen when growing in an aerobic environment. So when they are growing in highly aerated stirred-tank reactors, small amounts of inexpensive fertiliser-grade ammonium sulfate and potassium phosphate are added to ensure that sufficient nutrients are present. Biomining bacteria have other advantages. They tolerate moderate to high levels of many metal ions (Tuovinen *et al.*, 1971) and because few other organisms are capable of growth in an inorganic low-pH environment, it is unnecessary to ensure the sterility of industrial processes which employ these bacteria.

Moderate thermophiles

A number of bacteria are capable of oxidising iron or sulfur compounds at temperatures of between 45-55°C. These include the Gram-negative bacterium, *Thiobacillus caldus* (Hallberg and Lindström, 1994), which is capable of oxidising sulfur optimally at 45°C. *T. caldus* strains rapidly oxidise sulfur within the temperature range 35-50°C and occur in greater numbers in biooxidation plants than *T. thiooxidans* (Gardner and Rawlings, unpublished). *T. caldus* appears to be the moderately thermophilic equivalent of *T. thiooxidans*. A comparison of 16S rRNA sequences from *T. caldus* and *T. thiooxidans* indicates that the two bacteria are phylogenetically closely related (Hallberg and Lind-

ström, 1994). In contrast to the mesophilic iron-oxidising bacteria, the iron-oxidising moderate thermophiles are more commonly Gram-positive spore-forming bacteria belonging to the genus *Sulfobacillus* (Norris, 1997). *Sulfobacillus thermosulfidooxidans* has been reported to be the most efficient iron-oxidising moderate thermophile and its growth and ability to oxidise iron is stimulated in the presence of yeast extract and carbon dioxide-enriched air. *Acidimicrobium ferrooxidans* is a moderate thermophile which grows well, even in the absence of added carbon dioxide, but its ability to oxidise iron is less than *Sulfobacillus thermosulfidooxidans*, even when grown in carbon dioxide-enriched air. Mixed cultures of both bacteria are efficient oxidisers of iron in the absence of added carbon dioxide (Norris, 1997). It has been suggested that the reason for this is that *Acidimicrobium* is more efficient at fixing carbon dioxide than *Sulfobacillus thermosulfidooxidans* and when it is grown in mixed culture, *Acidimicrobium* secretes small amounts of organic nutrients which are used by the *Sulfobacillus*. This enables the *Sulfobacillus* to oxidise iron rapidly.

CURRENT INDUSTRIAL PROCESSES

Although several minerals are amenable to commercial-scale bioleaching/biooxidation, currently only two metals are recovered using this technology; these are copper and gold Rawlings and Silver 1995). In the 1980s substantial amounts of uranium were mined using *in situ* bioleaching technology (McCready and Gould, 1990). During 1988 approximately 300 tons of uranium with a value of over US$25 million was recovered from a single mine (Dennison mine, Lake Elliot district, Canada). However, with the reduction in demand for uranium in recent years, this mine has stopped production.

COPPER BIOLEACHING

In terms of tonnages copper is the most important metal to be recovered by biomining (Brierley, 1997). Bioleaching of copper involves the conversion of water-insoluble copper sulfides to water-soluble copper sulfates. Copper-containing minerals such as chalcocite (Cu_2S) or covellite (CuS) are crushed, acidified with sulfuric acid and agglomerated in rotating drums to bind fine material to coarser particles (Schnell, 1997). The agglomerate is stacked in heaps onto lined pads on which aeration piping may be placed. The stacked heaps are irrigated with an iron-containing solution (usually recycled spent leach liquor) through a second system of pipes laid on or just below the heap surface. Aeration may be enhanced by forcing air through the heap from the bottom using low-pressure fans. The solution percolates through the heap and bacteria growing on the surface of the ore and in solution catalyse the release of copper. Small amounts of inorganic nutrients in the form of fertiliser-grade ammonium sulfate and potassium phosphate may aid microbial growth and copper dissolution. The ferric iron generated by the bacteria plays an important role in the production of copper sulfate.

$$2\,FeSO_4 + 1/2\,O_2 + H_2SO_4 \xrightarrow{\text{bacteria}} Fe_2(SO_4)_3 + H_2O$$

$$Cu_2S + 1/2\,O_2 + H_2SO_4 \xrightarrow{\text{bacteria}} CuS + CuSO_4 + H_2O$$

$$CuS + 2\,O_2 \xrightarrow{\text{bacteria}} CuSO_4$$

$$Cu_2S + 2\,Fe_2(SO_4)_3 \longrightarrow 2\,CuSO_4 + 4\,FeSO_4 + S$$

$$CuS + Fe_2(SO_4)_3 \longrightarrow CuSO_4 + 2\,FeSO_4 + S$$

The pregnant leach solution containing 1.5 to 6 g/l soluble copper and up to 20 g/l iron is collected and sent to a recovery plant. The most common methods for copper recovery are by means of precipitation using iron filings (cementation), electrowinning or solvent extraction followed by electrowinning. The latter procedure produces the highest grade of copper. The most important copper-producing country in the world is Chile and the largest heap bioleaching operations are located in that country. Copper mines at Quebrada Blanca (Figure 4) and Cerro Colorado use bioleaching to produce 75,000 and 60,000 metric tons of copper per annum respectively (Brierley, 1997).

Figure 4. The copper mine at Quebrada Blanca, Northern Chile, where copper is leached from ore by irrigating crushed ore that has been stacked on pads. The ore is sprayed with a raffinate leaching solution (8 g sulfuric acid plus 0.3-0.5 g residual copper per l) through a system of pipes and nozzles placed on top of the ore. The bacteria grow on the surface of the crushed ore where they assist in the conversion of insoluble copper sulfides to soluble copper sulfate.

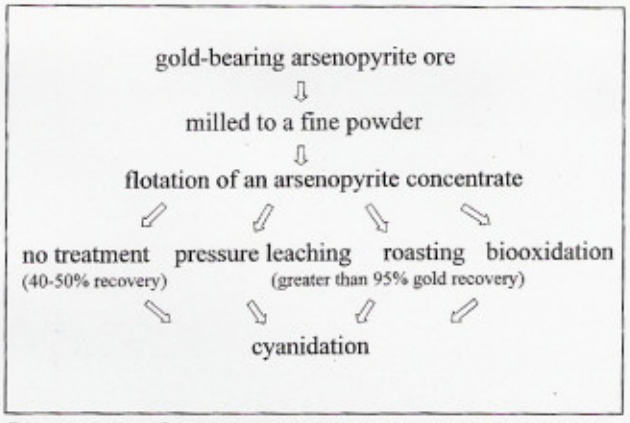


Figure 5. Steps required to prepare gold-bearing arsenopyrite ores for gold recovery. A concentrate is prepared to reduce the tonnage of ore required to be treated. This concentrate contains essentially all of the gold. Prior to 1986, roasting or pressure leaching were used as pretreatment processes, but since 1990 biooxidation has almost entirely displaced these technologies.

GOLD BIOOXIDATION

The potential of microbes to assist in the extraction of gold from some recalcitrant ores was first realized in the early 1980s. Much of the credit for this belongs to the late Eric Livesey-Goldblatt, who at the time was director of the Gencor Process Research Laboratory in Krugersdorp, South Africa. Gold is usually extracted from ores using cyanide. Recalcitrant ores are those in which gold is encased in a matrix of arsenopyrite/pyrite, so that even after fine milling, the gold cannot be efficiently recovered. Pretreatment of the ore is required to open up the molecular structure of the ore, which permits cyanide to make contact with and extract the gold. Since the quantities of ore to be treated are huge and most of the gold is present in a small pyrite/-arsenopyrite fraction, the ore is crushed and a gold-bearing concentrate is prepared by flotation (Figure 5). Prior to 1986 concentrate pretreatment processes were physico-chemical. For example, the concentrate was roasted at 700°C in the presence of oxygen or digested with acid under pressure in an oxygen-enriched atmosphere (autoclaved). In contrast, biomining bacteria decompose ores and concentrates at atmospheric pressure and at temperatures that are closer to ambient.

$$2\ FeAsS\ (arsenopyrite) + 7\ O_2 + H_2SO_4 + 2\ H_2O \xrightarrow{bacteria} 2\ H_3AsO_4 + Fe_2(SO_4)_3$$

Without pretreatment, only 30-50% of the gold is recovered depending on the concentrate, while after biooxidation more than 95% of the gold is recoverable. Since gold-bearing concentrates are valuable substrates relative to copper, it is economically viable to carry out the pretreatment biooxidation processes in efficient, aerated, temperature- and pH-controlled vat-type fermenters. Unlike the bioleaching of copper ores, where the copper is solubilised in the biological process, the gold is not solubilised by microbial action, but a separate chemical process is required. Since gold is not leached from the ores, the term 'biooxidation' is used to describe the treatment of gold ores as opposed to the term 'bioleaching'. Bioleaching strictly refers only to processes in which uranium and base metals such as copper, nickel, zinc or lead are solubilised as a result of microbial activity.

Development of the Biox process

Early experiments on gold-biooxidation were carried out in a series of three or four continuous-flow, aerated, stirred-tank reactors (Rawlings and Woods, 1995). As these reactors are expensive to construct and operate, the rate of concentrate decomposition has an important effect on the economics of the process. The initial processes were very slow because unadapted cultures of biooxidation bacteria were sensitive to the arsenic released from the arsenopyrite. Concentrate was decomposed until the build up of arsenic inhibited further microbial activity. Arsenic toxicity was reduced by the introduction of an arsenic precipitation step between each of the aerated reactors. This process was unworkable and uneconomical. A retention time of over 12 days was required for sufficient biooxidation to allow more than 95% gold recovery. However, a continuous-flow process provides strong selection for arsenic tolerance because slow-growing cells were preferentially washed out and the faster growing cells retained. Over a period of two years the arsenic resistance of the cultures increased from less than 1 g to over 13 g total arsenic/l. Largely because of this increase in arsenic resistance, the retention time of concentrate in the reactors was reduced to 7 days. During 1986 the first full-scale continuous biooxidation plant designed to treat 10 tons of gold-bearing arsenopyrite concentrate per day was built at the Fairview mine (Figure 6) in South Africa. By 1989 the growth rate of the bacteria had improved still further so that the retention time was reduced to 3.5 days. At the same time the solid concentration in the liquor was increased from 10 to 18% so that the same equipment could be used to treat almost four times the amount of concentrate per day than formerly. This process, which had been developed by Gencor SA (van Aswegen *et al.*, 1991; Dew *et al.*, 1997), was registered as the Biox process. The Biox

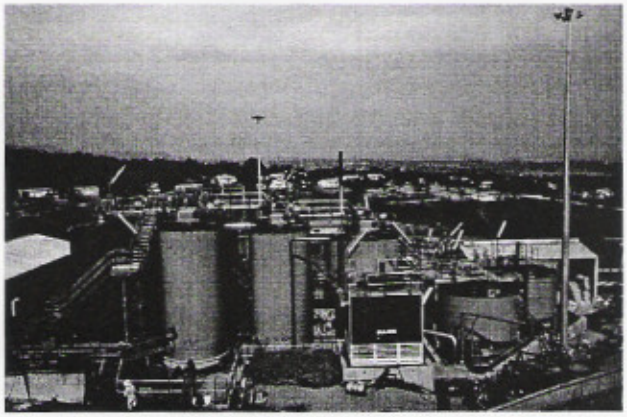


Figure 6. A photograph of the first production-scale biooxidation plant for the treatment of gold-bearing arsenopyrite ore. The plant was commissioned at the Fairview mine, Barberton, South Africa and was originally designed to treat 30% of the mine's production. In 1991 this demonstration plant was extended to treat the full production of approximately 40 tons of concentrate per day.

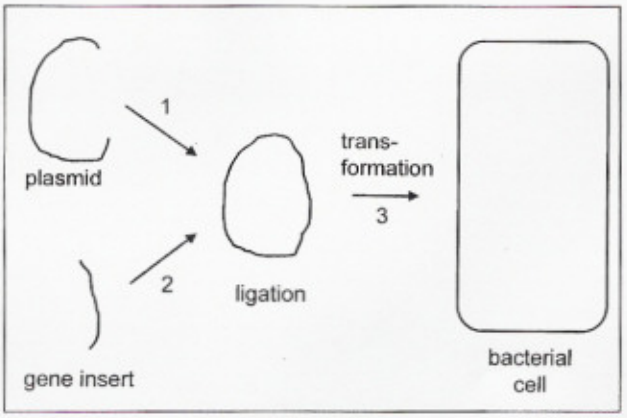


Figure 7. The basic requirements of a genetic system for a bacterium like *T. ferrooxidans* are: 1) a plasmid that will replicate in the organism to be modified, 2) a gene encoding some desirable property that will be expressed in the bacterium and 3)a method for introducing the new DNA construct into *T. ferrooxidans*.

process has proved to be highly robust and since 1990 plants using Biox technology have been commissioned at Sao Bento, Brazil (150 tons/day), Harbour Lights (40 tons/day) and Wiluna (115 tons/day), both in Western Australia, and Sansu, Ghana (1000 tons/day). The latter plant consists of twenty-four 1000 m^3 aeration tanks and is the largest fermentation plant in the world. A typical Biox plant operates at 40°C and a considerable amount of energy for cooling is required to maintain the process at this temperature.

Advantages and disadvantages of biooxidation

A major advantage of biooxidation is that relatively little of the ore need be decomposed to allow near complete gold recovery. The gold particles create a weakness in the pyrite/arsenopyrite crystal lattice and biooxidation takes place preferentially in these areas of weakness (Claasen *et al.*, 1993). Capital costs for biooxidation have been reported to be about 2-fold lower than roasting or pressure oxidation, and operating costs are also lower (Dew *et al.*, 1997). Waste disposal following biooxidation is comparatively easy. The pH of the ferric arsenate effluents is raised to above pH 5 with lime. This precipitates arsenic as $FeAsO_4$, which is almost insoluble (<0.2 mg/l arsenic) and may be disposed of to a tailings dam (Dew *et al.*, 1997). Biooxidation of gold-bearing ores has proved to be a remarkably reliable process. Early concerns about the possibility of phage infection and process instability proved to be unfounded. A major drawback of the biooxidation compared with the physico-chemical pretreatment processes is that in many cases the consumption of cyanide in the subsequent gold-recovery process is considerably higher following biooxidation.

DEVELOPMENT OF GENETIC SYSTEMS FOR BIOMINING BACTERIA

There are several challenges in the development of genetic systems for the highly acidophilic, obligately autotrophic bacteria involved in biomining. These are suitable cloning vectors, selectable genetic markers and methods for getting DNA into the cells (Figure 7).

Plasmids

Because of their unique physiology and specialised ecological niche, there was a concern that these bacteria may have been genetically isolated from the more commonly studied bacteria for which cloning vectors are available. As a result, replicons from plasmids considered to be broad-host-range might not have been functional in these bacteria. Early studies concentrated on the use of indigenous plasmids (which are widespread in the thiobacilli) as cloning vectors. Several *T. ferrooxidans* plasmids have been cloned and some completely or partially sequenced (reviewed in Rawlings and Kusano, 1994). In general *T. ferrooxidans* plasmids have replicons that are closely related to those of more

commonly studied bacteria (Dorrington and Rawlings, 1990) and the broad-host-range plasmid replicons that have been tested in *T. ferrooxidans* and *T. thiooxidans* have been functional (Jin *et al.*, 1992; Peng *et al.*, 1994). Mobilisation functions of *T. ferrooxidans* plasmids are likewise related to those of plasmids of hospital bacteria isolates (Rawlings and Kusano, 1994). There are no reports on the frequency of plasmid occurrence in the leptospirilli and no broad-host-range plasmids have been tested in these bacteria.

Heterologous gene expression and selectable markers

It was not initially known whether genes from other bacteria would be expressed in the autotrophic thiobacilli or which genetic markers would be suitable. Phylogenetic studies based on 16S rRNA (Lane *et al.*, 1992), RecA (Karlin *et al.*, 1995) and ATP synthase sequence data (Brown and Rawlings, 1993) have indicated that *T. ferrooxidans* is closely related to bacteria such as *E. coli*, more so than might be expected given the differences in their physiology. Gene expression studies of *T. ferrooxidans* genes cloned in *E. coli* have confirmed that the majority of housekeeping genes from *T. ferrooxidans* that have been tested complement corresponding *E. coli* mutants (reviewed in Rawlings and Kusano, 1994). In one study the transcription start of the cloned *T. ferrooxidans* thioredoxin (*trxA*) gene in *E. coli* was found to be identical to the start of the same gene in *T. ferrooxidans* (Powles *et al.*, 1995). No complementation or chromosomal gene expression studies have been reported for either *T. thiooxidans* or members of the genus *Leptospirillum*.

There are strong indications that expression of heterologous genes in *T. ferrooxidans* and *T. thiooxidans* should not be difficult. In spite of this, finding suitable selectable markers for the genetic manipulation of *T. ferrooxidans* and *T. thiooxidans* has been problematic. One reason for this would appear to be that the low pH produced (when growing on sulfur media) and the presence of metal ions (when growing on iron media) results in the inactivation of most antibiotics. Another is that the bacteria are slow growing and the antibiotic becomes inactive during the 10- to 14-day incubation period before colonies appear. Kanamycin is the only selectable marker that has been successfully used on sulfur (Jin *et al.*, 1992; Peng *et al.*, 1994) and mercury resistance has been used on iron media (Kusano *et al.*, 1992). However, selection for mercury resistance was not completely effective and only about 50% of the colonies that grew on mercury plates possessed the gene for the selected marker. Presumably the long incubation period allowed for the non-biological reduction in mercury toxicity. Clearly, the choice of selectable markers is less than satisfactory. No selectable markers for use with the leptospirilli have been identified.

Gene delivery systems

Gene delivery systems have been reported for only two of the bacteria involved in bioleaching, *T. ferrooxidans* and *T. thiooxidans*. After many unsuccessful attempts to transform *T. ferrooxidans* by conjugation either directly from *E. coli* or via a less acidophilic facultatively heterotrophic *Thiobacillus* intermediate, the first report of success in transformation of the bacterium was by electroporation (Kusano *et al.*, 1992). More than 30 isolates of *T. ferrooxidans* were tested, only one of which was successfully transformed at a very low frequency using a mercury resistance marker. After many years work in several laboratories the transformation of both *T. ferrooxidans* (Peng *et al.*, 1994) and *T. thiooxidans* (Jin *et al.*, 1992) by conjugation directly from *E. coli* at a low frequency was eventually achieved. IncP or IncQ plasmids were used with kanamycin as the selective marker on solid media with a rather high initial pH of 4.6-4.8. The high plate pH was almost certainly the secret of success as the mating frequency from *E. coli* and the stability of kanamycin decreases rapidly with pH. No experiments on the transformation of leptospirilli have been reported. Since leptospirilli will only grow on iron media at a very low pH, conjugation directly from *E. coli* is unlikely to be successful and electroporation is the most promising option.

MOLECULAR STUDIES OF UNIQUE ASPECTS OF THE PHYSIOLOGY OF BIOMINING BACTERIA

An investigation into the molecular genetics and biochemistry of the systems associated with the ability of biomining bacteria to oxidise iron and sulfur in a highly acidic environment has yielded interesting findings (Appia-Ayme *et al.*, 1997; Blake *et al.*, 1992; Blake *et al.*, 1993; Cavazza and Bruschi, 1995; Yamanaka and Fukumori, 1995). These are also the properties of the bacteria that make them valuable from a commercial viewpoint. Since DNA probes for these genes or defined mutants lacking them were unavailable, these studies have been highly challenging. In

spite of this, considerable success has been achieved in an investigation of the iron oxidation system of *T. ferrooxidans*. Most if not all of the components comprising the iron oxidation electron transport chain have been identified (Figure 8), although the exact role of each component remains to be settled (Yamanaka and Fukumori, 1995). For example, the extensively studied small copper protein rusticyanin is considered to form part of the electron transport chain, but it was reported recently that the aporusticyanin acts as specific receptor which stimulates the adhesion of the bacterium to pyrite (Ohmura and Blake, 1997). Several of the genes involved in iron oxidation have been cloned and sequenced (Appia-Ayme *et al.*, 1997; Rawlings and Kusano, 1994; Yamanaka and Fukumori, 1995). A particularly interesting finding was that the ability to oxidise iron may have evolved independently on more

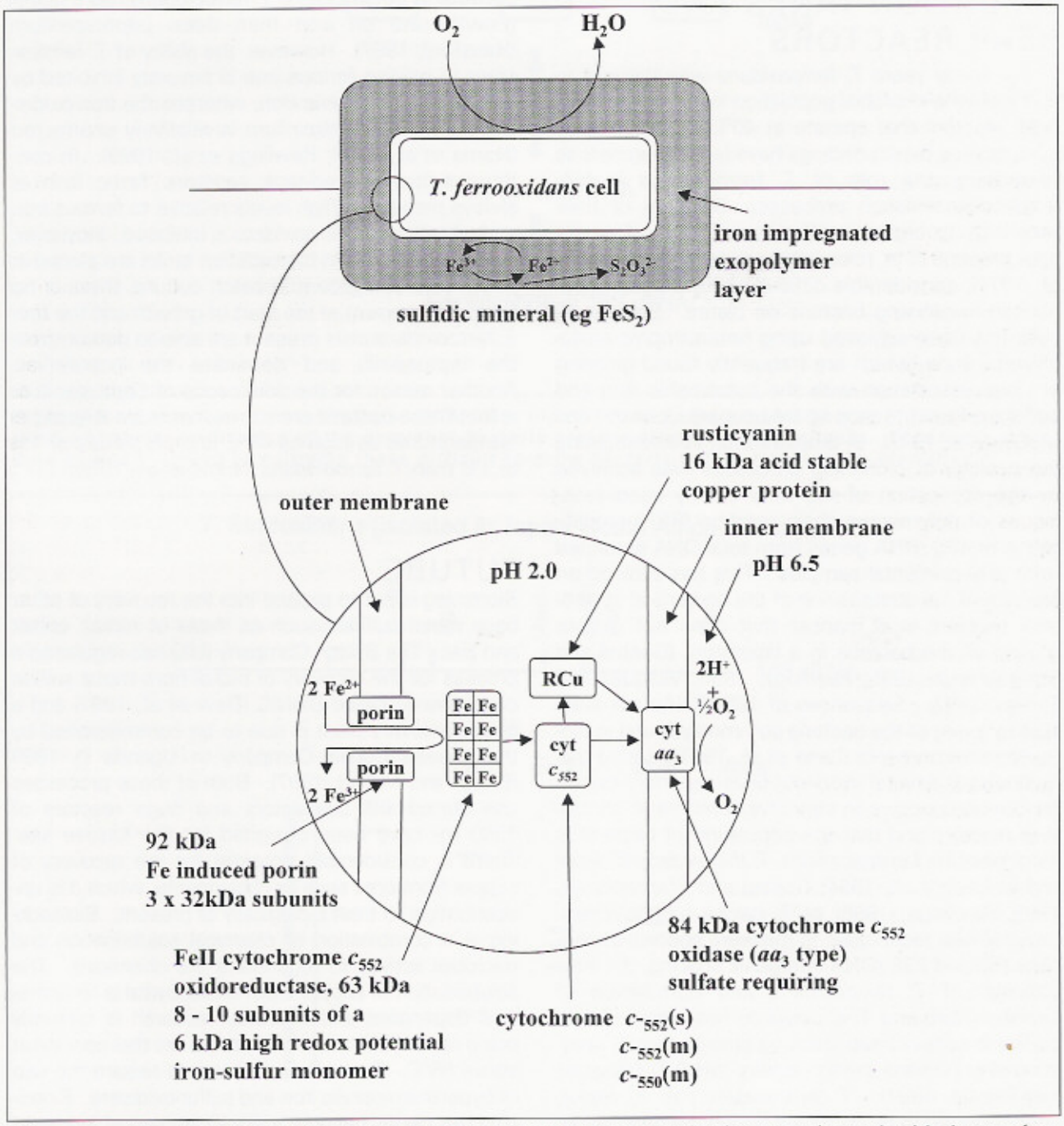


Figure 8. Diagram showing the cycling of ferrous and ferric iron in the exopolysaccharide layer of an iron-oxidising bacterium like *T. ferroxidans* attached to a mineral particle. A model of the path of electron transport from ferrous iron into the cell is shown in the expanded region.

than one occasion (Blake *et al.*, 1992; Blake *et al.*, 1993), since there seems to be very little in common between the components of the iron oxidation systems of *T. ferrooxidans*, *L. ferrooxidans* and some of the moderately thermophilic iron-oxidising bacteria.

ORGANISMS IN BIO-OXIDATION TANKS AND HEAP REACTORS

For many years *T. ferrooxidans* was thought to dominate the microbial population in stirred-tank and heap reactors that operate at 40°C or less. However, several recent findings have led researchers to re-evaluate the role of *T. ferrooxidans* in bioleaching/biooxidation processes. Because of their sensitivity to organic matter, including the free sugars present in or released from agar (Tuovinen *et al.*, 1971), considerable difficulties have been experienced in growing bacteria on plates. Some success has been achieved using heterotrophic acidophilic bacteria (which are frequently found growing in close association with the autotrophic iron and sulfur oxidisers) to mop up free sugars (Johnson and McGinness, 1993). A breakthrough in investigating the ecology of biomining processes was achieved by the application of the now widely used techniques of polymerase chain reaction (PCR) amplification of 16S rRNA genes from total DNA extracted from environmental samples. This has allowed an analysis of the composition of the bacteria in stirred-tank reactors in a manner that does not require growth of the bacteria in a laboratory (Goebel and Stackebrandt, 1995; Rawlings, 1995; Vásquez and Espejo, 1997). Sequences of 16S rRNAs are available for most of the bacteria commonly found in biomining environments (Lane *et al.*, 1991). Using this technology, several workers have reported that *T. ferrooxidans* occurs in very low numbers in stirred-tank reactors and that the population of bacteria is dominated by *Leptospirillum*, *T. thiooxidans* (Goebel and Stackebrandt, 1994; Goebel and Stackebrandt, 1995; Rawlings, 1995) or *T. caldus* (unpublished). Using similar techniques to measure species-dependent 16S and 23S rDNA intergenic spacing, the near absence of *T. ferrooxidans* and dominance of *Leptospirillum* and *T. thiooxidans* has also been reported in copper heap leaching environments, operating in conditions of high acidity (pH 0.7) (Vásquez and Espejo, 1997). *T. ferrooxidans* has, however, been reported to be dominant during the heap leaching of copper ores to which ferrous iron had been added (Pizarro *et al.*, 1996) and is frequently the dominant bacterium in the drainage from mining wastes (Schippers *et al.*, 1995).

An important question is why researchers believed that *T. ferrooxidans* was the dominant bacterium in stirred-tank reactors for so long. Part of the answer is that when samples from biooxidation tanks were grown on soluble iron media in batch culture, *T. ferrooxidans* outgrew its iron-oxidising competitors and dominated the population. This is because in batch culture *T. ferrooxidans* has a faster growth rate on iron than does *Leptospirillum* (Hansford, 1997). However, the ability of *T. ferrooxidans* to oxidise ferrous iron is severely inhibited by the presence of ferric iron, whereas the iron-oxidising ability of *Leptospirillum* is relatively unaffected (Norris *et al.*, 1998; Rawlings *et al.*, 1999). In continuous-flow stirred-tank reactors ferric iron is always present at high levels relative to ferrous iron and growth of *T. ferrooxidans* is inhibited. However, when samples from biooxidation tanks are placed in iron media and grown in batch culture, there is no ferric iron present at the start of growth and the few *T. ferrooxidans* cells present are able to outcompete the leptospirilli and dominate the population. Another reason for the dominance of *Leptospirillum* is that these bacteria are somewhat more tolerant of temperatures as high as 40°C and pH values of 1.4 to 1.6 than *T. ferrooxidans* (Norris *et al.*, 1988).

FUTURE

Biomining is set to expand into the recovery of other base metal sulfides such as those of nickel, cobalt and zinc. The Billiton Company (UK) has registered a process for the recovery of nickel from metal sulfide concentrates called BioNIC (Dew *et al.*, 1997) and a cobalt recovery plant is due to be commissioned by the Kasese Cobalt Company in Uganda in 1999 (Briggs and Millard, 1997). Both of these processes use stirred-tank bioreactors and huge reactors of 1380 m^3 have been designed for the Kasese site. There is considerable potential for the recovery of copper from ores such as chalcopyrite, which it is uneconomical to treat biologically at present. Bioleaching is a combination of chemical solubilisation and microbial activity to regenerate the chemicals. The solubilisation of copper from chalcopyrite is temperature dependent and intensive research is currently being carried out to develop processes that operate at above 65°C. Such processes would require the use of hyperthermophilic iron and sulfur-oxidisers. Biomining has clearly become a significant part of industrial microbiology and its application is likely to increase still further during the coming century.

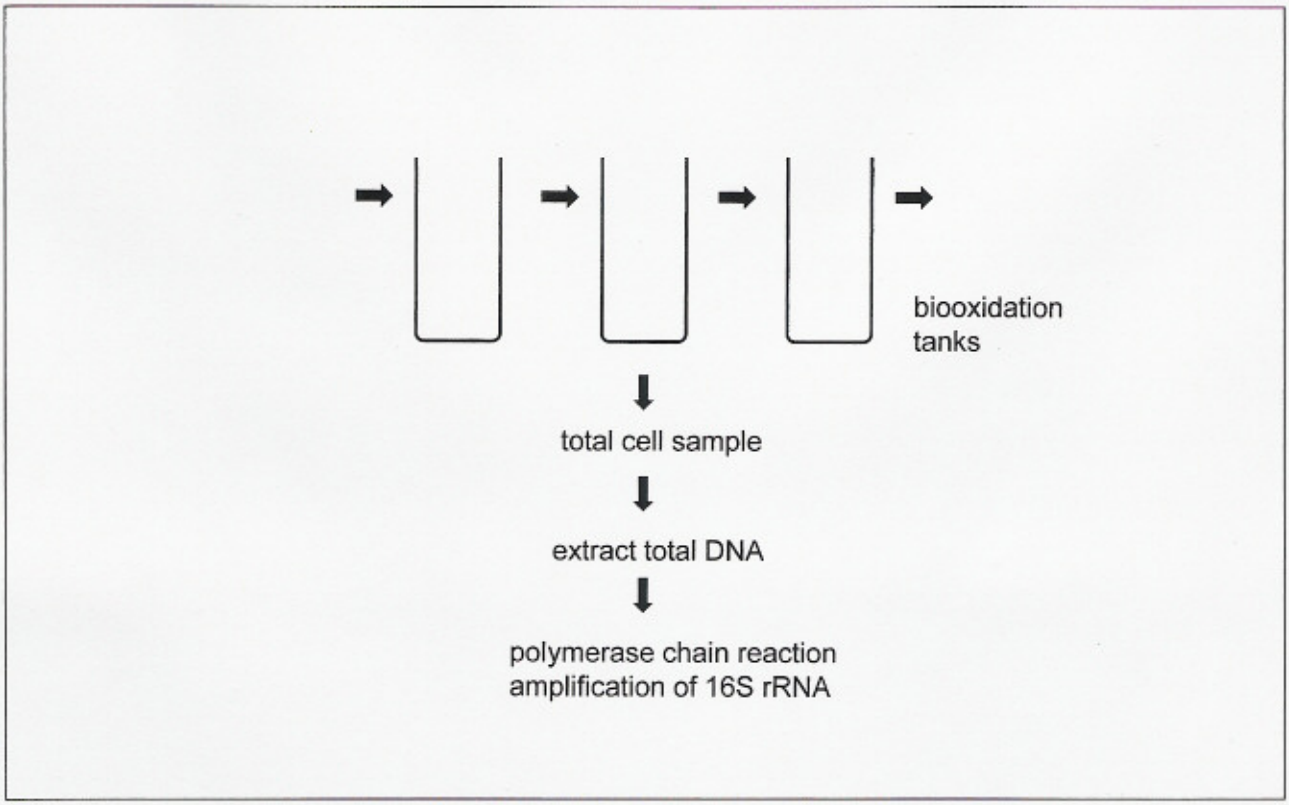


Figure 9. Extraction of total cellular DNA from biooxidation tanks enables the amplification of 16S rRNA and the identification of the dominant bacteria present directly from the tanks. This rapid procedure avoids the need to cultivate these difficult-to-grow bacteria before they can be identified.

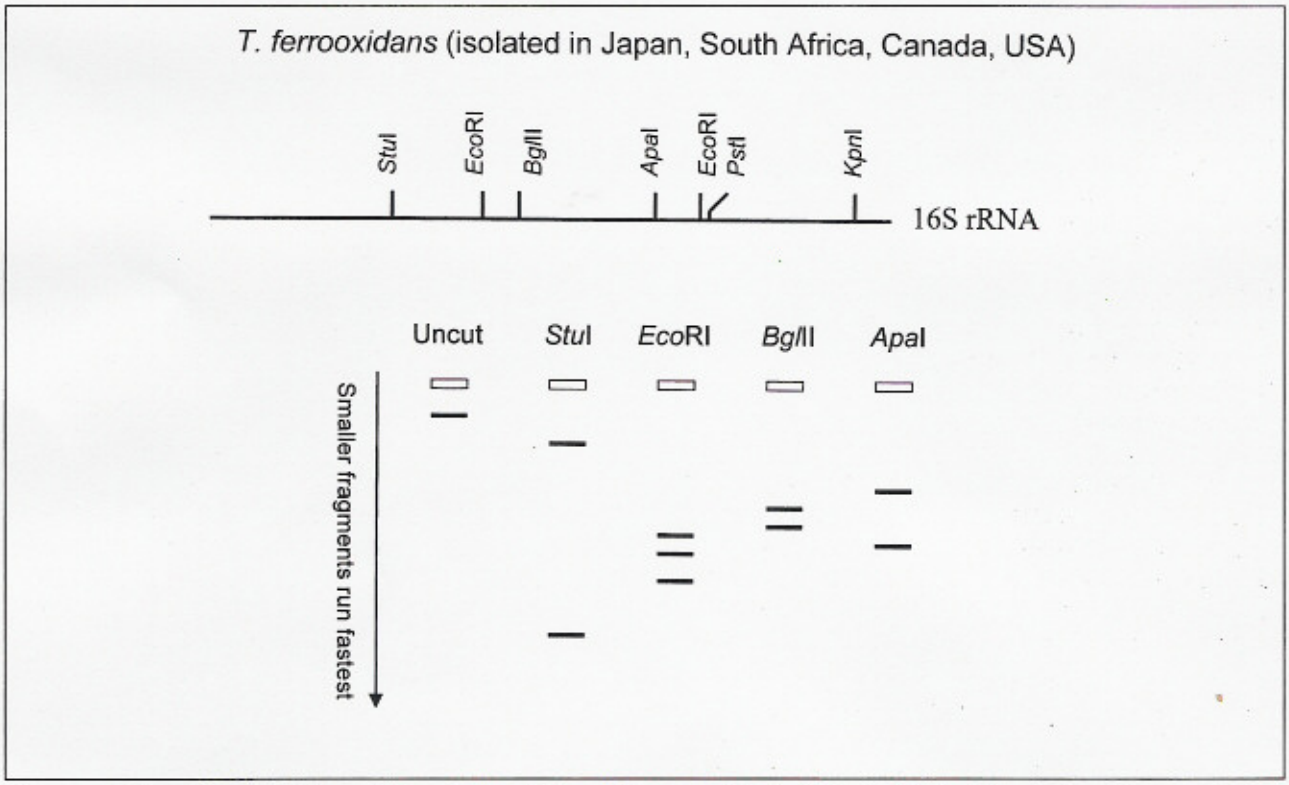


Figure 10. Identification of bacteria. When the amplified 16S rRNA is digested with restriction enzymes and the fragments separated according to size, a pattern of fragments characteristic of a particular type of bacterium is obtained.

ACKNOWLEDGEMENTS

No person is an island and numerous people have had a significant impact on my development as a scientist. In particular I wish to thank Professor David Woods, current Vice-Chancellor of Rhodes University, for his great enthusiasm for the subject of microbiology and microbial genetics. From him I learned what research was about, how to write scientific articles and we later had some wonderful years together as colleagues.

I also wish to thank colleagues such as Frank Robb, Dave Jones, Jenny Thomson, Ed Rybicki, Helen Garnett, Ralph Kirby and several others who provided important stimulation as I learned from them and as we learned together. I wish to thank past PhD research students who worked with me on the subject of biomining bacteria, including Eugenia Barros, Inge-Martine Pretorius, Raj Ramesar, Rosemary Dorrington, Dave Berger, Jurg Rohrer, Lynn Brown, Ros Powles, Cliff Dominy and Ant Smith. I must also acknowledge past Masters students such as Maureen Dennehy, Carol-Anne Kilkenny and Joseph Oppon, and current students Murray Gardner, Nicolette Coram and Bronwyn Butcher. I also thank post-doctoral research associates Nozibeli Mjoli and Shelly Deane, particularly Shelly who has worked with me for over 8 years.

No research is possible without financial support and in this regard I wish to thank my long-term industrial sponsors, whose name has changed from Genmin to Gencor and back on more than one occasion before being bought by Billiton in 1997, who are my current sponsors. I also thank the FRD, the University of Cape Town and now the University of Stellenbosch for the start-up funds required to re-establish a research group here. I thank this University for appointing me as a Professor of Microbiology and I trust that it will not be long before this group is as productive as it has been in the past.

A simple thanks is insufficient to express my appreciation of my wife Janet, our children Barry, Kim and Sarah-Jane, my parents as well as other family and friends for their never-ending support. Finally, I acknowledge that I have been blessed by God in ways that are too many to count.

LITERATURE CITED

APPIA-AYME C, BENGRINE A, CHIPPAUX M & BONNEFOY V, 1997. C4-type cytochrome *c*552 encoding gene from *Thiobacillus ferrooxidans*: cloning, characterization and expression in *Escherichia coli*. In: IBS Biomine '97. Australian Mineral Foundation, Glenside, Australia. pp. P2.1-10.

BLAKE RC & MCGINNESS S, 1993. Electron-transfer proteins of bacteria that respire on iron. In: TORMA AE, APEL ML & BRIERLEY CL (eds.) Biohydrometallurgical Technologies, Vol II. TMS Press, Warrendale, Pennsylvania. pp. 616-628.

BLAKE RC, SHUTE EA, WASKOVSKY J & HARRISON AP, 1992. Respiratory components in acidophilic bacteria that respire on iron. *Geomicrobiol. J.* **10**: 173-192.

BRIERLEY CL, 1982. Microbiological mining. *Scientific American* **247** (2): 42-51.

BRIERLEY CL, 1997. Mining biotechnology: research to commercial development and beyond. In: Biomining: Theory, Microbes and Industrial Processes. RG Landes & Springer-Verlag, Berlin. pp. 3-17.

BRIGGS A & MILLARD M, 1997. Cobalt recovery using bacterial leaching at the Kasese project, Uganda. In: IBS Biomine '97. Australian Mineral Foundation, Glenside, Australia. pp. M2.4.1-12.

BROWN LD & RAWLINGS DE, 1993. A comparison of the structure of the H^+-translocating ATP synthase from *Thiobacillus ferrooxidans* with those of other organisms. In: TORMA AE, ML APEL & BRIERLEY CL (eds.) Biohydrometallurgical Technologies, Vol II. TMS Press, Warrendale, Pennsylvania. pp. 519-528.

CAVAZZA C & BRUSCHI M, 1995. Iron oxidation by *Thiobacillus ferrooxidans*: characterization of two electron transfer proteins. In: JEREZ CA, VARGAS T, TOLEDO H & WIERTZ JV (eds.) Biohydrometallurgical Processing, Vol II. University of Chile Press, Santiago. pp. 97-107.

CLAASEN R, LOGAN CT & SNYMAN CP, 1993. Biooxidation of refractory gold-bearing arsenopyritic ores. In: TORMA AE, WEY JE & LAKSHMANAN VI (eds.) Biohydrometallurgical Technologies, Vol I. TMS Press, Warrendale, Pennsylvania. pp. 479- 488.

DEW DW, LAWSON EN & BROADHURST JL, 1997. The Biox process for biooxidation of gold-bearing concentrates. In: RAWLINGS DE (ed.) Biomining: Theory, Microbes and Industrial Processes. RG Landes & Springer-Verlag, Berlin. pp. 45-80.

DEW DW & MILLER DM, 1997. The BioNIC process; bioleaching of mineral sulphide concentrates for recovery of nickel. In: IBS Biomine '97. Australian Mineral Foundation, Glenside, Australia. pp. M7.1.1-9.

DORRINGTON RA & RAWLINGS DE, 1990. Characterization of the minimum replicon of the broad-host-range plasmid pTF-FC2 and similarity between pTF-FC2 and the IncQ plasmids. *J. Bacteriol.* **172**: 5697-5705.

DROLET M, ZANGA P & LAU PCK, 1990. The mobilization and origin of transfer regions of a *Thiobacillus ferrooxidans* plasmid: relatedness to plasmids RSF1010 and pSC101. *Mol. Microbiol.* **4**: 1381-1391.

FRANZMANN PD AND WILLIAMS TL, 1997. Biomass and microbial community composition in an arsenopyrite oxidation bioreactor determined by phospholipid-derived fatty acid methyl ester composition and content. In: IBS Biomine '97. Australian Mineral Foundation, Glenside, Australia. pp. QP1.1-10.

GOEBEL BM & STACKEBRANDT E, 1994. Cultural and phylogenetic analysis of mixed microbial populations found in natural and commercial bioleaching environments. *Appl. Environ. Microbiol.* **60**: 1614-1621.

GOEBEL BM & STACKEBRANDT E, 1995. Molecular analysis of the microbial biodiversity in a natural acidic environment. In: JEREZ CA, VARGAS T, TOLEDO H & WIERTZ JV (eds.) Biohydrometallurgical Processing, Vol II. University of Chile, Press, Santiago. pp. 43-51.

HALLBERG KB & LINDSTRÖM EB, 1994. Characterization of *Thiobacillus caldus* sp nov., a moderately thermophilic acidophile. *Microbiology* **140**: 3451-3456.

HANSFORD GS, 1997. Recent developments in modelling the kinetics of bioleaching. In: RAWLINGS DE (ed.) Biomining: Theory, Microbes and Industrial Processes. RG Landes & Springer-Verlag, Berlin. pp. 153-175.

JIN SM, YAN WM & WANG ZN, 1992. Transfer of IncP plasmids to extremely acidophilic *Thiobacillus thiooxidans*. *Appl. Environ. Microbiol.* **58**: 429-430.

JOHNSON DB & MCGINNESS S, 1991. A highly efficient and universal solid medium for growing mesophilic and moderately thermophilic, iron-oxidizing, acidophilic bacteria. *J. Microbiol. Methods* **13**: 113-122.

KARLIN S, WEINSTOCK GM & BRENDEL V, 1995. Bacterial classifications derived from RecA protein sequence comparisons. *J. Bacteriol.* **177**: 6881-6893.

KELLY DP & HARRISON AP, 1989. Genus *Thiobacillus* Beijerinck, Vol 3. In: STALEY JT, BRYANT MP, PFENNIG N & HOLT JG (eds.) Bergey's Manual of Systematic Bacteriology. The Williams & Wilkins Co., Baltimore. pp. 1842-1858.

KELLY DP, NORRIS PR & BRIERLEY CL, 1979. Microbiological methods for the extraction and recovery of metals. In: BULL AT, DG ELLWOOD & C RATLEDGE (eds.) Microbial technology: Current State and Future Prospects. Cambridge University, Press, Cambridge. pp. 263-308.

KUSANO T, SUGAWARA K, INOUE C, TAKESHIMA T, NUMATA M & SHIRATORI T, 1992. Electrotransformation of *Thiobacillus ferrooxidans* with plasmids containing a *mer* determinant as the selective marker by electroporation. *J. Bacteriol.* **174:** 6617-6623.

KUSANO T, TAKESHIMA T, SUGAWARA K, INOUE C, SHIRATORI T, YANO Y, FUKUMORI Y & YAMANAKA T, 1992. Molecular cloning of the gene encoding *Thiobacillus ferrooxidans* Fe(II) oxidase. *J. Biol. Chem.* **267**: 11242-11247.

LANE DJ, HARRISON AP Jr., STAHL D, PACE B, GIOVANNONI SJ, OLSEN GJ & PACE NR, 1992. Evolutionary relationships among sulfur- and iron-oxidizing eubacteria. *J. Bacteriol.* **174**: 269-278.

LIVESEY-GOLDBLATT E, NORMAN P & LIVESEY-GOLDBLATT DR, 1983. Gold recovery from arsenopyrite/pyrite ore by bacterial leaching and cyanidation. In: ROSSI G & TORMA AE (eds.) Recent Progress in Biohydrometallurgy. Azzociazione Mineraria Sarda, Iglesias, Sardinia. pp. 627-641.

LUNDGREN DG & SILVER M, 1980. Ore leaching by bacteria. *Annu. Rev. Microbiol.* **34:** 263-283.

MCCREADY RGL & GOULD WD, 1990. Bioleaching of uranium. In: ERLICH HL & BRIERLEY CL (eds.) Microbial mineral recovery. McGraw-Hill, New York. pp. 107-126.

MILLER PC, 1997. The desgn and operating practice of a bacterial oxidation plant using moderate thermophiles (the BacTech process). In: RAWLINGS DE (ed.) Biomining: Theory, Microbes and Industrial Processes. RG Landes & Springer-Verlag, Berlin. pp. 81-102.

NORRIS PR, 1990. Acidophilic bacteria and their activity in mineral sulfide oxidation. In: ERLICH HL & BRIERLEY CL (eds.) Microbial mineral recovery. McGraw-Hill, New York. pp. 3-27.

NORRIS PR, 1997. Theromphiles and bioleaching. In: RAWLINGS DE (ed) Biomining: Theory, Microbes and Industrial Processes. RG Landes & Springer-Verlag, Berlin. pp. 247-258.

NORRIS PR, BARR DW & HINSON D, 1988. Iron and mineral oxidation by acidophilic bacteria: affinities for iron and attachment to pyrite. In: NORRIS PR & KELLY DP (eds.) Biohydrometallurgy, Science and Technology Letters. Kew, Surrey. pp. 43-59.

NORRIS PR, MURRELL JC & HINSON D, 1995. The potential for diazotrophy in iron- and sulfur-oxidizing acidophilic bacteria. *Arch. Microbiol.* **164:** 294-300.

OHMURA N & BLAKE II R, 1997. Aporusticyanin mediates the adhesion of Thiobacillus ferrooxidans to pyrite. In: IBS Biomine '97. Australian Mineral Foundation, Glenside, Australia. pp. PB.1-10.

PENG J-B, YAN W-M & BAO X-Z, 1994. Plasmid and transposon transfer to *Thiobacillus ferrooxidans*. *J. Bacteriol.* **176:** 2892-2897.

PIZARRO J, JEDLICKI E, ORELLANA O, ROMERO J & ESPEJO RT, 1996. Bacterial populations in samples of bioleached copper ore as revealed by analysis of DNA obtained before and after cultivation. *Appl. Environ. Microbiol.* **62:** 1323-1328.

POWLES RE, DEANE SM & RAWLINGS DE, 1995. Molecular genetic analysis of a thioredoxin isolated from *Thiobacillus ferrooxidans*. *Microbiology* **141**: 2175-2181.

PRETORIUS I-M, RAWLINGS DE & WOODS DR, 1986. Identification and cloning of *Thiobacillus ferrooxidans* structural *nif* genes in *Escherichia coli*. *Gene*. **45**: 59-65.

RAWLINGS DE, 1995. Restriction enzyme analysis of 16S rRNA genes for the rapid identification of *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans* and *Leptospirillum ferrooxidans* strains in leaching

environments. In: JEREZ CA, VARGAS T, TOLEDO H AND & WIERTZ JV (eds.) Biohydrometallurgical Processing, Vol II. University of Chile Press Santiago. pp. 9-17.

RAWLINGS DE, 1997. Mesophilic, autotrophic, bioleaching bacteria; description, physiology and role. In: RAWLINGS DE (ed.) Biomining: Theory, Microbes and Industrial Processes. RG Landes & Springer-Verlag, Berlin. pp. 229-245.

RAWLINGS DE, TRIBUTSCH H & HANSFORD GS, 1999. Reasons why *Leptospirillum*-like species rather than *Thiobacillus ferrooxidans* are the dominant iron-oxidizing bacteria in many commercial processes for the biooxidation of pyrite and related ores. *Microbiology* **145**: 5-13

RAWLINGS DE & KUSANO T, 1994. Molecular genetics of *Thiobacillus ferrooxidans*. *Microbiol. Rev.* **58**: 39-55.

RAWLINGS DE & SILVER S, 1995. Mining with microbes. *Bio/Technology* **13**: 773-778.

RAWLINGS DE & WOODS DR, 1995. Development of improved biomining bacteria. In: GAYLARDE CG & VIDELA HA (eds.) Bioextraction and biodeterioration of metals. Cambridge University Press, Cambridge. pp. 63-84.

SCHIPPERS A, R HALLMANN, S WENTZIEN & W SAND, 1995. Microbial diversity in uranium waste heaps. *Appl. Environ. Microbiol.* **61**: 2930-2935.

SCHNELL HA, 1997. Bioleaching of copper. In: RAWLINGS DE (ed.) Biomining: Theory, Microbes and Industrial Processes. RG Landes & Springer-Verlag, Berlin. pp. 21-43.

SUGIO T, DOMATSU C, MUNAKATA O, TANO T & IMAI K, 1985. Role of a ferric ion-reducing system in sulfur oxidation of *Thiobacillus ferrooxidans*. *Appl. Environ. Microbiol.* **49**: 1401-1406.

TUOVINEN OH, NIEMELÄ SI & GYLLENBERG HG, 1971. Tolerance of *Thiobacillus ferrooxidans* to some metals. *Antonie van Leeuwenhoek* **37**:489-496.

TUOVINEN OH, NIEMELÄ SI & GYLLENBERG HG, 1971. Effect of mineral nutrients and organic substances on the development of *Thiobacillus ferrooxidans*. *Biotech. Bioeng.* **13**: 517-527.

WHITLOCK JL, 1997. Biooxidation of refractory gold ore, the Geobiotics process. In: RAWLINGS DE (ed.) Biomining: Theory, Microbes and Industrial Processes, RG Landes & Springer-Verlag, Berlin. pp. 117-127.

VAN ASWEGEN PC, GODFREY MW, MILLER DM & HAINES AK, 1991. Developments and innovations in bacterial oxidation of refractory ores. *Minerals and Metallurgical Processing* **8**: 188-192.

YAMANAKA T & FUKUMORI Y, 1995. Molecular aspects of the electron transfer system which participates in the oxidation of ferrous iron by *Thiobacillus ferrooxidans*. *FEMS Microbiol. Rev.* **17**: 401-413.

VÁSQUEZ M & ESPEJO RT, 1997. Chemolithotrophic bacteria in copper ores leached at high sulfuric acid concentration. *Appl. Environ. Microbiol.* **63**: 332-334.