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Biodiversity and stress response to extremophilic prokaryotes isolated from the Escondida Copper Mine, Chile

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Biodiversity and stress response of extremophilic prokaryotes isolated from the Escondida copper mine, Chile.

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by

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Abstract

Heap bioleaching technology has been successfully applied at an industrial scale for over 30 years, and is generally considered to be the most effective engineering option to bio-process low-grade copper sulfide ores. Heap bioleaching was established at the Escondida mine in Chile in 2006, and culture-independent analysis of liquid samples had previously provided some information of the composition of the indigenous microbial communities. In the current project, six prokaryotes belonging to three bacterial genera and one archaeal genus were isolated from the heap bioleaching plant at Escondida and studies carried out comparing their responses to those of known species and strains of mineral-degrading bacteria to some environmental stress factors, in order to understand how the indigenous microflora are able to successfully colonize and exploit the bioheaps. Stress factors examined including elevated concentrations of heavy metals and osmotic potentials. One of the isolates, identified as a strain of *Acidithiobacillus ferrooxidans,* was able to grow at more extreme conditions of pH and temperature than reported for most other strains of this species. Copper tolerance observed in a *Leptospirillum ferriphilum* isolate was found to be comparable with other strains of this important mineral-oxidizing acidophile, and far greater than in strains of other *Leptospirillum* spp. (*L. ferrooxidans* and "*L. ferrodiazotrophum"*), which helps to explain why only *L. ferriphilum* rather than other species is found in this (and many other) copper bioleach operation, even though the relatively low temperatures might favour other species. Studies carried out on osmotic stress response by several mineral-degrading acidophiles identified the disaccharide trehalose as a major organic osmo-protectant synthesized by several unrelated species of bacteria, including *L. ferriphilum, At. ferrooxidans, Sulfobacillus thermosulfidooxidans* and *Acidiphilium* sp. SJH. In contrast, the major organic solute that accumulated within the sulfur-oxidizer *At. thiooxidans* in response to salt stress was the monosaccharide glucose, together with smaller amounts of proline. Micro-representational difference analysis (MRDA) was used to elucidate the genetic response of a *L. ferriphilum* isolate to copper-induced and osmotic stress. Data obtained suggested that certain metabolisms related to lipopolysacharide synthesis, transport systems and nucleic acid processing were activated in response to these stress factors.

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To my family and the memory of my grandparents and friend.

Content

List of Tables

Table 6.2. Analysis of the translated gene products of differentially expressed transcripts identified by MRDA analysis using BLASTP at NCBI and BLASTP against a private database of the annotated genome sequence of *L. ferriphilum* Sp-Cl.

List of Figures

Figure 5.3 Overlay of chromatograms obtained using the AminoPac PA10 column of trehalose (blue line), glucose (pink line) and the hypo-osmotic shock extracts from *L. ferriphilum* IESL25 grown in the presence of 300 mM of magnesium sulfate (black line). The latter shows a peak with the same retention time of glucose, and was not detected in ethanol extracts as shown in Figure 5.2.

Figure 5.4. Overlay of chromatograms (using the CarboPac MA1 column) of ethanol extracts obtained from *At. thiooxidans* IESL33 cultures grown at increasing concentrations of magnesium sulfate. (HBS indicates non-supplemented medium).

Figure 5.5 Overlay of chromatograms of extract obtained of *At. thiooxidans* IESL33 grown in the presence of 500 mM of magnesium sulfate (black line) and a chromatogram of the same extract after acid hydrolysis (blue line).

Figure 5.6 Overlay of chromatograms of trehalose standard 0.1 mM (pink line) and hypo-osmotic extracts obtained from cultures of *At. ferrooxidans* IESL32 containing 300 mM magnesium sulfate (blue) and in non-amended medium (black). Chromatograms were obtained from cultures grown with ferrous iron (A) or sulfur (B) as electron donors.

Figure 5.7 Overlay of chromatograms obtained from hypo-osmotic shock extracts of cultures of *Sb. thermosulfidooxidans*^{T} grown in iron-yeast extract medium (blue line), and in the same medium amended with 300 mM of magnesium sulfate (black line).

Figure 5.8 Overlay of chromatograms of hydrolyzed extracts obtained from cultures of *At. ferrooxidans* IESL32 (black line) and *Sb. thermosulfidooxidans*^{T} (blue line) grown in media amended with 300 mM magnesium sulfate.

Figure 5.9 Overlay of chromatograms obtained from cultures of *Acidiphilium* sp SJH grown in galactose medium (black line), and the same medium amended with 200 mM sodium chloride (blue line) or 300 mM of magnesium sulfate (pink line).

Figure 5.10 Chromatograms obtained of ethanol extracts of cells harvested from samples from: (A) PLS from the full-scale heap bioleaching process of Escondida; (B) PLS from a column leach test; (C) Culture from a 7 L pilot-scale reactor.

Figure 6.1 Growth doubling times calculated for cultures of *L. ferriphilum* IESL25 in standard laboratory medium and medium amended with either copper sulfate or magnesium sulfate at the same concentrations (160 mM).

Figure 6.2. Agarose (2% w/v) gel electrophoresis stained with ethidium bromide of nested PCR products obtained after application of MRDA protocol. The tester cDNA used for each subtractive hybridization experiment is indicated above the respective lane. The bands indicated by lettering are described in the text and correspond to the initial amplicons obtained by MRDA, sequences were later confirmed not to be false positives by quantification by qPCR (6.3.3).

Figure 6.3 Expression of gene transcripts detected by MRDA during growth of *L. ferriphilum* IESL25 under a variety of stresses as indicated relative to expression in cells grown under control conditions (e.g. without Cu or Mg stress).

Figure 6.4. Genomic context found in *L. ferriphilum* Sp-Cl, "*L. rubarum"*, *Leptospirillum* sp. "5 way CG" and "*L. ferrodiazotrophum"*, including the protein annotated as major facilitator family transporter, similar to peptide deduced from sequence of band c from *L. ferriphilum* IESL25 (*).

Figure 6.5. Genomic context found in *L. ferriphilum* Sp-Cl, "*L. rubarum"*, *Leptospirillum* sp. "5 way CG" and "*L. ferrodiazotrophum"*, including the protein annotated as Transcription-repair coupling factor, similar to peptide deduced from sequence of band d retrieved from *L. ferriphilum* IESL25 (*). Genes indicated with discontinuous arrow lines differed in the annotation of "*L. rubarum"*, *Leptospirillum* sp. "5 way CG" and "*L. ferrodiazotrophum"*. In the latter genomes the sequence annotated PpiC-type peptidyl-prolyl cis-trans isomerase and Cytidylyltransferase family protein were annotated instead of SurA and ADP-heptose synthase, respectively.

Figure 6.6. Genomic context found in *L. ferriphilum* Sp-Cl, "*L. rubarum"*, *Leptospirillum* sp. "5 way CG" and "*L. ferrodiazotrophum"*, including the protein annotated as Polyribonucleotide nucleotidyl transferase, similar to peptide deduced from sequence of band e retrieved from *L. ferriphilum* IESL25 (*).

Figure 6.7. Genomic context showing the possible peptide deduced from strain IESL25 band a (*) in *L. ferriphilum* Sp-Cl (A), "*L. rubarum"* and *Leptospirillum* sp. "5 way CG" (B) and "*L. ferrodiazotrophum"* (C).

Figure 6.8. Genomic context showing the possible peptide deduced from band b of IESL25 (*) in *L. ferriphilum* Sp-Cl (A), "*L. rubarum"* and *Leptospirillum* sp. "5 way CG" (B).

Figure 6.9. Genomic context found in *L. ferriphilum* Sp-Cl, "*L. rubarum"*, *Leptospirillum* sp. "5 way CG" and "*L. ferrodiazotrophum*," including the protein annotated as hypothetical protein, similar to peptide deduced from sequence of band c retrieved from *L. ferriphilum* IESL25. * indicates the protein assumed similar to *L. ferriphilum* IESL25 is indicated.

Abbreviation List

16S rRNA	Small subunit ribosomal ribonucleic acid
A.	*Acidiphilium*
ABS	Autotrophic basal salts
AD	*Anno Domini*
ADP	Adenosine diphosphate
Am	*Acidimicrobium*
AMD	Acid Mine drainage
At.	*Acidithiobacillus*
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
BLASTP	Basic Local Alignment Search Tool for protein similarity
bp	base pair
cDNA	copy DNA
DGGE	Denaturing Gradient Gel Electrophoresis
dNTP	Deoxyribonucleic triphosphate
DDHV	DNA-DNA hybridisation value
DOC	Dissolved organic carbon
DNA	Deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DTSC	Dye Terminator Cycle Sequencing
e.g.	*exempli gratia* (in example)
Eq.(s)	equation(s)
Feo	Ferrous iron overlay solid media
FeSo	Ferrous iron/tetrathionate overlay solid media
Fig.	Figure
Fp.	*Ferroplasma*
g.	gram
-GC	GC clump
g/L	grams per litre
h	hour
HBS	Heterotrophic basal salts
IESL	Isolate from Escondida sulphide leach plant
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
iFeo	Inorganic iron overlay solid media
kV	Kilovolts
kg	Kilogram
Ltd.	Limited
L.	*Leptospirillum*
M	Molar
MALDI-TOF	Matrix-assisted laser desorption/ionization time of flight
MFS	Major facilitator superfamily
mg	milligram
min.	minute
ml	millilitre
mM	millimolar
MRDA	Micro-Representational difference analysis
mRNA	messenger RNA
mV	millivolt

m/z	Mass charge ratio
µg	microgram
µl	microlitre
µM	micromolar
NCBI	National Center for Biotechnology Information
ND	Not determined
nM	nanomolar
NS	no signal
PAR	4-(2-pyridylazo) resorcinol
PC	Personal computer
PCR	Polymerase chain reaction
PFGE	Polymorphism fragment gel electrophoresis
PF	Pregnant leach solution from strip
PLS	Pregnant leach solution
p.s.i.	pounds per square inch
qPCR	Quantitative real time PCR
r^2	Linear correlation coefficient
RAPD	Randomly amplified polymorphic DNA Analysis
rcf	Relative centrifugal force
RNA	Ribonucleic acid
rpm	Revolutions per minute
Sb.	*Sulfobacillus*
sp./spp.	species
sec.	second
SX-EW	Solvent extraction-electrowinning
TE.	Trace elements
TSB	Tryptone soy broth
U.K.	United Kingdom
U.S.A.	United States of North America
v	volume
V	Volts
w	weight
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter I: General Introduction

I.1 The development of mineral bioprocessing technologies ("biomining")

Biomining has a long history, although the early miners were not aware that microorganisms were involved in the process. One of the earliest records of the practice of copper leaching is from the island of Cyprus. Galen, a naturalist and physician, reported in AD 166 the operation of what is now known as "*in situ"* leaching of copper. In the process, water was allowed to percolate through the permeable rock, and collected in amphorae (large earthenware vessels). The percolation solution was allowed to evaporate until copper sulfate was crystallised. Similar practice was carried out in Spain, as reported by Pliny (23-79 AD). An ancient mine in which microorganisms seemed to play a role in leaching metals is the deposit located in the Iberian pyrite belt in the south-west of Spain and southern Portugal, the most famous area of which is located in the Huelva province - the Río Tinto ("red river"). In this site Bronze-age people recovered silver and copper, and later Romans recovered copper and gold. The name derived from the colour imparted to the water by the high concentration of ferric iron. The ferric iron dissolved in the Río Tinto is due, at least in part, to natural phenomena, as acidic, iron-rich water is found upstream of the large (and currently abandoned) Rio Tinto mine complex. The microbial dissolution of sulfide minerals generates acidity and results in transition metals and aluminium being present in solution, causing the water to be toxic to animals (and many other life forms), as evidenced by early records of the Río Tinto being undrinkable and devoid of fish (Hazen and Tabak, 2005; Rawlings 2004).

An early form of heap leaching (though not bioleaching as such) of copper sulfides on an industrial scale was carried out at the Rio Tinto mine in Spain in about 1752. The ore was crushed and laid on a gently sloping impervious pad. Layers of

ore were alternated with beds of wood. Once the heap was constructed, the wood was ignited, resulting in the roasting of copper and iron sulfides. Water was then distributed over the top of the heap. As the water percolated through the heap, the copper and iron dissolved, forming copper and iron sulfates. In 1888, this method of extraction was prohibited by law, because of serious environmental damage caused by the clouds of sulfur dioxide formed. The reason for the success of this technique was unknown, but it was thought to be due to 'some obscure quality either of the Rio Tinto ore or the Spanish climate'.

In the late 1940s, a novel bacterium was isolated from acidic mine water draining a coal mine in the Appalachian mountains in the U.S.A.. The bacterium was found to be acidophilic and capable of oxidizing reduced sulfur. However, in contrast to a bacterium (*Acidi)thiobacillus thiooxidans* that also had these physiological characteristics, the new isolate was also shown to oxidize ferrous iron. The bacterium was named as *Thiobacillus ferrooxidans* (Colmer et al., 1950), and its characterisation was followed by the isolation of bacteria that had similar physiological traits (*Ferrobacillus ferrooxidans* and *Ferrobacillus sulfooxidans*), though these were later re-classified as strains of *Thiobacillus ferrooxidans* (Kelly and Tuovinen, 1972) and the species itself reclassified as *Acidithiobacillus (At.) ferrooxidans* from phylogenetic analysis by (Kelly and Wood, 2000). *At. ferrooxidans* was shown to be able to accelerate the oxidative dissolution of pyrite (FeS_2) and, in 1954, of copper sulphides (Beck, 1967). Shortly after this, the first deliberate (as opposed to inadvertent) application of biomining was devised. In 1958, the Kennecott Mining Company patented the use of *At. ferrooxidans* for copper extraction using the acidophile to extract copper from low-grade run-of-mine copper ore at the Bingham mine, Utah, U.S.A, in a "dump leaching" operation (Zimmerley et al., 1958). Dump leaching low grade (typically less than 0.5% by wt.) copper-containing rocks that would otherwise be classified as waste has since that time proliferated in many parts of the world (Brierly, 2008). In the 1970s, *in situ* leaching of worked-out uranium

mines was practised in Canada (Johnson, 2010). This approach has much in common with “pre-1950s” biomining, in that it involves deliberate flooding of underground mines, removal of the metal-enriched flood water and recovery of the target metal that has been solubilised by bacterial activity (*via* ion exchange in the case of uranium, though for copper this was usually by cementation: $Cu^{2+} + Fe^{0} \rightarrow Cu^{0} + Fe^{2+}$).

Major advances in the engineering aspects of biomining came in the 1980s with the development of thin-layer heaps, and stirred-tank bioleaching/bio-oxidation processes (Fig. 1.1). The first industrial bioprocess designed for bioleaching ore containing 1 to 2 % copper exploited solely by bacterial technology was initiated in the mid-1980s by Minera Pudahuel (Chile), and produced 14,000 tonnes of fine copper per year. This process involved crushing and agglomeration of the ore which was stacked on waterproof pads as heaps 6-10 m in height, which were aerated (from below) and irrigated from above. After the start-up of Pudahuel process, several other copper bioleaching operations were established in Chile. Heap bioleaching has also been applied successfully in other countries such as Peru, Australia, United States, Myanmar and China (Watling, 2006). Usually mineral leaching heaps are built as discrete units on pads or by continuous addition. Ore stacked as a single irrigation unit is known as a “lift”, and heaps or lifts are often stacked on top of one another. Heap/lift heights vary, and are typically between 6 and 12 m. The ore coming from the mine is typically crushed to 25 mm and may be agglomerated with acid and/or raffinate, (solution coming from solvent extraction step) and stacked. Aeration is injected through pipes laid out underneath the heap, aeration rates vary from 0.08 to 2 m^3 of air/h per m^2 of heap. Irrigation using raffinate solution is typically done through drip lines spread over the heap surface, and irrigation rates vary from 5 to 20 L of raffinate/h per m^2 of heap. The (copper)-enriched liquor generated (“pregnant leach solution”: PLS) is usually processed by solvent extraction and electrowinning to recover the metal (Fig. 1.1).

In operations at high altitude in the desert (e. g. in the Chilean Andes) the heap is covered with plastic sheets as radiation shields for heat retention during the cold nights. Thermo-blankets are used to conserve heat in the Talvivaara heap leaching operation in Finland. Heap cycle times vary depending on operational practice, mineral grade and composition and usually are in the order of 8 months to 2 years. Copper recovery depends, among other things, on the mineralogy of the ore, and for ore containing predominantly chalcocite, the recovery is usually around 80%. Active inoculation of heaps with bacteria is not a common practice, but occurs usually in the agglomeration step with bacteria included in the raffinate. Alternatively bacterial populations present on the ore develop and thrive as the heap progresses.

Stirred-tank bio-processing of minerals was developed in South Africa, and is now used in many parts of the world. This engineering approach is the most efficient currently used in biomining, in terms of both speed and efficiency, though the greater costs involved in constructing and maintaining the vast bioreactors (up to 1,350 m^3) means that they have are mostly been operated as full-scale commercial systems for recovering gold rather than base metals. A notable exception is a bioleaching plant in Kasese, Uganda, where cobalt is the target metal. Most commercial stirred tank biomining systems operate at pH of about 1.5 and temperatures between 40 ° and 45 °C. Since the oxidative dissolution of sulfide minerals is an exothermic reaction, cooling rather than heating of the tanks is required, constituting (along with stirring of the dense mineral slurries - 20 % pulp density is typical) a significant part of the running costs of any single operations. Stirred tank configurations can vary from single to multiple tank systems. Nutrients (N and P) are added to promote growth of the mineral-oxidizing microorganisms.

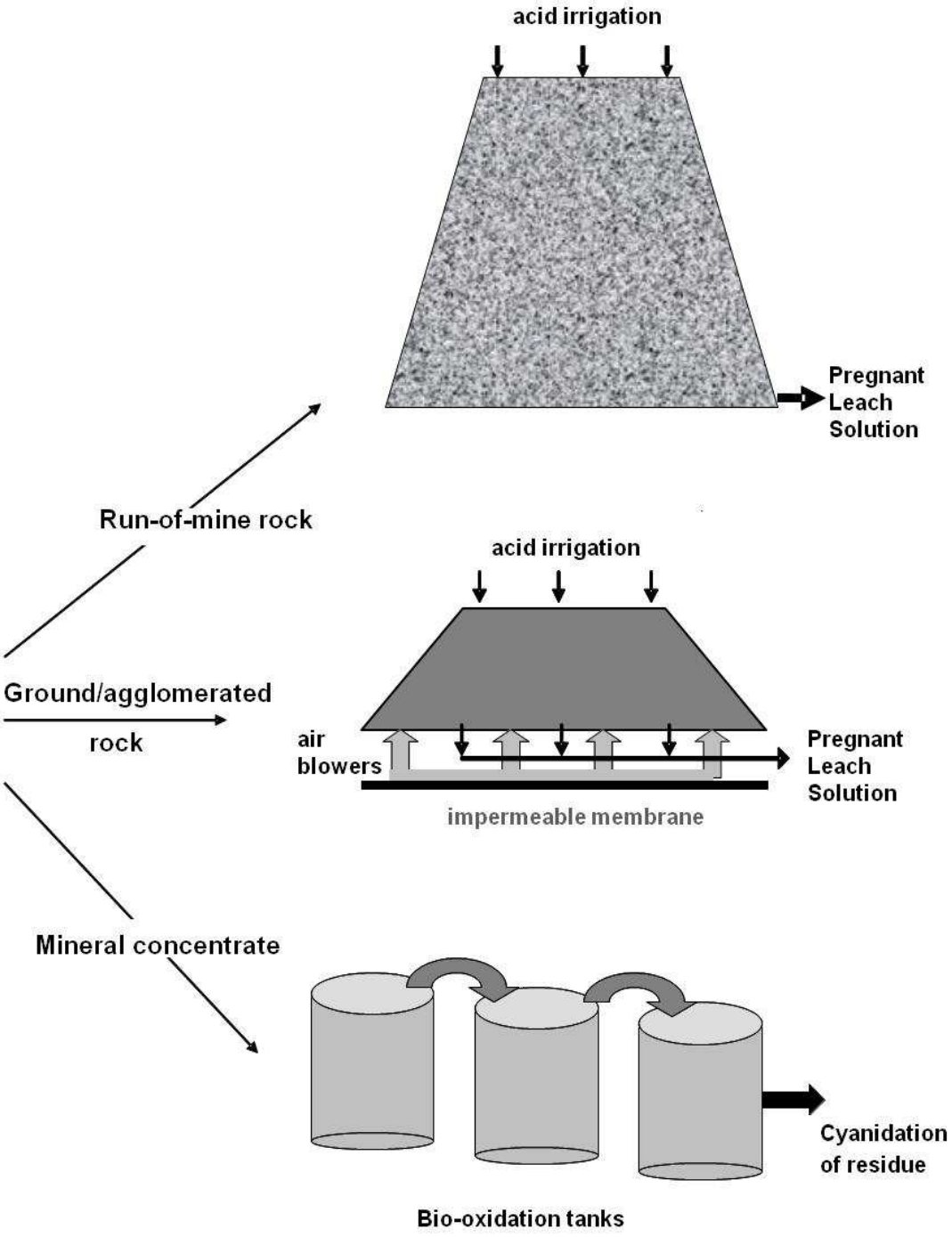


Figure 1.1. Major process options used in biomining. Top, dump bioleaching; middle, heap bioleaching; bottom, bio-oxidation in stirred-tanks (Johnson, 2010).

The isolation of thermophilic sulfur-oxidizing microorganisms from hot springs by James Brierley and by Tom Brock in the late 1960's provided the opportunity of operating stirred tank reactors at more elevated temperatures (60-80 ℃). Virtually all bioleaching species able to thrive at high temperature are archaea rather than bacteria. Archaea found in industrial mineral bioprocessing operations include *Sulfolobus* spp., *Metallosphaera* spp., and *Acidianus* spp. In the copper mining industry the thermophile bioleaching process represents an alternative to treat chalcopyrite and arsenic-bearing concentrates. Due to environmental restrictions imposed to smelters, concentrates containing levels above 5,000 mg/kg of As are not accepted for production of copper or other metals. The bioleaching of a chalcopyrite concentrate using acidophilic thermophiles has been successfully demonstrated at Chuquicamata, Chile (Batty and Rorke, 2006). High temperatures can also develop in bioheaps, which improves their performances particularly in cold locations. One such example is the Talvivaara Sotkamo Mine, located in central Finland, which has been operating since 2008 in sub-arctic environmental conditions. Here the ore body being processed is a polymetallic black schist that contains Ni, Zn, Co and Cu as sulfides, and about 13% pyrrhotite ($Fe_{(1-x)}S$ (x = 0 to 0.2)). The oxidation of pyrrhotite proceeds relatively rapidly in the presence of oxygen and is highly exothermic. In a test heap set up at Talvivaara, the temperature of the PLS was found to quickly be >50 ^{o}C. The microbial population in the Talvivaara heaps includes mesophilic acidophiles as well as some moderate thermophiles *At. caldus*, *Sulfobacillus thermosulfidooxidans*; the role of extreme thermophiles has not, as yet, established (Riekkola-Vanhanen, 2007).

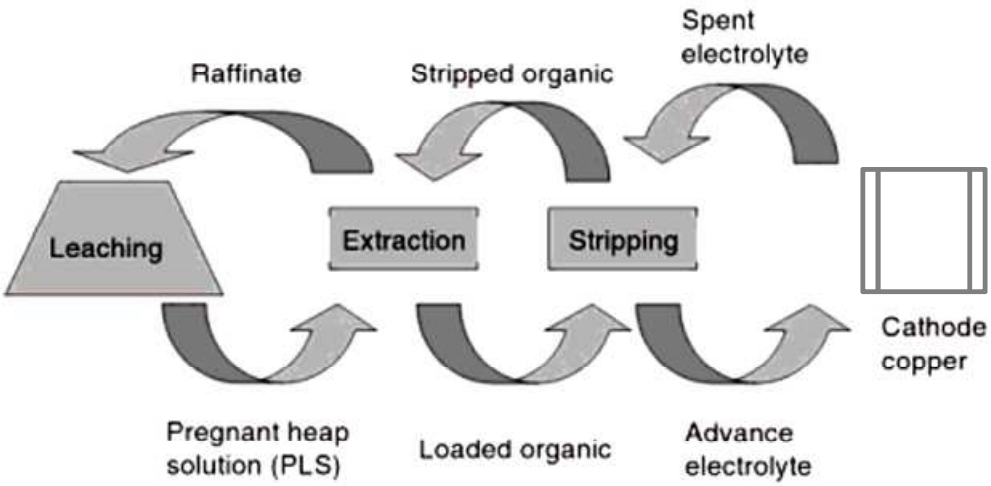


Figure 1.2. Stages in the recovery of bioleached copper in a typical heap leaching operation process (taken from Johnson, 2010).

I.2 World demand and production of copper

Global demand for copper has grown by just below 4%, while global copper mine supply was expected to grow by less than 2% in 2011 (*Wall Street Journal Commodities*, *"Xstrata Says Copper Demand Continues to Outpace Supply"* September 19, 2011). This increased demand is due to the fact that more people are able to acquire goods such as cars, refrigerators, air conditioners and others containing copper. In addition, countries with rapid developing economies such as China require copper for infrastructure growth. After two decades of copper prices just exceeding production costs, it is expected that actual and future copper prices will be highly profitable, at least until 2020. According to preliminary data from the *International Copper Study Group*, global growth in copper demand for 2011 is expected to exceed global growth in copper production, with a production deficit of about 200,000 metric tonnes of refined copper expected for the year (The Daily Telegraph, *"Copper demand continues to exceed supply*", 9th of October 2011). The

increase of copper production during the past decades has consumed most of the high-grade copper ore, and thus low-grade ore is increasingly becoming commonly mined. In this scenario bioleaching becomes a suitable technology to treat such type of ores.

I.3 Heap bioleaching as a technology to treat low-grade copper ore

Since the 1980's, the use of heap bioleaching to extract copper from low-grade sulfide ore has been adopted and developed in Chile and at number of other countries. However, the efficiency of the process is relatively low (Brierly and Brierly, 1999). The growing interest in heap bioleaching process of copper can be explained by the fact, that large amounts of low-grade copper ore contain secondary sulfides, such as chalcocite (Cu_2S) and conventional (pyrometallurgical) processing of these ores is not economically viable.

Bioleaching has been applied successfully to mixed low-grade sulfide ores notably in Chile at the mines Lo Aguirre, Ivan, Punta del Cobre, Andacollo, Dos Amigos, Lomas Ballas, Lince II, Cerro Colorado, Quebrada Blanca, Zaldivar, Chuquicamata (as dump leaching), at and Escondida. Since 1990, 100 million tons of low-grade copper ore has been stored at Escondida which would be considered as non productive material without heap bioleaching technology. The successful of the application of this technology is based on the low investment and relatively simple operation. In addition, metal-rich solutions from copper bioleaching processes (PLS) are readily connected to the circuit solution of oxide heap leach processes, and afterwards the soluble copper is easily recovered by solvent extraction and electrowinning (SS-EW; Fig. 1.2).

Despite the importance of this process, the microbial ecology and physiology involved in this are poorly understood and few attempts have been made to

understand and to control the biological components (Brierly and Brierly, 2001). It has been acknowledged that the understanding of the ecological-microbiological processes involved in heap bioleaching is the key to advance in commercial operations (Brierly and Brierly, 2001).

I.4 The industrial bioleaching process at the Escondida copper mine, Chile

The huge reserve and characteristics of the low-grade sulfide deposit present at the Escondida mine resulted in heap bioleaching being chosen as the most appropriate approach to extract copper from this ore. Prior to the establishment of this technology at an industrial scale (in 2006), the process had been studied (since 2000) at two preliminary stages including a pilot plant and a demonstration heap. After six years of development, the first copper cathode was produced at the industrial plant using bioleaching. Although the bioleaching process at Escondida uses heaps, as does the leaching of the high-grade oxide ore, the design of the bioleaching heaps does not involve crushing or curing of the run-of-mine ore, which has an important bearing on the establishment of the microbial population in the process. Crushing produces homogeneity in the size of the ore particles, increases the permeability and liquid-solid contact area, and also improves the hydraulic characteristics of the heap. On the other hand, curing prevents acid consumption by gangue materials, so that once irrigation of the heap commences, the acid contained in the irrigation solution is intended to facilitate the solubilisation of the copper sulfide ore, instead of gangue. Despite the benefits of crushing and curing, application to the low-grade sulfide ore at Escondida was considered to be uneconomical, due to the vast quantity of material that would have to be processed. The resulting

characteristics of the run-of-mine ore heaps at Escondida have influenced the composition and dynamics of the microbial population within them.

While the bioleaching operation at the Escondida mine is considered to be successful as an economic enterprise, and has achieved a significant reduction in the cost of producing metallic copper, the efficiency of copper extraction is still rather low, often recovering (depending on the mineralogy of the ore) less than 50% of total copper contained in the ore, whereas dump operations, such as Chuquicamamta dump leaching recovers less than 15% (Domic, 2007).

I.5 Mechanisms involved in the bio-oxidation of sulfide minerals

The principal mechanism involved in bioleaching is the oxidation of ferrous iron to ferric iron and the reduction of oxygen to water in a proton-consuming reaction (eq. 1). This process is used as energy source by some microorganisms, such as *Acidithiobacillus* spp., *Leptospirillum* spp. and others.

$$4Fe^{2+} + O_2 + 4H^+ \longrightarrow 4Fe^{3+} + 2H_2O \quad \text{(eq. 1).}$$

Ferric iron is a powerful oxidizing agent, which can mediate the oxidative dissolution of sulfide minerals, such as pyrite. In this reaction, the ferric iron is reduced to ferrous and an oxidized form of sulfur (e.g. the oxy-anion, thiosulfate) is formed. Re-generation of the ferric iron by iron-oxidizing bacteria allows the process to continue. The reduced sulfur product(s) are oxidized by sulfur-oxidizing bacteria and archaea (some of which also oxidize ferrous iron) ultimately to sulfuric acid maintaining the acid levels that are required for the microorganisms to thrive. Carbon-degrading (heterotrophic and mixotrophic) acidophiles are also usually found in association with the iron- and sulfur-oxidizing acidophiles (which are mostly autotrophic) giving rise to bioleaching consortia (Rawlings and Johnson, 2007) (Fig. 1.3).

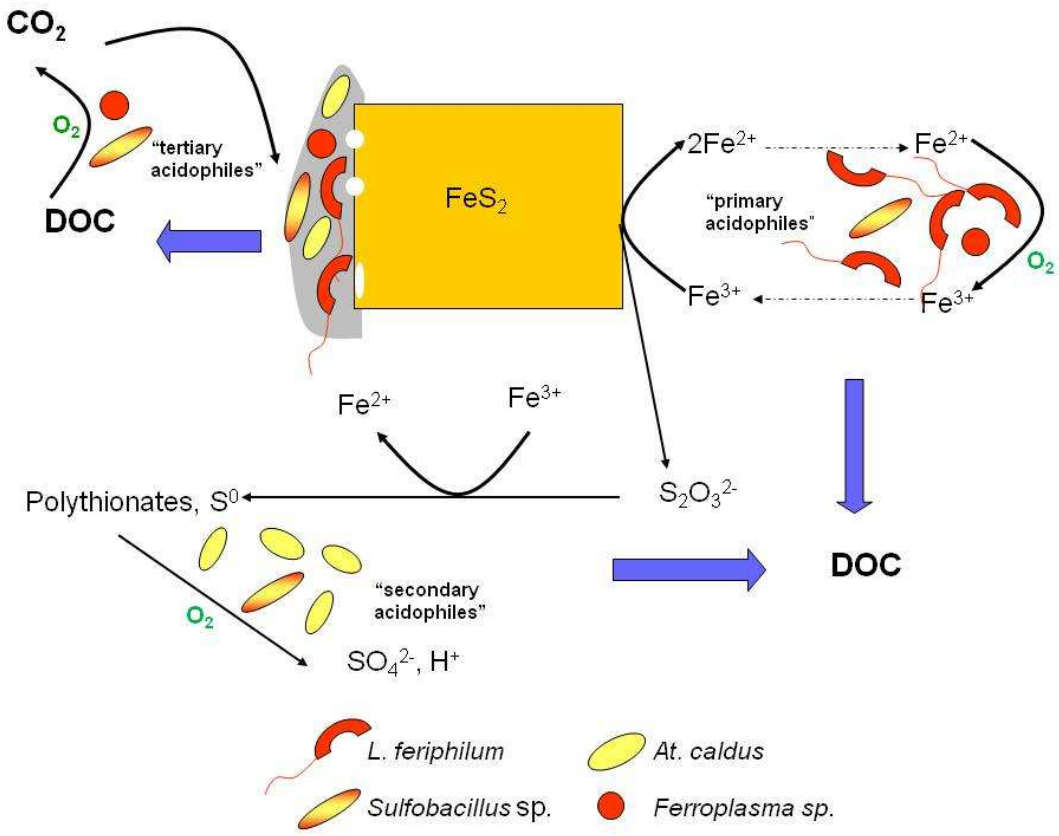


Figure 1.3. The oxidative dissolution of pyrite in acidic liquors, showing the major microorganisms identified in the biooxidation of refractory gold concentrates and the bioleaching of a cobaltiferous pyrite concentrate processed at *ca*. 40˚C. Those reactions that consume oxygen are highlighted; "DOC" is dissolved organic carbon (Johnson, 2010).

Another sulfide mineral of importance in copper mining is chalcocite and it can also be oxidized by ferric iron. The oxidation of chalcocite occurs in two stages (eqs. 2 and 3).

$$Cu_2S + 1.6Fe^{3+} \longrightarrow 0.8Cu^{2+} + 1.6Fe^{2+} + (Cu_{1.2}S) \quad \text{(eq. 2)}$$

In the first reaction, about 40% of initial copper is released and a covellite (CuS)-like material is produced. However this is not a stable compound under such conditions, and a number of intermediates are formed, from $Cu_{1.97}S$ to $Cu_{1.12}S$, as the reaction progresses. Subsequently the covellite-like material reacts as follows:

$$(Cu_{1.2}S) + 2.4Fe^{3+} \longrightarrow 1.2\ Cu^{2+} + 2.4Fe^{2+} + S^0 \quad \text{(eq. 3)}$$

The kinetics of the two stages are quite different. The first stage (eq. 2) occurs rapidly even at ambient temperature while the rate of stage 2 (eq. 3) is slow at ambient temperature (Petersen and Dixon, 2007).

There are many other primary and secondary sulfides with more complex oxidation mechanisms, Table 1.1 listed some of the more important metal sulfides with economical and environmental importance. Wolfgang Sand and co-workers have subdivided metal sulfides into those that are “acid-soluble” and which can, at least in theory, be solubilised by bacteria, such as *At. thiooxidans* and *At. caldus,* that oxidize reduced forms of sulfur to sulfuric acid, and “acid-insoluble” sulfides that are attacked by powerful oxidants such as ferric iron, and thereby are susceptible to being degraded only by iron-oxidizing prokaryotes, such as *Leptospirillum* spp. and *Ferroplasma* spp.. Acid-soluble sulfide minerals include sphalerite, galena, arsenopyrite, chalcopyrite, and hauerite (ZnS, PbS, FeAsS, $CuFeS_2$, and MnS_2, respectively), while acid-insoluble metal-sulfides include pyrite, molybdenite, and tungstenite (FeS_2, MoS_2, and WS_2, respectively) (Rohwerder et al., 2003).

Table 1.1. Metal sulfides of economic and environmental importance.

Iron sulfides	
Pyrite	FeS_2
Marcasite	FeS_2
Pyrrhotite	$Fe_{0.8}S$ - FeS
Greigite	Fe_3S_4
Other single metal/metalloid sulfides	
Argentite	Ag_2S
Chalcocite	Cu_2S
Cinnabar	HgS
Covellite	CuS
Digenite	Cu_9S_5
Galena	PbS
Millerite	NiS
Molybdenite	MoS_2
Realgar	AsS
Sphalerite	ZnS
Stibnite	Sb_2S_3
Mixed metal/metalloid sulfides	
Arsenopyrite	FeAsS
Bornite	Cu_5FeS_4
Chalcopyrite	$CuFeS_2$
Cobaltite	CoAsS
Pentlandite	$(Fe/Ni)_9S_8$

Although heap bioleaching appears to be simple in concept, several processes taking place as the solution trickles through the ore bed are complex, and their interactions are not well understood. Processes occurring in bioheap systems can be distinguished ranging from the macro- to microscales. The macro-scale is represented by the complete heap, where leaching is governed by transport of mass and energy into, through and out of the structure, including, solution flow, heat flow, gas flow.

The next level is represented by a cluster of particles, at this level three processes contribute to the overall rate of leaching, gas uptake (CO_2 and O_2), bacterial growth (propagation and oxidation) and inter- and intra-particle diffusion. Finally at the single grain level, leaching is governed by the chemical and electrochemical interactions at the mineral surface. The multiple processes at different levels tend to interact with each other in complex patterns and any of the listed process can become overall rate controlling under certain circumstances, and control can be switched from one domain to another in the course of one particular leach scenario. This makes diagnosing the effects of certain variables on overall heap performance virtually impossible without sophisticated modelling tools (Petersen and Dixon, 2007).

I.6 Microorganisms involved in copper heap bioleaching

It is known that some species of bacteria and archaea play an important role in the mobilization and mineralization of iron in the environment (Murad and Fisher, 1988). Microorganisms have participated in geochemical cycles of elements in the earth's crust since geologically ancient times. Some prokaryotes can gain energy from the oxidation of inorganic substrates and, as a consequence, the environment is modified, especially in its mineralogical composition. One important role of microorganisms in bioleaching process is the oxidation of ferrous iron. The average concentration of Fe in the earth´s crust is 5.09 mass%. Microorganisms currently used in commercial bioleaching operations (both stirred tank and bio-assisted heap leaching) are ubiquitous in nature. Wherever a suitable ore is exposed to the surface and water is present, such microorganisms are generally to be found occurring naturally. Microorganisms used in commercial mining operations are the same as those found in nature; the only difference is that in some cases they have been

selected for rapid growth on the ore or concentrate concerned (Rawlings and Johnson, 2007). It is known that growth of biomining microorganisms can be inhibited or prevented in the presence of organic matter (Alexander et al., 1987; Fang and Zhou, 2005). Some heterotrophs can be found (Goebel and Stackebandt, 1994; Bruhn et al., 1999; Demergasso et al., 2005; Hawkes et al., 2006; Bryan et al., 2007) that use the organic carbon derived from the primary-producers (mostly the chemolithophic population) as well as extraneous sources. The microorganisms involved in biomining operations require iron or reduced sulfur as an energy source and grow optimally at pH < 2.5. These extreme conditions or requirements seemingly prevent the growth of the microorganisms on plants, insects or animals. They are therefore non-pathogenic and there have been no reports of illness due to these prokaryotes. Three groups of prokaryotic microorganisms involved in copper bioleaching can be distinguished on the basis of their temperature growth range and optima. This division is not exclusive and some microorganisms can show overlap between temperature ranges or to be active over a wide temperature range.

I.6.1 Mesophiles

Mesophilic acidophiles are generally considered to be those that grow at temperatures between 20 and 40 ^{o}C (Johnson and Hallberg, 2008). Important primary (iron-oxidizing) bacteria involved in mineral oxidation are iron-oxidizing *Acidithiobacillus* and *Leptospirillum* spp. , though some strains of the latter can grow above 40^{o}C and have been described as “thermo-tolerant” acidophiles. The *Acidithiobacillus* genus is the most studied of all acidophilic prokaryotes. Species belonging to this genus are Gram-negative, autotrophic, acidophilic, and all can use as elemental sulfur, sulfides and reduced inorganic sulfur compounds as energy sources (electron donors). Currently there are five validated species of this genus: *At. ferrooxidans*, *At. thiooxidans*, *At. caldus*, *At. albertensis* and *At. ferrivorans*. *At.*

ferrooxidans and *At. ferrivorans* differ from the other species in being able to oxidize ferrous iron as well as reduced sulfur. Based on 16S rRNA gene sequence, four phylogenetic groups of iron-oxidizing acidithiobacilli can be distinguished. Previous phylogenetic analysis (Karavaiko et al., 2003) had shown high similarity level between 16S rRNA gene sequences of species isolated from different regions of the world, indicating a common evolutionary history. The same report showed that strains of this species isolated from different habitats exhibited considerable genotypic diversity, which manifested itself as a noticeable divergence of their 16S rRNA gene sequences, a low level of similarity for total DNA and significant differences in the structure of chromosomal DNA revealed by PFGE. Figure 1.4 illustrates the phylogenetic groups of iron-oxidizing acidithiobacilli, according to Amouric et al. (2011). Group I iron-oxidizing acidithiobacilli are all strains of the well-known species *At. ferrooxidans* and those of group III are from the psychro-tolerant species *At. ferrivorans* (Hallberg et al. 2010), while Groups II and IV have not yet been delineated as separate species and are generally still referred to as strains of *At. ferrooxidans*.

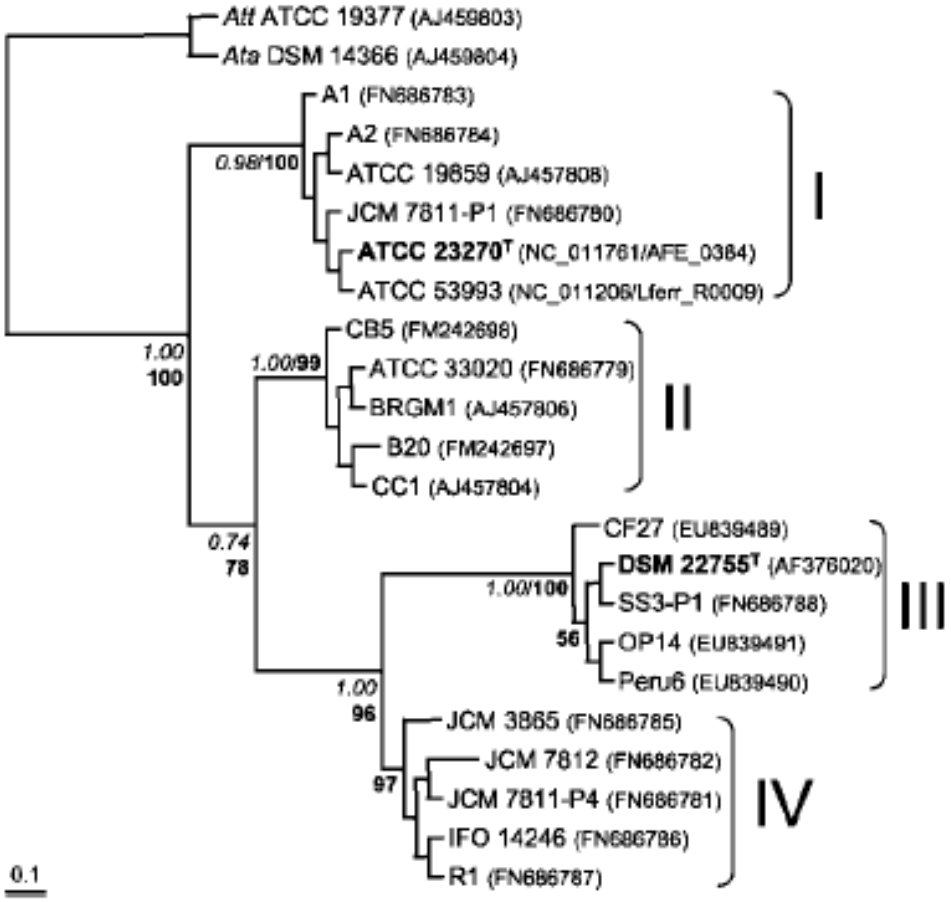


Figure I.4. Phylogenetic affiliation of 16S rRNA gene sequences of iron-oxidizing *Acidithiobacillus* (Amouric et al., 2011). Four phylogenetic groups can be distinguished. Organisms are shown by strain name and 16S rRNA sequence accession number for NCBI database are indicated between brackets.

Leptospirillum spp. are Gram-negative, strictly aerobic, chemolithoautotrophic and acidophilic. Based on phylogenic analyses they belong to the *Nitrospira* phylum, a deeply-branching group within the domain *Bacteria*. *Leptospirillum* spp. are known only to be able to couple the oxidation of ferrous iron to the reduction of oxygen in acidic liquors. However, they display higher substrate (ferrous iron) affinities and tolerance to ferric iron (and therefore high redox potentials) than *At. ferrooxidans* which, together with their generally greater tolerance to moderately thermal

temperatures, enables them to outcompete the latter in many mineral bio-processing operations (Rawlings et al., 1999). Recent phylogenic studies based on 16S rRNA sequences of *Leptospirillum* spp. have shown that these may be divided into three groups: Group I (*L. ferrooxidans*), Group II (*L. ferriphilum* and the candidate species "*L. rubarum*"), and Group III, currently represented by the non-validated species "*L. ferrodiazotrophum"* (Goltsman et al., 2009). *L. ferrooxidans* and *L. ferriphilum* differ in the copy numbers for *rrn* genes, with 3 copies of the gene present in *L. ferrooxidans* and 2 copies in *L. ferriphilum*. The G+C content and the similarity of 16S rRNA sequences also show clear differences between those two species. Strains of *L. ferriphilum* are able to grow to pH 1.4 to 1.8 and temperatures between 30 to 37 ºC, and some species can grow at 45 ºC (Coram and Rawlings, 2002). Using genomic and proteomic information the species name "*Leptospirillum rubarum*" has been used to refer a subgroup of sequences very similar to group II (Goltsman et al., 2009). Group III of the *Leptospirillum* genus is represented by a single, currently non-validated species, "*L. ferrodiazotrophum"*, which was isolated from acid mine drainage at Iron Mountain using a combination of the information obtained from metagenomes and culture conditions. Metagenome information indicated the presence of nitrogen fixation genes, related to *Leptospirillum* Group III, then medium containing no soluble N sources was used to enrich and to obtain a pure culture in liquid phase available at the *American Type Culture Collection* as strain ATCC BAA-1181 (Tyson et al., 2005). *"L. ferrodiazotrophum*" was also isolated directly from Iron Mountain AMD by plating on a selective overlay medium (D. B. Johnson, unpublished data).

Recently, using genomic and proteomic tools, a fourth *Leptospirillum* group, related to Group III has been proposed. Members of this group have been speculated to play an active role in the anaerobic metabolism of an acidophilic community found at Iron Mountain in California U.S.A, due to unique hydrogenase proteins. However only 50% of the genome has been retrieved from samples (Goltsman, 2011).

Studies in bioleaching systems, such as percolation and steady-state continuous-flow tank, stacked column or heap-type reactors (Rawlings et al., 1999), and industrial heap bioleaching of copper (Demergasso et al., 2005), have shown the presence and, in some cases, the dominance of *Leptospirillum* Group II bacteria, rather than Groups I and III. *Leptospirillum.* Group I and III have been found in acid mine drainage and natural acidic environments such as Parys Mountain in Wales (Coupland and Johnson, 2004), Iron Mountain in United States (Edwards et al., 1999) and the Rio Tinto in Spain (González-Toril et al., 2003). One of the differences between acid mine drainage and copper bioleaching systems is the higher concentrations of metals, such as copper, in the latter, and it is possible that the lack of Group I and Group III *Leptospirillum* in mineral heaps and PLS is due to their greater sensitivity (than Group II) to one or more transition metals, such as copper.

Ferroplasma spp. are mesophilic and thermo-tolerant acidophilic archaea. *Ferroplasma* has been detected in, and sometimes isolated from, a range of metal-rich acidic environments, including geothermal environments, mine tailings and stirred tank leaching operations (Vasquez et al., 1999; Burton and Norris, 2000; Edwards et al., 2000; Golyshina et al., 2000; Demergasso et al., 2005; Hawkes et al. 2005), and its growth appears to be favored by high concentration of acid and the presence of organic carbon sources (Okibe et al., 2003). *Ferroplasma acidiphilum* is a mesophilic species originally isolated from a pilot plant processing pyrite/arsenopyrite, and grows optimally at pH 1.7 (Golyshina et al., 2000). In contrast, the pH optimum for the currently non-validated species "*Ferroplasma acidarmanus"* is 1.2. A moderately thermophilic *Ferroplasma* has also been isolated from a commercial process bioreactor, which shows optimum temperature just above 50 ºC (Norris, 2007).

Recently, new strains affiliated to the *Ferroplasmaceae* family have been isolated from an industrial mineral sulfide bioleach heap in Myanmar, strain BH2, (Hawkes et al., 2006b) and a hydrothermal pool on Vulcano Island, Italy, strain V^T,

(Golyshina et al., 2009). Based on comparisons of 16S rRNA gene sequences, physiological and chemical components of major glycosyl dibiphytanyl phosphoglycerol lipids, and the lack of similarity in peptide fragment masses in the whole-cell proteome, it was concluded that both strains were not members of *Ferroplasma,* but were designated as a new genus, *Acidiplasma*. Two species. *Acidiplasma cupricumulans* (Strain BH2) and *Acidiplasma aeolicum* (Strain V^T) have been described. Figure I.5 shows a neighbour-joining phylogenetic tree based on 16S rRNA gene sequences with these new species, and previously-described acidophilic euryarchaeotes.

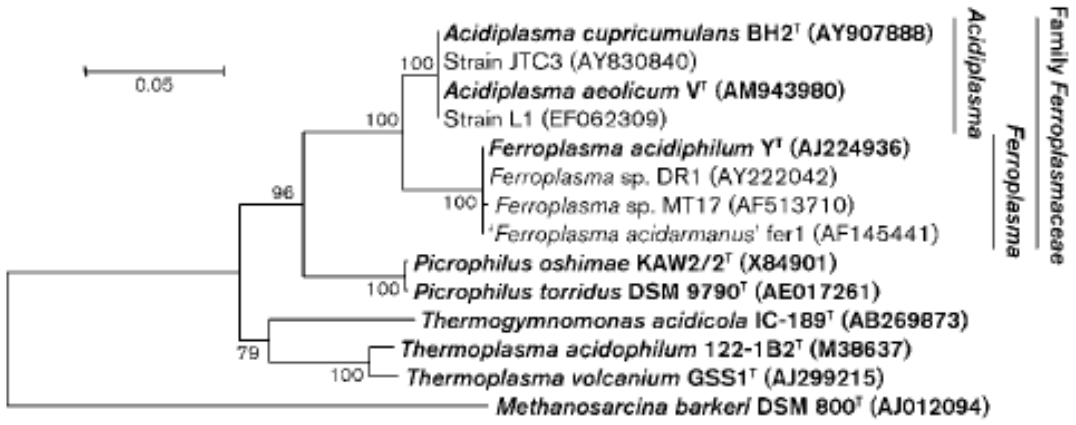


Figure I.5. Phylogenetic tree of acidophilic euryarchaeotes based on 16S rRNA gene sequences (Golyshina et al., 2009).

I.6.2 Moderate thermophiles

Moderately thermophilic acidophiles are generally considered to be those that grow optimally between 40 and 60 ℃ (Johnson and Ha llberg, 2008). Some species can grow over a wide temperature range, for example *At. caldus* and *Acidimicrobium (Am.) ferrooxidans* which are active from 25 ºC to about 55 ºC. In such cases, organisms overlap classifications for mesophiles and moderate thermophiles. One

genus of moderate thermophile often found in copper heap bioleaching process is *Sulfobacillus*. Species of this genus are Gram-positive spore-forming rods that oxidize reduced sulfur and ferrous iron. They can use organic substrates such as yeast extract and glucose and organic sulfur substrates such as glutathione, cysteine and cystine, as well as fix carbon dioxide (Johnson and Hallberg, 2008). As with *At. caldus*, species of this genus can have wide temperature ranges, with some strains growing at up to 65℃ (Norris, 2007). Another moder ately thermophilic acidophilic genus is *Acidimicrobium*. Currently this contains only one validated species, *Am. ferrooxidans,* which oxidizes ferrous iron but not reduced sulfur (Clark and Norris, 1996); however, other species related to *Am. ferrooxidans* but which also oxidize reduced sulfur, have been isolated and currently await classification; the genus "*Acidithiomicrobium*" has been proposed for these (Davis-Belmar and Norris, 2009).

I.6.3 Extreme thermophiles

Extremely thermophilic acidophiles are generally regarded as those that grow optimally at temperatures above 60 ℃ (Johnson and Hallberg, 2008). The upper growth temperature for acidophilic thermophiles is generally about 80 ℃, which is considerably lower than for their neutrophilic counterparts. Examples include *Sulfolobus metallicus*, which has been reported to be abundant in mineral bioprocesses operating at 65 ℃ to 70 ℃ (Gericke a nd Pinches, 1999). In processes operating at higher temperatures, such as 75 ℃ and above, *Acidianus brierleyi*, *Metallosphaera sedula* and various unclassified species tend to be more dominant (Dinkla et al., 2009). Although less work has been published on mineral leaching by extreme thermophiles, it is thought that the number of species involved in bioleaching at high temperatures might be similar to those moderate thermophiles at lower temperature (Norris et al., 2000; Plumb et al., 2002).

I.6.4 Heterotrophic acidophiles

A number of species of heterotrophic acidophilic prokaryotes have been isolated and detected in sulfide leach processes (Goebel and Stackebrandt, 1994; Bruhn et al., 1999; Hawkes et al., 2006; Bryan et al., 2007). However, these tend to be outnumbered by chemoautotrophic species due to the generally low concentrations of organic matter in those environments. The mesophile *Ferrimicrobium acidiphilum* is a Gram-positive actinobacterium, related to *Acidimicrobium ferrooxidans* and, like the latter, is able to oxidize ferrous iron but not sulfur. *Sulfobacillus* spp. are facultative autotrophs, but tend to grow far better when organic carbon is provided in laboratory cultures. The same is the case for *Alicyclobacillus* spp., some species of which are also found in mineral bioleaching environments (Johnson and Hallberg, 2008). Other heterotrophic species found in commercial processes include *Acidiphilium* spp. These are mesophilic, Gram-negative alphaproteobacteria, and all classified species are able to oxidize reduced sulfur, and to reduce, but not to oxidize iron. With one exception (*Acidiphilium acidophilum*) all *Acidiphilium* spp. are obligate heterotrophs. An *Acidiphilium* sp. has been detected in bioheap leaching at Escondida mine (Demergasso et al., 2005).

I.6.5 Salt-tolerant acidophiles

Some metal ores and heap leaching operations are located in, or close to, desert regions, where only saline or brackish water is available. Most known acidophiles are sensitive to high concentration of salt NaCl, though halotolerant, iron-oxidizing acidophiles have been isolated from marine geothermal sites (the "*Thiobacillus prosperus"* group; (Huber and Stetter, 1989), which also include some

moderately thermophilic isolates (Norris, 2007). Mesophilic strains have been found to be able to oxidize pyrite at a salt concentration twice that of sea water (Norris and Simmons, 2004). One strain (V6) was found to require 1 to 2% NaCl for optimal growth, and no growth was observed when NaCl was omitted from the medium. Strain V6 was also observed to grow in the presence of $MgCl_2$, but not in the presence of Na_2SO_4, indicating a specific requirement for chloride. Another characteristic found in strain V6 was the induction of a *rus* operon (as *At. ferrooxidans)* when grown in the presence of ferrous iron. However, in contrast to *At. ferrooxidans,* the gene cluster did not contain a homologue of the gene coding for cytochrome cyc (Nicolle et al., 2009). Other halotolerant bacteria related to both "*Thiobacillus prosperus"* (Kamimura et al., 1999) and salt tolerant, iron oxidizing *Alicyclobacillus*-like have been isolated from marine harbour sediments (Crane and Holden, 1999; Holden et al., 2001). Bioleaching of a copper ore by halo-tolerant acidophiles has been successfully demonstrated in column experiments (Davis-Belmar et al., 2008).

I.7 Stress and cellular response

Living organisms can respond to stress activating mechanisms to avoid or reduce the damage caused by unfavourable conditions. Stress response elicited by a given condition is dependent on the organism as well as the stressor. The stresses in general can be categorized into different groups as enlisted in Table 1.2. Physico-chemical parameters like high/low temperatures, pH, presence of toxic metal ions, osmolarity, and water content of the growth medium, among others are perceived as stress conditions by a variety of organisms. Cells can also respond to mechanical stress as sensed by the cell membranes/cell walls exerted internally by the turgor pressure or externally by increased atmospheric pressure. It has been shown that

cells need to maintain the integrity of their envelopes (membranes, walls) for survival. Trans-membrane signal-transducing protein factors monitor perturbations and respond appropriately by modulating gene expression (Wecke et al., 2006).

Table I.2. Broad categories of cellular stress

Type of stress	Parameters
Physical/chemical	Temperature, pH, presence of toxic metal ions,osmolarity, water level, oxygen level.
Mechanical	Atmospheric or turgor pressure leading to mechanical stress on membranes.
Starvation	Absence of one or more nutrients

It has been noted in several organisms that, when an organism responds to one stress, it often shows increased ability to cope with other stresses and indeed a particular stress is able to cause induction of genes required to function in response to an unrelated stress. This may be well justified from an evolutionary perspective, since in nature stresses are not encountered in isolation. While instantaneous, or rapid response to a stress may appear to be best achieved at the level of activation of functional molecules like proteins, to have the protein molecules required for a stress specific function synthesized before stress condition is encountered, is not likely to be economical or efficient. So, the cells resort to mechanisms which would enable them to synthesize the rescue operators rapidly on sensing stress. Most often this is achieved by regulating the synthesis of the required proteins at the transcriptional level.

Conceptually, a wider control over wider spectrum of genes could be achieved if the regulator is close to the core of the transcription machinery and it would enable the system to rapidly and simultaneously respond to a number of stresses at the transcriptional level. The process of transcription is highly regulated at the level of transcription initiation. The differential transcription initiation is brought

about by altered promoter selectivity by the transcription machinery. In prokaryotes, the sigma subunit of the RNA polymerase dictates specificity towards the promoter. The gene specific transcription factors and other ancillary factors further fine tune the level and specificity of expression. Studies have been carried out to elucidate the mechanism of how gene specific transcription factors function both in prokaryotes and in eukaryotes, and the role of the basal transcription machinery in transcriptional regulation in stress is well studied mainly in prokaryotes (Sadhale et al., 2007).

Acidophilic microorganisms are able to grow under very different conditions to most living organisms, such as low pH and (very often) elevated concentrations of transition metals, aluminium and some metalloids (e.g. arsenic). Despite these characteristics being of particular importance in commercial biomining processes, the genetic basis of their particular phenotypic characteristics mostly remain unknown. Some efforts have been made in order to understand some genetic response in acidophiles. Gene expression was studied in *At. ferrooxidans* strain LR (isolated from acid effluent of uranium leaching columns) in response to copper (200 mM) by using differential display technique (Paulino et al., 2002). The researchers found 104 differentially expressed genes using eight arbitrary primers; 70 % of these genes were confirmed to be expressed differentially, with seventeen showing highest induction or repression level. The RAP-PCR sequence identified to have highest induction was related to *At. ferrooxidans* cytochrome *c*, also sequences related to thiamin biosynthesis gene *thiC* in *Caulobacter crescentus* were found to have high induction with copper. An RAP-PCR product repressed by copper showed significant similarity with the carboxysome operon that includes the ribulose-1,5-bisphosphate carboxylase/oxygenase complex from *At. ferrooxidans* and another copper-repressed product was significantly similar to the XyIN outer membrane protein from *Pseudomonas putida*. In addition RAP-PCR products of unknown similarities were found (Paulino et al., 2002) in a similar study in which RNA was extracted from PLS samples from the Escondida sulfide bioleach plant, and a gene related to pyruvate

dehydrogenase complex E1 beta of *A. ferrooxidans* was found to have differential expression during the leach cycle (Galleguillos et al., 2007). Using proteomic techniques to study the differential expression of protein of *At. ferrooxidans* ATCC 19859, growing in ferrous iron, sulfur, tetrathionate and metallic sulfides, it was found that a set of proteins changed their synthesis level, the identification of these proteins by N-terminal amino acid sequencing and mass spectroscopy allowed the localization of the corresponding genes in the genomic sequence of the type strain of *At. ferrooxidans* (ATCC 23270), the genomic context of the genes related to the proteins suggests their involvement in the energetic metabolism of *At. ferrooxidans* (Ramirez et al., 2004).

I.8 Stress conditions for microorganisms in copper heap bioleaching

The design of bioheaps must consider two main aspects to favour the copper leaching process. On the one hand, the design has to allow microorganisms to thrive in the system (heap and the circuit solutions) but, on the other hand, the design has to maintain certain physico-chemical conditions demanded by subsequent processes (solvent extraction and electrowinning) involved in the production of metallic copper. Among the conditions for a good practice of the biohydrometallurgy process in industrial standard plants are:

i) Copper concentration in the PLS: the concentration of this heavy metal usually ranges around 4 to 5 g/L. This concentration is toxic for most living organisms (including most microorganisms) and many acidophiles cannot thrive in liquors containing such concentrations of copper (Cabrera et al., 2005).

ii) Low pH: Usually the pH in copper hydrometallurgy solutions ranges from 1.0 to 1.5. Iron oxidizing acidophiles vary in their pH optima and

minima. For example, *At. ferrooxidans*^{T} has a pH optimum and minimum of ~2.3 and 1.4, while and *L. ferriphilum* (strain MT6) has corresponding values of 1.5 and 0.8 (Okibe et al., 2003).

In addition, the continuous re-circulation of solutions through the system produces solutions with elevated concentration of ions, decreasing the availability of water and resulting in increasing osmotic stress to the indigenous microbial communities. Other variable stress factors include concentrations of soluble iron, variable temperature and concentrations of dissolved organic carbon (e.g. from solvent extraction).

Despite the important role played by the microbial community in heap leaching, few reports has been published regarding aspects such as composition and dynamics (Demergasso et al., 2005; Hawkes et al., 2005). Recently it has been shown that populations vary in the solutions during the bioleaching cycle, probably depending on the availability of ferrous iron and the increase of ionic concentration (Demergasso et al., 2005; Remonsellez et al., 2009).

I.9 Scope and objectives of the current project

The current project is intended to gain new insights into the physiological and genetic responses of prokaryotes that play important roles in the industrial bioleaching process at the Escondida copper mine. During the leaching cycle, changes in physico-chemical parameters can produce extreme conditions for microorganisms and many questions arise about how microbial communities respond and change when faced with such challenges, and also how modifications in the operation of the plant could affect the microbial population and its performance. In addition, Escondida, and many other major copper mines in Chile are located in the

Atacama Desert and therefore water availability is a major issue, which will impact bioleaching operations in future years.

Therefore, the major objectives of this project were:

- To isolate important members of the microbial community from the Escondida bioheap operation, previously detected by molecular tools, and to characterise those in terms of optimal growth conditions.

  (This topic is addressed in Chapter 3).

- To study the tolerance of isolates to some transition metals and sodium chloride, and to assess the potential for bioleaching of copper concentrate in saline liquors.

  (This topic is addressed in Chapter 4).

- To study the effect of elevated concentration of magnesium sulfate and sodium chloride on growth of different isolates and to identify the major osmo-protectants used by different mineral degrading bacteria.

  (This topic is addressed in Chapter 5).

- To identify potential genes induced when organisms are grown at elevated concentrations of copper and sulfate.

  (This topic is addressed in Chapter 6).

CHAPTER 2: MATERIAL AND METHODS

This chapter describes the standard material and methods that were used throughout this Ph.D. project. Modifications or additional methods used for specific experiments are described in respective chapters. All chemicals used in this project were of analytical grade quality and supplied by Merck (Germany), VWR (U.S.A) or Sigma Aldrich (U.S.A).

2.1 CULTIVATION TECHNIQUES

Liquid and solid media were used to cultivate acidophilic microorganisms during this project. Incubations were mainly performed in the range of 30-45 ℃ and under aerobic conditions. Liquid media were used in shake flask and bench scale bioreactor experiments, whereas solid media were used for microbial isolation and enumeration. All media were prepared using reverse osmosis grade water (RiOs^{TM}, Millipore UK) and were sterilized by autoclaving at 121 ℃ for 20 min utes.

2.1.1 Stock solutions and supplements for media preparation

Liquid media were prepared using a basal salt stock solution, supplemented with a suitable energy source and trace elements. Three main basal salts solutions were used during this project: autotrophic basal salts, heterotrophic basal salts and plate basal salts. The compositions of these three main basal salts media are shown in Table 2.1.

Table 2.1 Compositions of basal salts solutions used in this project.

	Components (per litre)					
Basal salt medium	$(NH_4)_2SO_4$	$Na_2SO_4 \cdot 10H_2O$	KCl	$MgSO_4 \cdot 7H_2O$	KH_2PO_4	$Ca(NO_3)_2 \cdot 4H_2O$
"Autotrophic" Basal Salts (ABS)	0.15	0.15	0.05	0.5	0.05	0.014
"Heterotrophic" Basal Salts (HBS)	0.45	0.15	0.05	0.5	0.05	0.014
"Plate" basal salts	1.25	-	-	0.5	-	-

2.1.2 Electron donors

All media contained at least one energy source (electron donor) including: ferrous iron, tetrathionate, sulfur, pyrite or a combination of these. The preparatory procedure for each energy source is described below.

Ferrous iron stock solution: prepared by dissolving $FeSO_4 \cdot 7H_2O$ in acidified (1 M H_2SO_4) reverse osmosis-grade (RiOs) water. The solution was vacuum-filter-sterilized and stored at 4 ℃. Final media concentration usual ly ranged between 20 and 50 mM.

Tetrathionate stock solution: prepared by dissolving potassium tetrathionate in RiOs-grade water. It was filter-sterilized and stored at 4 ℃. The final concentration of tetrathionate in media ranged from 1 to 10 mM.

Sulfur: supplied to culture media as a fine powder. It was heat-sterilized at 110 ℃ for 40 minutes in universal tubes and added to liquid media at 1% w/v.

Pyrite: supplied as a powder and was heat-sterilized in an oven at 160 ℃ over night in glass tubes covered with aluminium foil. Plastic tube caps were sterilized separately in an autoclave (121 ℃ for 20 minutes) to seal the tubes, under sterile conditions. Pyrite was used as the energy source in several experiments and during this project it was also added to cultures of ferrous iron-oxidizing bacteria for temporary storage.

2.1.3 Trace elements (TE)

A trace elements stock solution was used in order to supply micronutrients in both liquid and solid culture media. A x1000 concentrated stock solution was prepared in 0.01 M H_2SO_4 which contained [g/L]: $ZnSO_4 \cdot 7H_20$ [10.0], $CuSO_4 \cdot 5H_2O$ [1.0], $MnSO_4 \cdot 4H_2O$ [1.0], $CoSO_4 \cdot 7H_2O$ [1.0], $Cr_2(SO_4)_3 \cdot 15H_2O$ [0.5], H_3BO_3 [0.6], $NaMoO_4 \cdot 2H_2O$ [0.5], $NiSO_4 \cdot 6H_2O$ [1.0], $Na_2SeO_4 \cdot 10H_2O$ [1.0], $Na_2WO_4 \cdot 2H_2O$ [0.1], $NaVO_3$ [0.1]. The pH was adjusted to pH 2.0 with sulfuric acid and the solution was heat-sterilized in an autoclave (121 ℃ for 20 minutes) and stored at 4 ℃. The required volume of trace elements stock solution was added to media to result in a normal strength solution.

2.2 LIQUID MEDIA

Various liquid media were prepared using the basal salts solutions described in Table 2.1. Each medium type was prepared using particular basal salt (Table 2.2), adding trace elements and adjusting the pH (1.0 - 2.5) using a previously calibrated combination pH electrode (pHase; VWR, U.K.). Iron- and tetrathionate-free media were heat-sterilized in autoclave at 121℃ for 20 minutes. After sterilization the medium was cooled to room temperature and one, or a combination of energy sources was added. The liquid media used in this project are listed in Table 2.2.

Table 2.2 The composition and characteristics of liquid media used in this project.

Liquid media	Content
Low N - iron	ABS, TE 1x, $FeSO_4$ (20-50 mM), pH = 1.0 – 2.5
Low N - tetrathionate	ABS, TE 1x, $K_2S_4O_6$ (2-10 mM), pH = 1.0 – 2.5
High N - iron	HBS, TE 1x $FeSO_4$ (20-50 mM), pH = 1.0 – 2.5
High N - tetrathionate	HBS, TE 1x, $K_2S_4O_6$ (2-10 mM), pH = 1.0 – 2.5

2.3 SOLID MEDIA

Solid media were used to isolate microorganisms by picking off individual colonies growing on the surface of solid media. They were used later in the project in order to enumerate active acidophilic microorganisms from mineral leaching and stress tests.

Type I agarose (Sigma-Aldrich) was used at 0.5 % (w/v) in all acidic solid media. The agarose was prepared in RiOs water and heat-sterilized separately from the remaining acidic salts solution to avoid any hydrolysis resulting from the acidity and heating. After sterilization, the agarose solution was allowed to cool down to 50 ℃ before it was combined with the acidic salts solution. Heat-labile components, such as ferrous sulfate or potassium tetrathionate were filter-sterilized and added to the media at less than 50℃.

2.4 OVERLAY PLATES

Overlay solid media plates to isolate and cultivate acidophilic prokaryotes were developed at Bangor University by D.B. Johnson and co-workers (Johnson, 1995). These plates are formed of two layers and were shown to have high efficiency when isolating and enumerating autotrophic acidophilic microorganisms from industrial and mine-related samples (Okibe et al., 2003; Galleguillos et al., 2009). Overlay plates are composed of two layers, the bottom layer containing an acidophilic heterotroph (*Acidiphilium* sp. strain SJH) and the top layer of sterile gelled medium. The heterotroph in the lower layer plays an important role metabolizing compounds released by agarose hydrolysis at low pH, such as pyruvic acid or other organic acids, and other by-products which have been shown to inhibit the growth of autotrophic acidophilic microorganisms. The detoxification of the overall medium contained in overlay plates by the acidophilic heterotroph in the bottom layer is a key factor for the growth of acidophilic autotrophic organisms, especially those species sensitive to organic acids, such as *Acidithiobacillus* spp. or *Leptospirillum* spp., on the plate surface. The composition of overlay media

plates, including the heterotrophic microorganism included in the lower layer, are shown in Table 2.3.

Table 2.3 Composition of solid media plates.

Overlay medium	Components	Heterotroph
"Inorganic" iron overlay (iFeo)	HBS (1x), TE (1x), $FeSO_4$ (25 mM).	*Acidiphilium* strain SJH
Iron overlay (Feo)	$(NH_4)_2SO_4$ (1.25 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), TE (1x), tryptone soy broth (0.25 g/L), $FeSO_4$ (25 mM).	*Acidiphilium* strain SJH
Iron/tetrathionate overlay (FeSo)	$(NH_4)_2SO_4$ (1.25 g/L), $MgSO_4 \cdot 7H_2O$ (0.5 g/L), TE (1x), tryptone soy broth (0.25 g/L), $FeSO_4$ (25 mM), potassium tetrathionate (2.5 mM).	*Acidiphilium* strain SJH

2.5 DETERMINATION OF MICROBIAL BIOMASS

Biomass measurements were a critical parameter determined in a number of experiments preformed during this project. A number of methodologies were used depending on the nature of individual samples. These standard methods are described below.

2.5.1 Total cell counts

The number of bacterial cells in liquid culture media were determined using a Thoma counting chamber (Weber Scientific International, England) under a phase contrast microscope (Labolux light microscope fitted with a Phaco 2 objective lens and a

phase contrast condenser; Leitz, Germany). After mixing the sample, about seven microlitres were placed in the center of the chamber and covered with a cover slip. The chamber was placed on the phase contrast microscope and the cells were observed and counted using 400x magnification. To obtain the number of cells per ml of test sample, at least ten squares were counted and the mean value was multiplied by the factor $2 \cdot 10^7$.

2.5.2 Protein determination

The protein contents of liquid culture samples were determined using the Bradford assay (Bradford, 1976). Bradford reagent was prepared by dissolving 50 mg of Coomassie Brilliant Blue G in 25 ml of 95% ethanol. Once dissolved 50 ml of 85% (w/v) phosphoric acid was added and finally RiOs water added to a final volume of 500 ml. Cells contained in liquid culture samples were harvested by centrifugation (10,000 rpm, 20 minutes). The pellet obtained after centrifugation was resuspended in 0.5 ml of 0.5 M NaOH and incubated at room temperature for 15 minutes to obtain a cell lysate. After incubation, 100 μL of cell lysate was mixed with 1 ml of Bradford reagent and incubated in the dark for 2 minutes. Finally, the absorbance at 595 nm was determined using a Cecil CE1011 spectrophotometer (Cecil instruments Ltd. England). To obtain the concentration of protein in the sample a standard curve (concentration vs. absorbance) was built using bovine serum albumin (BSA) as standard.

2.5.3 Wet weight

In some cases biomass was determined by weighting the pellet obtained after centrifugation of a certain volume of culture. To obtain the wet mass value a sterile

empty microfuge tube was weighed using an analytical scale. This same tube was then used to centrifuge the sample. After centrifugation the supernatant was discarded and the tube containing the cell pellet was weighed again. The wet weight was calculated from both values.

2.6 ANALYTICAL TECHNIQUES

2.6.1 Spectrophotometric techniques

Determination of soluble ferrous and total iron. Ferrous iron in solution was determined using the Ferrozine assay described previously (Lovley and Phillips, 1987). Ferrozine reagent was prepared by dissolving 1 gram of ferrozine (3-(2-pyridyl)5,6-bis(4-phenyl-sulfonic acid)-1,2,3-triazine) in 1 L, 50 mM HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid) buffer, adjusted to pH 7.0 with KOH. Spectrophotometric determination was carried out by adding 950 µl of ferrozine reagent to 50 µl of sample (usually previously diluted between 20 and 100 times). The absorbance for each sample was recorded at 562 nm using a blank (950 µl of ferrozine and 50 µl of distilled water) as the reference value on a Cecil CE1011 spectrophotometer (Cecil instruments Ltd., England). A standard curve was constructed using concentrations of $FeSO_4$ ranging between 0.01 to 1 mM and this was used to calculate the concentration of ferrous iron in test samples.

Determination of Sulfate. Sulfate was measured in test samples using the turbidimetric assay described by Kolmert et al. (2000). For the assay, one ml of sample and one ml of conditioning reagent (150 g NaCl, 100 ml glycerol, 60 ml concentrated HCl and 200 ml of 95% ethanol, adjusted with distilled water to 1L) were

mixed thoroughly. Finally, 60.0 mg of barium chloride was added and it was mixed by vortexing for 30 seconds before the absorbance was measured at 420 nm against a blank (prepared with distilled water) using a Cecil CE1011 spectrophotometer (Cecil instrument Ltd., England). Sulfate standards were prepared using K_2SO_4 in a range of concentration between 0.1 to 2.5 mM and a standard curve was built using these standards in order to determine the concentration of sulfate in test samples.

2.6.2 Liquid chromatography

Determination of concentrations of transition metals. Total iron, copper and zinc in solution were determined using ion chromatography (Dionex, U.S.A). A Dionex system equipped with a an Ion Pac® CS5A column, an AD25 absorbance detector and IP25 isocratic pump was used. The flow rate was set to 1.2 ml/minute, column pressure, *ca*. 1600 p.s.i. and 50 µl of sample were injected. The eluent for this analysis contained PDCA (pyridine-2,6-dicarboxylic acid; 1.4 mM), KOH (13.2 mM), K_2SO_4 (11.2 mM) and formic acid (14.8 mM). PDCA forms anionic complexes with transition metals and these complexes are differentially retained in the column. The post column reagent consisted of 0.12 g 4-(2-pyridylazo) resorcinol (PAR) dissolved in 1 L of diluents solution (1 M 2-dimethylaminoethanol, 0.5 M ammonium hydroxide and 0.3 M sodium bicarbonate. The concentrations of iron, copper and zinc in solutions were then determined by measuring changing in absorption at 520 nm and using standards for each metal at three different concentrations at the beginning and end of each run.

Identification and determination of sugars and amino acids. Sugars were analyzed using a Dionex ICS 3000 system equipped with a Carbo Pac™ Pa10 separation column, including guard column PA10, and an ED40 amperometric detector.

The separation column was kept at 30℃ during the analyses. The flow rate used was 0.4 ml/minute and 50 µL of sample were injected. Column pressure was 1,600 p.s.i. with a lower limit of 200 p.s.i. and a upper limit of 3,000 p.s.i.. A 0.25 M solution of sodium hydroxide was used as eluent and the pH was maintained between 10 and 13 during the analysis. Sugars were identified by the retention time and also by mass spectrometry. Sixteen different sugars were run in the system as standards.

The same configuration was used for amino acid analysis, but an AminoPac PA10 column was used instead of CarboPac PA10. Various amino acids previously identified as osmo-protectants or as precursors of osmo-protectants were tested, including proline, lysine, arginine and glycine.

2.7 BIOMOLECULAR TECHNIQUES

2.7.1 Nucleic acids extraction

DNA was extracted from liquid culture samples for use in molecular analyses such as polymerase chain reaction (PCR), sequencing or cloning. Two different DNA extraction methodologies were used, depending on the subsequent analyses.

(i) **Crude cell lysate:** This procedure was usually carried out for pure liquid cultures. Cells were harvested from 10 ml of culture by centrifugation (10 minutes, 10,000 rpm). After centrifugation the pellet was resuspended in 20 µL of lysis solution (0.05 M NaOH, 0.25% SDS, sodium dodecyl sulfate) and transferred to a 0.5 ml PCR tube. The mixture was then heated at 95℃ for 15 minutes in a PCR thermocycler (Uvigene™ (UVItec

Ltd., U.K.) or Techne® TC-312 (Midwest Scientific, U.S.A.)). After the heating step, 180 µL of nuclease free water was added. Cells lysates, or dilutions of these, were used as template for PCR amplification of 16S rRNA genes and/or other functional genes.

(ii) **Commercial kit:** The UltraClean™ Soil DNA kit (MoBio Laboratories Inc., USA) was used to extract DNA from solid phases or mixed culture samples, following the manufacturer´s instructions. The concentration of DNA was determined in a Nanodrop spectrophotometer. This method was used in order to optimize the DNA recovery in experiments containing potential PCR inhibitors (e.g. copper concentrate leaching experiments).

RNA was extracted from liquid culture samples to study gene expression of isolates grown under several different stress conditions. Cells were harvested from liquid culture samples at late exponential phase by centrifugation (20 minutes, 10,000 rpm, 4℃) and RNA was extracted using the Qiagen RNeasy kit (Qiagen, Germany), according to manufacturer´s instructions. The concentration of RNA was determined in a Nanodrop spectrophotometer. Special care was taken during the RNA extraction process, including the use of ice in order to keep samples at low temperature during all the extraction process and wearing of gloves to avoid contamination. Finally, samples were stored in RNALater® reagent (Qiagen, Germany) at -80 ℃ in order to stabilize the RNA and avoid any degradation. The RNA extracted using this method was of high quality, with no visible degradation of rRNA and no visible DNA contamination. To eliminate any trace of DNA, RNA samples were treated with “RQ1 RNase-Free DNase” (Promega, U.S.A.) and success was determined as the lack of amplification of 16S rRNA genes by PCR (Section 2.7.2).

2.7.2 ANALYSIS OF NUCLEIC ACIDS

DNA amplification by polymerase chain reaction (PCR). During the first part of the project, PCR amplification was carried out using the 16S rRNA gene, in order to identify the strains isolated from industrial samples. After amplification, the 16S rRNA gene fragment was sequenced and compared against the NCBI database using the BLAST tool. In the later phases of the project PCR was used to amplify and subsequently sequence fragments of genes obtained from gene expression experiments and also for the detection of *nifH* genes in leptospirilli. PCR reactions were performed using the GoTaq® green master mix (Promega, U.S.A.). The PCR reactions contained 4 µl of 5x buffer, magnesium chloride (1.5 - 2.5 mM), 5-10 *p*mol of each primer (forward and reverse), dimethylsulfoxide (DMSO) at 2% (v/v), and 1 µl of DNA template, the total volume was adjusted to 20 µl by adding nuclease free water. Thermal cycling was performed in an Uvigene™ (Uvtec ltd., UK) thermocycler. PCR was generally carried out using the following steps: initial denaturation at 95℃ for 5 minutes; 30 cycles of denaturation at 95℃ for 30 seconds, annealing 55℃ for 30 seconds and polymerisation at 72℃ for 90 seconds, the 30 cycles were followed by an extension period at 72℃ for 10 minutes, before being kept at 4℃. The annealing step was modified depending on the primer pairs and the size of the amplicon. Table 2.4 lists the primers used in this project. The primer pair 341f-GC and 907r were used to analyse communities by Denaturing Gradient Gel Electrophoresis (DGGE).

Table 2.4 Description of primer pairs used in this project.

Target gene	Primer name	Sequence	Reference
Bacterial 16S rRNA	27F	AGAGTTTGATCMTGGCTCAG	Lane et al. 1991
	1392R	ACGGGCGGTGTGTRC	
	341F-GC*	CCTACGGGAGGCAGCAG	Muyzer et al. 1993
	907R	CCGTCAATTVCMTTTGAGTTT	
Nitrogenase reductase (NifH)	nifH19F	GCIWTYTAYGGIAARGGIGG	Ueda et al. 1995
	nifH407R	AAICCRCCRCAIACIACRTC	

*GC clamp at 5´ end = CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG
M= A or C, R= G or A, V= A or G or C, W= A or T, Y= T or C, I= inosine

Agarose gel electrophoresis. PCR products were analysed by agarose gel electrophoresis. The agarose concentration in the gel was 0.7% to 3% (w/v), and was prepared in 0.5 x TBE buffer (5.4 g tris(hydroxymethyl) methylamine, 5.5 g boric acid per litre in 0.1 M ethylenediaminetetra-acetic acid (EDTA) pH=8.0). Agarose was dissolved by heating, in a microwave oven, allowed to cool for ten minutes on the bench and ethidium bromide was added at a final concentration of 0.5 µg/ml. Then the gel was cast in the gel tray with a comb to form loading wells. PCR products were directly loaded into the wells when 5x-green GoTaq buffer was used for PCR, in the case of using normal buffer the product was mixed in a ratio of 5:1 with 6x DNA loading buffer (30% v/v glycerol, 0.05% w/v bromophenol blue and 60 mM EDTA). To perform the electrophoresis the samples were loaded into the wells and 1 Kb DNA ladder (New England Biolabs, Inc, U.S.A) was loaded as molecular size reference. The tray was placed into the chamber and the migration of the nucleic acid was produced by applying

a constant voltage during the time necessary to produce a good separation indicated by the bromophenol blue dye included in the loading buffer and in the ladder. The gel was then removed from the chamber and tray and imaged under UV illumination.

Reverse transcription. Reverse transcription is a technique used to convert RNA into cDNA in order to apply to cDNA similar analysis carried out on normal DNA. In this project total RNA was extracted from *L ferriphilum* strain IESL25 when it was grown in different conditions and transcribed to cDNA for further analysis. The ImProm-II™ Reverse Transcription System (Promega, U.S.A) and random hexamers (Qiagen, Germany) were used to perform the reverse transcription. First, 0.1 to 1 µg of experimental RNA was mixed with 0.5 µg of random hexamers (5 µl, final volume) and preheated for 5 minutes at 70 ℃ and immediately chilled in ice-water for at least 5 minutes. Next, this was transferred to a microfuge tube containing 4.0 µl of ImProm-II™ 5X Reaction Buffer, $MgCl_2$ (final concentration 2.5 mM), dNTP Mix (final concentration 0.5 mM each dNTP) and 1.0 µL ImProm-II™ Reverse Transcriptase. The tube was incubated at 25℃ for 5 minutes and up to two hour at 42 ℃. Finally to inactivate the reverse transcriptase the tube was incubated at 70°C for 15 minutes. The cDNA was maintained frozen (-80℃) for long term storage.

Denaturing Gradient Gel Electrophoresis (DGGE). DGGE was used to determine the temporal composition of the microbial population during leaching experiments when a consortium of microorganisms (able to thrive in high concentration of NaCl) was tested for their ability to leach copper concentrate in laboratory scale reactors. The DNA was obtained from liquid-sludge samples using the UltraClean™ Soil DNA kit (MoBio Laboratories Inc., USA) and used as template to amplify a fragment of the 16S rRNA gene (341f-GC and 907r primers). DGGE facilitates the separation of

fragments based on their base composition as they migrate through a polyacrylamide gel containing a gradient of denaturing compounds (urea and formamide). Individual bands/fragments visualised on the gel were excised, re-amplified and sequenced in order to identify each member of the community. A denaturing gradient between 40 and 80% of urea/formamide was used to obtain an optimal separation of 16S rRNA gene fragments from acidophilic bacteria, as described previously for the analysis of heap leaching (Demergasso et al., 2005). A Bio-Rad DCode system was used for electrophoresis and 6% acrylamide gels were prepared using a peristaltic pump to produce a linear chemical gradient of urea and formamide from 40 to 80%. Between 3 to 10 µL of PCR product were loaded to the gel using TAE buffer (40 mM Tris [pH 8.0], 20 mM acetic acid, 1 mM EDTA) as running buffer. The system was heated at 60℃ and run for 12 h at 100 V. After electrophoresis, the gel was stained with SYBR gold (Invitrogen Cat. S11494) for 45 minutes at room temperature, rinsed with filter-sterilized distilled water and imaged under UV illumination using a QICAM 10-bit mono camera (QIMAGING, Canada). The visible bands were excised, transferred to microfuge tubes containing 15 µL of nuclease free water and stored over night at -20 ℃. The tubes were thawed and the bands were re-amplified using 2 µl of solution. PCR products were submitted for sequencing to Macrogen Inc. (Korea).

2.7.3 Gene cloning and sequencing

Cloning was used in this project to identify genomic sequences obtained by Micro-representational Difference Analysis (see next section). Differentially expressed cDNA fragments were cloned as follows.

PCR product and ligation reaction. Fresh PCR products were used throughout this procedure. In order to eliminate the reagents used in the PCR, the PCR products

were firstly purified using the QIAquick PCR Purification Kit (Qiagen, Germany). For the ligation reaction, 3 µl of purified PCR product were mixed with 5 µl of Rapid Ligation buffer, 1 µl T4 DNA Ligase (3 Weiss units μL^{-1}) and 1 µl pGEM®-T Easy Vector plasmid (all reagents supplied with the pGEM®-T Easy vector kit, Promega, U.S.A.). The reactions were incubated at 4℃ overnight to proceed with the transformation step.

Transformation of *Escherichia coli* JM-109. For the transformation step an aliquot of competent cells of *E. coli* strain JM-109 was thawed on ice for 5 minutes, then 50 µL of cells were added to an microfuge tube containing 2 µl of ligation reaction and incubated on ice for 20 minutes. In order to produce the transformation, the cells mixed with the ligation product were heat-shocked for 50 seconds in a water bath at exactly 42 ℃ and immediately placed on ice again for two minutes. Then 900 µl of SOC medium (containing 20 g tryptone, 5.0 yeast extract, 0.58 g NaCl, 0.19 g KCl, 2.03 g $MgCl_2 \cdot 6H_2O$, 2.47 g $MgSO_4 \cdot 7H_2O$, 3.6 g glucose per litre, and adjusted to pH=7.0 with NaOH) were added to the tube containing the cells and incubated at 37 ℃ for two hours in orbital shaker (150 rpm).

Plating and screening of colonies: After the incubation in SOC medium 50 and 100 µL of transformation culture was spread onto LB agar plates (10 g tryptone, 5 g yeast extract and 5 g NaCl per litre and 2% w/v agar) containing 100 µg ml^{-1} ampicillin, 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and 80 µg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (Melford Laboratories Ltd., U.K.). Plates were incubated at 37℃ for 16 hours. The easy vector plasmid confers ampicillin resistance to those transformed cells, therefore only cells containing this plasmid were able to grow on the plates. White colonies are formed by cells containing plasmid with insert and blue

colonies are formed by cells containing plasmid without insert. Therefore only white colonies were selected for further analysis.

Screening of clones: Crude cell lysates of white colonies were prepared and the presence of insert was confirmed by PCR using the suitable primers pair. Only clones containing the insert produced DNA fragments visualized after electrophoresis in agarose gels. For each experience 5 clones were selected and transferred to 4 ml of LBamp broth (LB medium containing 100 µg of ampicillin per litre) and incubated at 37 ℃ overnight. Plasmid were purified from active cul tures using the Plasmid Midi Kit (Qiagen, Germany). Finally the purified plasmids were submitted for sequencing to Macrogen Inc. (Korea).

2.7.4 MICRO-REPRESENTATIONAL DIFFERENCE ANALYSIS (MRDA)

MRDA was used to find specific sequences and genes involved in the genetic response of the strain *L. ferriphilum* IES25 when it was grown in stress conditions. This method consists of four major steps: Extraction of total RNA and cDNA synthesis, generation of cDNA fragments by restriction enzyme and linking of adaptors, subtractive hybridization, and selective amplification by PCR. RNA extraction and cDNA synthesis were explained previously (see section 2.7.2)

The PCR-Select™ cDNA Subtraction Kit Bacterial Genome (Clontech, U.S.A.) was used to perform the analysis. This kit includes the reagents shown in Table 2.5.

Table 2.5 Reagents supplied by The PCR-Select™ cDNA Subtraction Kit Bacterial Genome (Clontech, U.S.A.)

Reagents supplied by the kit	**Step**
RsaI enzyme Restriction enzyme buffer	Preparation of cDNA
Adaptors 1 and 2R T4 DNA ligase Ligation buffer	
Hybridization buffer Dilution buffer	Subtractive Hybridization
Primer 1 Nested primer 1 Nested primer 2R	Selective Amplification

After the reverse transcription of RNA obtained from two selected growth conditions, special adaptors are linked to the cDNA. The cDNA obtained from the reference growth condition is referred as “driver” and from the stress condition is referred as “tester” (stress). After the linking of adaptors, systematic hybridizations rounds between the tester and the driver cDNAs (with driver in excess) are performed in order to allow only the amplification of cDNA differing from the reference condition. The Figure 2.1 shows the main steps to perform the MRDA analysis.

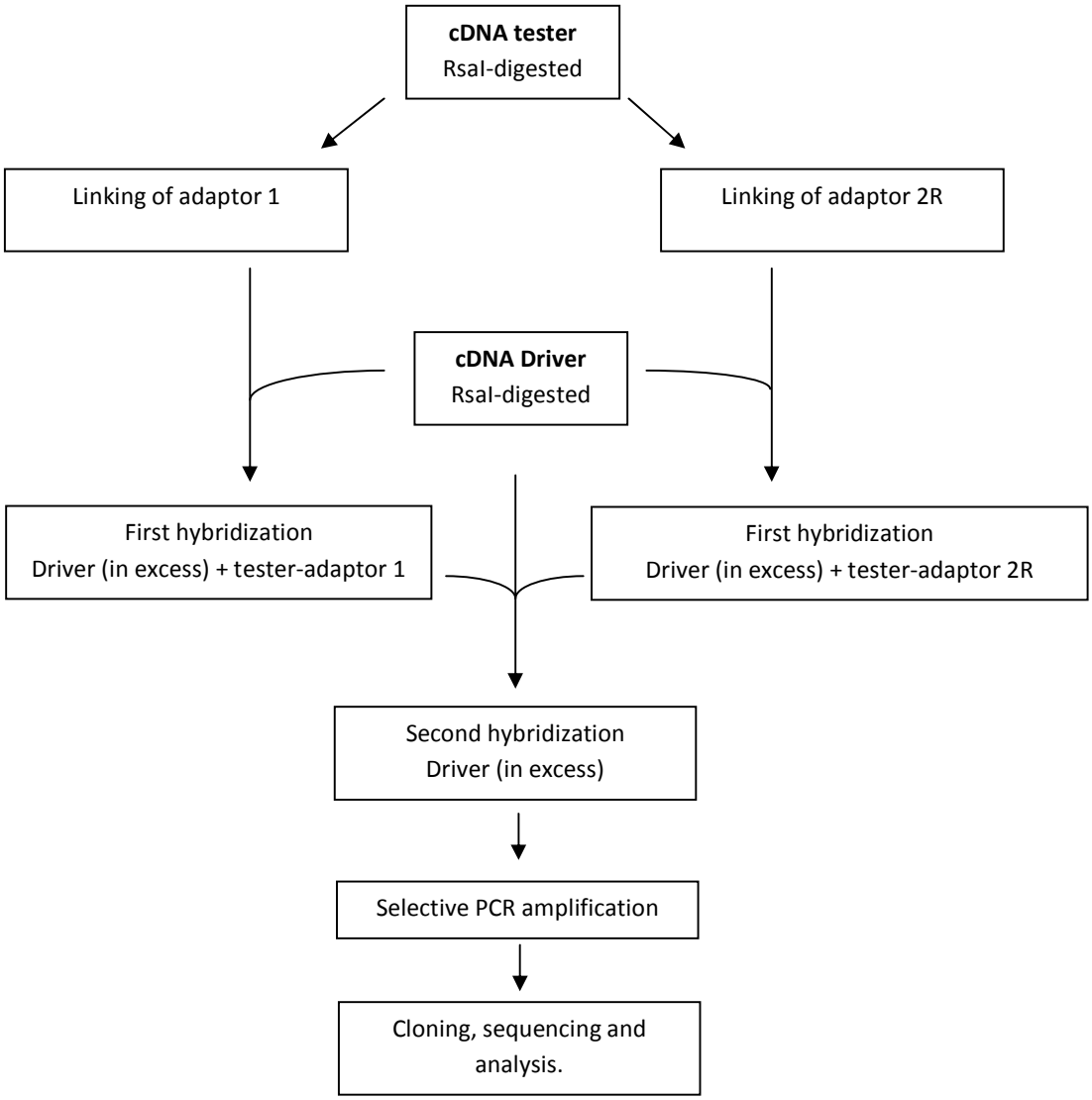


Figure 2.1 Overview of the main steps to perform MRDA analysis.

2.7.5 REAL TIME PCR EXPERIMENTS

Real time (or quantitative) PCR was used in this project to determine the level of expression in the Escondida heap samples of transcripts identified in the MRDA and also to quantify the temporal abundance of microorganisms in bioleaching experiments. For this analysis the RNA was previously transcribed to cDNA as described in section 2.7.2.

Transcript specific primers were designed using the online-tool Primer-BLAST, available on the NCBI web site (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). This online-tool combines the programs Primer3 and BLAST to supply as outcome pairs of oligo-nucleotides that can used as target-specific primers. In order to estimate the copy numbers of target sequences, relative standard curves were constructed from a serial dilution of plasmids containing the target sequence and plasmids containing a fragment of 16S rRNA gene. The DNA concentration in ng per µl was transformed to each specific gene copy number per µl by calculating the molecular weight of the plasmid plus the insert assuming that 660 g/mol is the average molecular weight of a base pair. Then this value is divided by Avogadro´s number (6.024×10^{23}) to obtain the number of molecules per gram. Finally to obtain the number of molecules per ng the last value is divided by 10^9. A Rotor-GeneTM 6200 (Corbett Research Pty Ltd, Australia) using The Quantimix Easy SYG Kit (Biotools, Spain) were used to perform real-time PCR, according to the manufacturers' instructions. An optimized volume of cDNA and the corresponding specific primers were used for the amplification of each gene. The following PCR program was used to amplify all genes: 95 ℃ - 3 min; 40 cycles: 95 ℃ - 30 sec, 60 ℃- 30 sec, 72 ℃ - 20 sec, followed by 4 ℃ hold. The melting curve was measured for the

amplification program with a ramp raising the temperature from 50 to 95ºC by 1℃ every 5 seconds (Demergasso et al., 2004).

2.7.6 PHYLOGENETIC ANALYSES

Phylogenetic analyses were applied to the 16S rRNA gene sequences obtained from the microorganisms isolated from industrial solutions of Escondida mine. These analyses were performed using the package ARB (Wolfgang et al., 2004). This package is available on ww.arb-home.de and it was downloaded and installed on a PC with the Ubuntu operating system. Consensus sequences were used for the phylogenetic analysis. These sequences were obtained by aligning the sequences of at least 3 sequences obtained from the same organism using the software ClustalX2 (http://university-college-dublin.software.informer.com). The 16S rRNA gene sequences not included in ARB database and similar to those obtained from the isolated microorganisms were downloaded from nucleotide database of *National Center for Biotechnology Information* website (http://www. ncbi.nlm.nih.gov) and included in the local ARB database. The ARB_EDIT tool was used for automatic sequence alignment. The alignments were looked through and improved manually. During tree construction, several trees differing in the set of alignment positions as well as reference sequences were used. Phylogenetic trees were built by performing maximum likelihood (fastDNAml), maximum parsimony and neighbour-joining analysis included in the ARB package.

CHAPTER 3: ISOLATION AND IDENTIFICATION OF MICROORGANISMS FROM THE COPPER HEAP BIOLEACHING OPERATION AT THE ESCONDIDA MINE, CHILE.

3.1 INTRODUCTION

At the time of writing, *Minera Escondida Limitada* is the biggest copper producer in the world, with its production accounting for ~8% of copper produced globally by mining. Copper is produced at Escondida as both copper concentrate and copper cathodes. Bioleaching is applied to leach low grade sulfide ores (containing ~ 0.6 % w/w Cu). More than half (55%) of the copper cathodes produced at the mine come from the bioleaching operation, *via* solvent extraction and electrowinning of the pregnant leach solutions (PLS) generated in the heaps. The microbial populations inhabiting the bioleaching system at Escondida mine have been studied by culture-independent techniques and have revealed the phylogenetic characteristics and dynamics of a microbial community involved in heap bioleaching of low-grade copper ore (Demergasso et al., 2005). In the pilot-scale heap, the abundance of each member of the population varied during the leaching period, probably due to changes in physico-chemical parameters as the operation evolved. Demergasso et al. (2005) found that *Acidithiobacillus ferrooxidans* was the most abundant species in the PLS solution during the first stage of bioleaching (0-250 days). As the bioleaching process continued, physico-chemical changes within the heap resulted in changes in the PLS, including temperature, sulfate concentration, copper concentration, electrochemical potential (E_h) and pH. These variations impacted the microbial consortia, and changes in the relative abundance of the species present were apparent as the operation progressed. During

the final stages of the leaching cycle (600 days) *Leptospirillum ferriphilum* was the most abundant species (Demergasso et al., 2005; Remonsellez et al., 2007). Many questions arose from these first observations regarding how each species was able to cope with different kinds of stress produced by changes of the physico-chemical parameters in the process. A first approach to answer these questions involved the isolation and characterisation of microorganisms detected in the process. This chapter describes the isolation of six microbial strains from the solutions of the industrial bioleaching plant at Escondida. The identity of these isolates was compared with previous results obtained from the same system by culture-independent techniques. In addition, the effects of pH and temperature on the growth of primary (iron-oxidizing) isolates obtained from the heaps were examined.

3.2 MATERIALS AND METHODS.

3.2.1 DESCRIPTION OF THE PROCESS AND ORIGIN OF SAMPLES

The Escondida Mine is located 170 km south-east of Antofagasta, in northern Chile. Bioleaching technology is used at Escondida as part of the process to obtain metallic copper from low grade sulfide ore (approx. 0.6 % w/w Cu). Figure 3.1 shows the location of relevant biohydrometallurgical processes at the Escondida mine. Low grade ore is used to build the heaps (Fig. 3.1-B), directly after blasting in the pit (Fig. 3.1-A), and it is not previously processed by crushing or curing as is the case for heap leaching of the higher grade oxide ore that is also present at Escondida. The PLS containing dissolved copper is transported through pipes from the bottom of the heap to the solvent extraction facility (Fig. 3.1-C), where copper cathodes are produced. The ore used in this

process is known as run-of-mine (ROM) and, of the major copper minerals present, ~50% is chalcopyrite ($CuFeS_2$), ~40% is chalcocite (Cu_2S) and ~10% is covellite (CuS).

Figure 3.1. Satellite image of Escondida mine showing the location of relevant processes. Pit (A), Bioheaps (B) and solvent extraction facility (C).

Figure 3.2 shows a schematic representation of the sulfide leach plant process at the Escondida mine. The first lift of the industrial heap is divided into twenty-one strips. Each strip is formed by a section 125 m wide, 2000 m long and 18 m high. The design of the process involves building a total of seven lifts. Air is injected into the heap through airlines located at 1 m intervals over the base of the heap, supplied by external blowers. The design for this bioleaching heap process uses the raffinate (effluent of solvent extraction facility) as the only irrigation solution for all the strips of the heap. The design also means that each strip generates its own PLS. The PLS of each strip is transported to a pond, which represents the “stock solution” for the solvent extraction process (referred to as the “PLS común”). Irrigation of the strips is carried out in succession at

intervals of one month, starting at strip 1. Liquid samples used to isolate the acidophilic microorganisms inhabiting the heap were taken in January 2007.

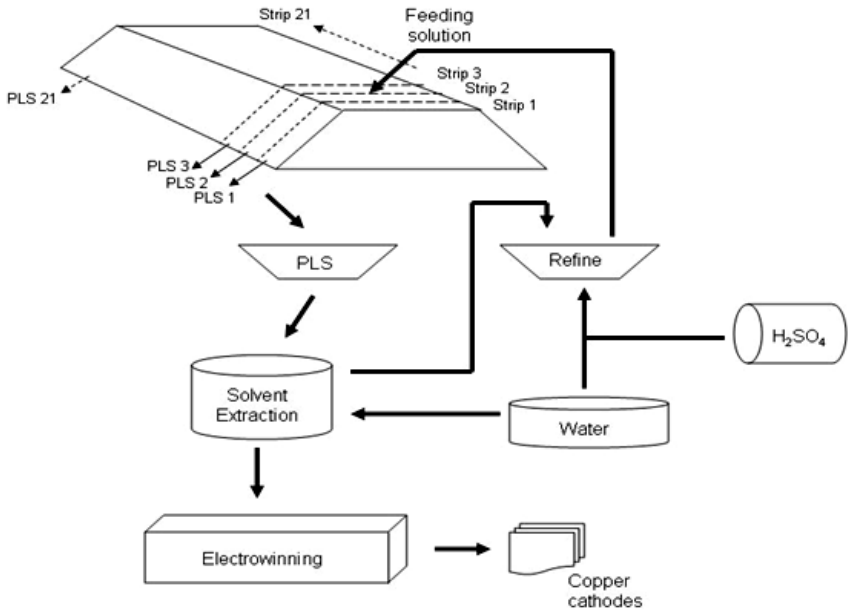


Figure 3.2. Simplified schematic representation of the industrial sulfide bioleach plant at the Escondida mine.

3.2.2 PLS SAMPLES

Four samples were used for the isolation of indigenous microorganisms from the industrial process, PLS from strips 1, 2, 3 and PLS from the Demonstration Plant (Table 3.1).

Table 3.1. PLS samples, origin and date of collection

Samples	Source	Date
PF1 PF2 PF3	Industrial Sulfide Leach Plant	13th January 2007
PLS-PD	Demonstration Sulfide Leach Plant	3rd October 2006

3.2.3 PHYSICO-CHEMICAL ANALYSIS

Physico-chemical data of the samples was kindly supplied by personnel operating the metallurgy section at the Escondida mine.

3.2.4 CULTURE-INDEPENDENT ANALYSIS

Quantitative polymerase chain reaction (qPCR) was used to determine the abundance of microorganisms in the PLS samples. Table 3.2 shows primers and targets used to quantify the population in the samples. A combination of one reverse primer with one specific forward primer was used to determine the copy numbers of the 16S rRNA gene for each target species. A full description of the protocol is given in section 2.7.5.

Table 3.2. Primers used for qPCR.

sense	Primer	Sequence	Target
Reverse	Bacteria	TTGTGCGGGCCCCCGTCAAT	Most bacteria
Forward	ATFD2	CGGGTCCTAATACGATCTGCT	*Acidithiobacillus* group II*
	ATFDM	TGGTTCCTAATACGAGCTACTG	*Acidithiobacillus* group I*
	ATT	GGGTGCTAATANCGCCTGCT	*Acidithiobacillus thiooxidans*
	LF1	GATGTCAGAACACGGCATTT	*Leptospirillum ferriphilum*
	LF2	CGTCAGAAIACGGCGCTTC	*Leptospirillum ferriphilum*
	Sulf-Esc	GGAGACCGTGCCGTCG	*Sulfobacillus* sp. Escondida
	Sulf-G1	AGTGGCGAAGGCGCCTTGCTCG	*Sulfobacillus* sp. G1
Reverse	Archaea	TGCTCCCCCGCCAATTCC	Most archaea
Forward	Ferr	GAAGCTTAACTCCANAAAGTCTG	*Ferroplasma* sp.

*****Group I and II represents phylogenetic divisions described for iron-oxidizing acidithiobacilli (Amouric et al., 2010).

3.2.5 ISOLATION OF ACIDOPHILIC MICROORGANISMS

Acidophilic microorganisms were isolated on overlay plates as described in section 2.4. Samples (~10 µL) were inoculated onto overlay plates and incubated at 30, 37, and 45 ℃ for 15 days. Three types of overlay p lates were used:

i. iFeo, “inorganic” iron-overlay, containing ferrous sulfate

ii. FeSo, iron sulfur-overlay, containing ferrous sulfate, potassium tetrathionate and tryptone soya broth

iii. Feo, iron/TSB-overlay, containing ferrous sulfate and tryptone soy broth.

Following incubation, several different colony morphologies were visible on the surfaces of the solid media. Single representative colonies were transferred to microfuge tubes containing 0.5 mL of liquid media. The liquid media contained trace elements and either ferrous iron (20 mM) or tetrathionate (2 mM) as an energy source (section 2.2). Incubation was performed at the same temperature as that of the corresponding plates from which the colonies were removed. Culture purity was checked by streaking grown liquid cultures onto solid media, and single colonies reselected, if more than one representative colony was found.

3.2.6 IDENTIFICATION OF ISOLATED MICROORGANISMS

Preliminary identification of the isolates was based primarily on the morphology of the colonies and the electron donor (ferrous iron or tetrathionate) used. To confirm their identities, 16S rRNA genes were sequenced from pure cultures grown in liquid media. Lysates were produced (Section 2.7.1) and the 16S rRNA gene of each of the isolates was amplified and sequenced. PCR was performed as described in section 2.7.2 using primers 27F and 1392R and lysates (or diluted lysates) from each liquid culture as DNA templates. The PCR conditions included an initial denaturation step at 95 ℃, 30 cycles of 95 ℃ for 30 seconds, 58 ℃ for 30 seconds and 7 2 ℃ for 90 seconds, and a final elongation step of 71 ℃ for 10 minutes.

For the sequencing reaction, the Dye Terminator Cycle Sequencing (DTCS) Quick start kit (Beckman Coulter, U.S.A.) and a CEQ 2000 capillary sequencer were used according to manufacturer's instructions. For the sequencing reaction, 5 *p*mol either of 27F or 1392R and 1 µL of purified PCR product were added. Cycling conditions consisted of 30 cycles of 95 ℃ for 30 seconds, 55 ℃ for 30 seconds and 60 ℃ for 4 minutes. The resulting sequence data were visualized using Chromas Lite version 2.01 and compared with gene sequences deposited in GenBank database using BLAST.

3.2.7 PHYLOGENETIC ANALYSIS

Phylogenetic analysis was carried out using the 16S rRNA gene sequences obtained from the isolates. Analysis of the sequences and construction of phylogenetic trees were performed using ARB software, as described in section 2.12.

3.2.8 EFFECT OF TEMPERATURE AND PH ON THE GROWTH OF ISOLATES

The effects of pH and temperature on growth of two of the isolates obtained, IESL25 (*L. ferriphilum*) and IESL32 (*At. ferrooxidans*), using ferrous iron as energy source, was examined using a 2 L bioreactor equipped with temperature, aeration, pH and stirring control (model P350, Electrolab, U.K.). Batch cultures were grown in pH- and temperature-controlled conditions. For both isolates, autotrophic basal salts (section 2.2) supplemented with 25 mM of ferrous iron as energy source, was used. Culture doubling times of exponentially-growing populations were calculated for each batch culture from semi-logarithmic plots of the iron oxidized against time.

3.3 RESULTS

3.3.1 PHYSICO-CHEMICAL CHARACTERISATION OF THE SAMPLES

Table 3.3 shows the physico-chemical parameters of the solutions (raffinate and PLS of each strip) at the time when samples were collected in January 2007. The mean ambient (air) temperature at the mine at this time was 14.7 ℃.

Table 3.3. Physico-chemical parameters and operation time (days) of solutions determined in January 2007. PLS solutions highlighted in bold were used for isolation of microorganisms.

Sample	Operation day	pH	Cu (g/L)	Eh (mV)	Sample temperature (℃)	Content of Pyrite
Raffinate	310	1.35	0.47	823	22,6	
Strip 1	309	1.68	2.54	830	19.5	2.1
Strip 2	289	1.75	2.82	829	16.0	2.0
Strip 3	250	1.78	3.42	831	16.6	2.3
Strip 4	207	1.92	3.46	841	17.8	2.5
Strip 5	142	1.96	3.46	845	17.5	2.4
Strip 6	55	2.15	3.76	775	14.5	2.4

In addition some other compounds which can impact bacterial activity were determined each month in samples from the PLS pond (Figure 3.2). Data obtained in January 2007 are shown in Table 3.4.

Table 3.4. Chemical characteristics of PLS pond sample.

Sample	Cl (g L^{-1})	Mg (g L^{-1})	Al (g L^{-1})	Mn (mg L^{-1})	SO_4^{2-} (g L^{-1})	NO_3^- (mg L^{-1})	Cu (g L^{-1})
PLS común	1.19	2.35	1.86	0.23	33.0	0.11	3.33

3.3.2 MICROBIAL POPULATIONS DETERMINED BY CULTURE-INDEPENDENT TECHNIQUES

The microbial population was studied at three scaling stages: pilot, demonstration and full (industrial) scale. Although each stage had a specific design differing in the solution circuits, operation size, and mineralogy of the ores, the microbial communities

were very similar, and the same species were detected by culture-independent techniques at the three scaling stages (data not shown).

A comprehensive study of the sequences retrieved by DGGE analysis from the industrial heap, revealed the presence of two strains of *At. ferrooxidans* (designated D2 and DM), two strains of *L. ferriphilum* (U and W), two strains of *Sulfobacillus* (G1 and Escondida), an a *Ferroplasma* sp.. During late 2009, *At. caldus* and *Sulfolobus metallicus* were also detected in the system in relatively low numbers (10^2-10^3 cells mL^{-1}). Figure 3.3 shows the spatial dynamics of the population in the system at the time when the samples were collected (January 2007) for the isolation of acidophilic microorganisms.

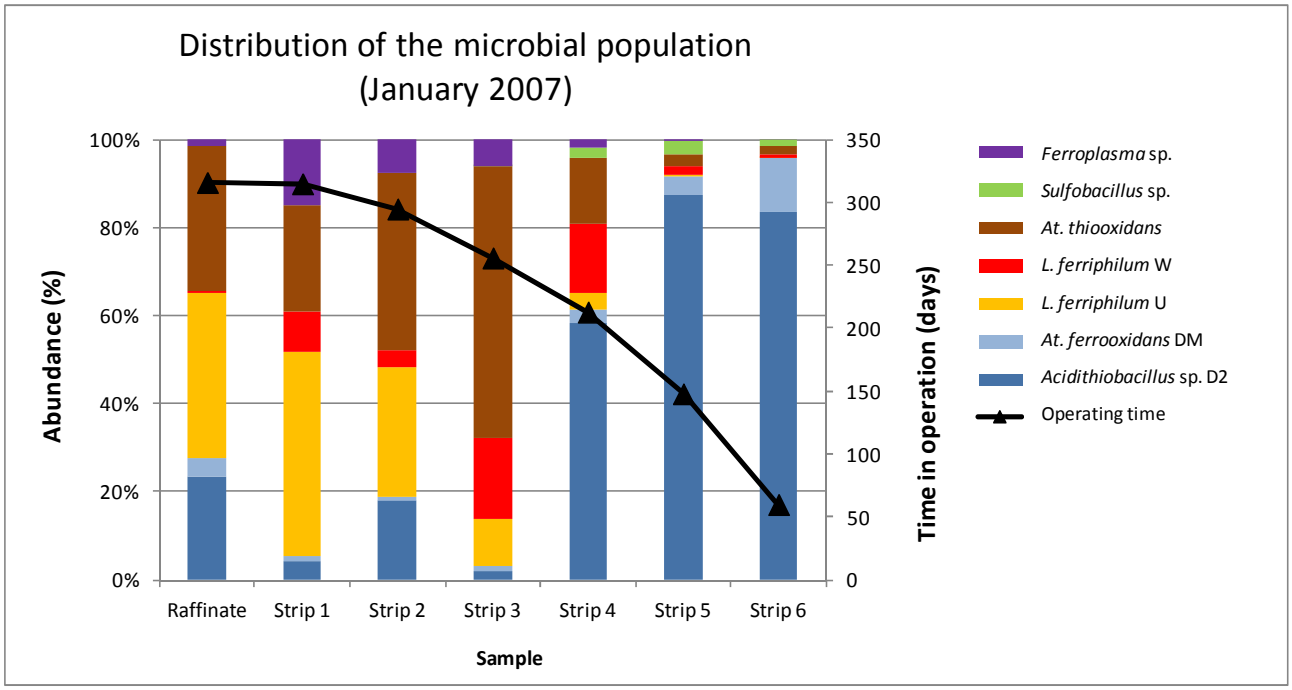


Figure 3.3. Microbial population determined by qPCR in samples from the full-scale industrial bioheap leach process, obtained in January 2007.

The relative abundance of each microorganism was different in the samples (PLS, Strip and raffinate solutions) probably as a result of differences in physico-chemical parameters of the leach solutions. Although qPCR results clearly showed the differences in the microbial populations of the different strips, it is important to bear in mind that the total number of microorganisms determined by this method will depend on the copy number of the 16S rRNA gene of each species. The species *At. ferrooxidans* have been described to have two copies of *rrn* genes (Venegas et al., 1988), while genomic data obtained from *At. thiooxidans* also indicated the presence of two *rrn* genes in the genome of this species. *L. ferriphilum* was shown to have 2 copies of rrn gene (Coram and Rawlings, 2002). Ferroplasma spp.and Sulfobacillus thermosulfidooxidans have shown to have 3 and 6 copies of rrn gene respectively (Zammit et al., 2008).

3.3.3 ISOLATION OF MICROORGANISMS

Preliminary identification of the isolates obtained from plating was made on the bases of the substrate oxidized on the solid media (e.g. iron-oxidizers were identified by their formation of ferric iron, producing orange/brown-coloured, encrusted colonies), and morphologies of the colonies. Figure 3.4 shows the morphologies of representative colonies obtained on solid media, and Table 3.5 shows the results of the classification and the process carried out to obtain liquid cultures of isolated microorganisms.

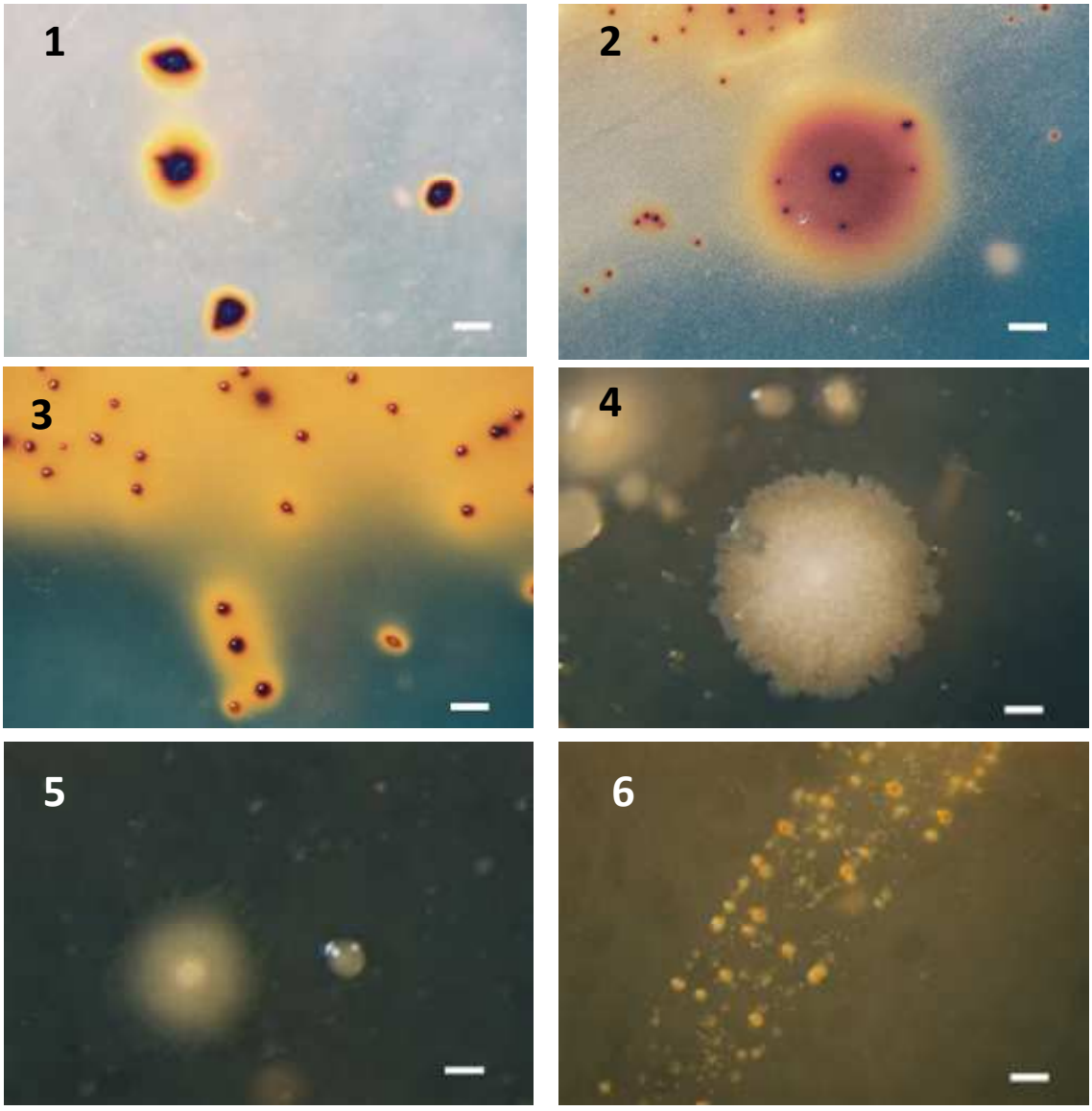


Figure 3.4 Morphologies of representative colonies obtained on solid media (bar= 1mm). Colonies shown in 1, 2, 3 and 6 are iron oxidizers representing respectively colonies 4, 13, 11 and 24 described in Table 3.5 and colonies shown in 4 and 5 are sulfur oxidizers representing respectively colonies 7 and 9 described in Table 3.5.

Table 3.5. Colonies, characteristics and growth in liquid media

Colony	Sample	Description	Incubation temperature (℃)	Plate	Transferred to:	Growth in liquid medium
1	PF1	Brown-orange, round	45	iFeo	Liquid autotrophic iron	-
2	PF1	Orange-yellow, round	45	iFeo	Liquid autotrophic iron	-
3	PF1	Orange, round	45	Feo	Liquid autotrophic iron	-
4	PF1	Brown, round	45	Feo	Liquid autotrophic iron	-
5	PF1	Orange, round, brown center, bright	45	FeSo	Liquid autotrophic iron	-
6	PF1	Orange, round	45	FeSo	Liquid autotrophic iron	-
7	PF1	White, round, regular border	30	FeSo	Liquid autotrophic tetrathionate	+
8	PF1	White, round, regular border	30	FeSo	Liquid autotrophic tetrathionate	+
9	PF1	White, round, regular border	30	FeSo	FeSo	+
10	PF1	White, round, irregular border	30	FeSo	FeSo	+
11	PF1	White, round, irregular border	30	iFeo	Liquid autotrophic iron	-
12	PF1	White, round, irregular border	30	iFeo	Liquid autotrophic iron	-
13	PF1	Brown, round dark center	45	FeSo	Liquid autotrophic iron	-
14	PF1	Brown, round dark center	45	FeSo	Liquid iron/YE	-
15	PF1	Brown, round, small colony (<1 mm)	45	FeSo	Liquid autotrophic iron	-
16	PF1	Brown, round, small colony (<1 mm)	45	FeSo	Liquid iron/YE	-
17	PF1	Brown, round, small colony (<1 mm)	45	FeSo	FeSo	+
18	PF1	Brown, round dark center	45	FeSo	FeSo	+
19	PF1	White, round, irregular border	30	FeSo	Feo	+
20	PLS-PD	White, irregular	45	FeSo	Liquid iron/YE	-
21	PF1	White, irregular	30	FeSo	FeSo	+
22	PF2	White, round	30	FeSo	FeSo	-
23	PF3	Brown, round	30	iFeo	iFeo	+
24	PLS-PD	Brown, white border	37	Fe/YE	Liquid iron/YE	+
25	PF1	Brown, round	30	iFeo	Liquid autotrophic iron	+
26	PF1	Brown, round, small colony (<1 mm)	45	FeSo	Liquid autotrophic iron	+
27	PF1	Brown, round dark center, big colony	45	FeSo	Liquid autotrophic iron	-
28	PF1	Brown, round dark center, big colony	45	FeSo	Liquid autotrophic iron	-
29	PF1	Brown, small colony	45	FeSo	Liquid autotrophic iron	-
30	PF1	Brown, round	30	iFeo	Liquid autotrophic iron	-
31	PF1	White, round, regular border	30	FeSo	Liquid autotrophic tetrathionate	+
32	PF1	Brown, round	30	Feo	Liquid autotrophic iron	+
33	PF1	White, round, irregular border	30	FeSo	Liquid autotrophic tetrathionate	+

3.3.4 IDENTIFICATION OF THE ISOLATES BASED ON 16S RRNA GENE SEQUENCES

Table 3.6 shows the identification of six the isolates obtained, based on their 16S rRNA gene sequences. Three isolates were identified as strains of *At. thiooxidans*, and the others identified as strains of *At. ferrooxidans*, *L. ferriphilum* and *Ferroplasma* sp.

Table 3.6. Identification of isolates obtained from the Escondida bioheap operation

Isolate	Designation	Identity	Similarity to type strain (%)	Type strain accession number (NCBI)
8	IESL8	*At. thiooxidans*	99.9	AJ224936
31	IESL31		99.9	
33	IESL33		99.7	
32	IESL32	*At. ferrooxidans*	99.6	AF465604
25	IESL25	*L. ferriphilum*	98.2	AF356829
24	IESL24	*Fp. acidiphilum*	99.0	Y11596

Figures 3.5, 3.6 and 3.7 show the phylogenetic affiliation of the isolates to known microorganisms.

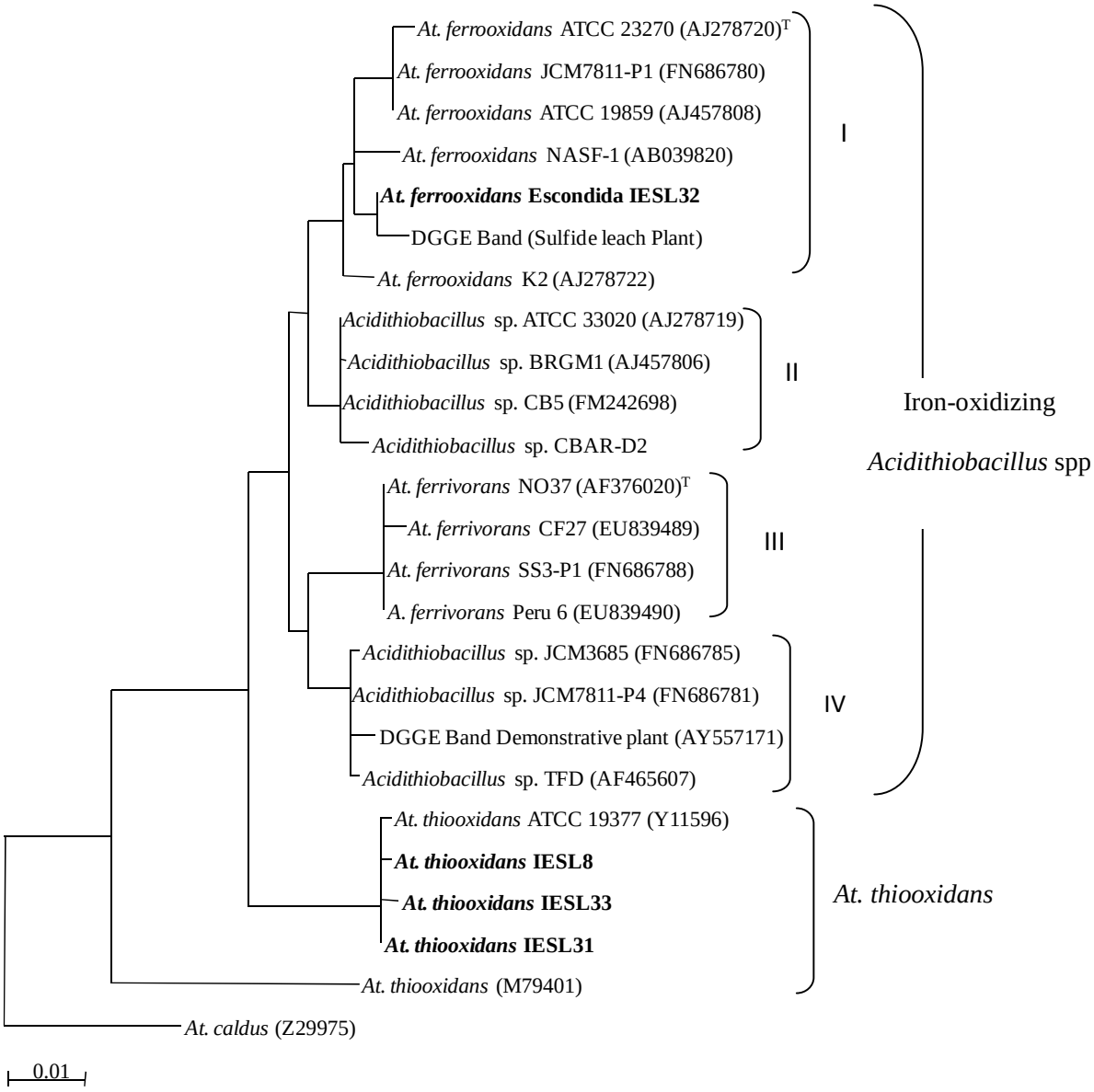


Figure 3.5. Phylogenetic affiliation of 16S rRNA gene sequences of isolates (in bold) from the Escondida mine, identified as members of the *Acidithiobacillus* genus. Groups I, II, III and IV were recently described as separate phylogenetic groups for iron oxidizer Acidithiobacilli (Amouric et al. 2010).Strain CBAR-D2 was isolated from PLS solution of column tests performed with ore from Escondida mine (Centro de Biotecnología, Universidad Católica del Norte, Antofagasta, Chile).

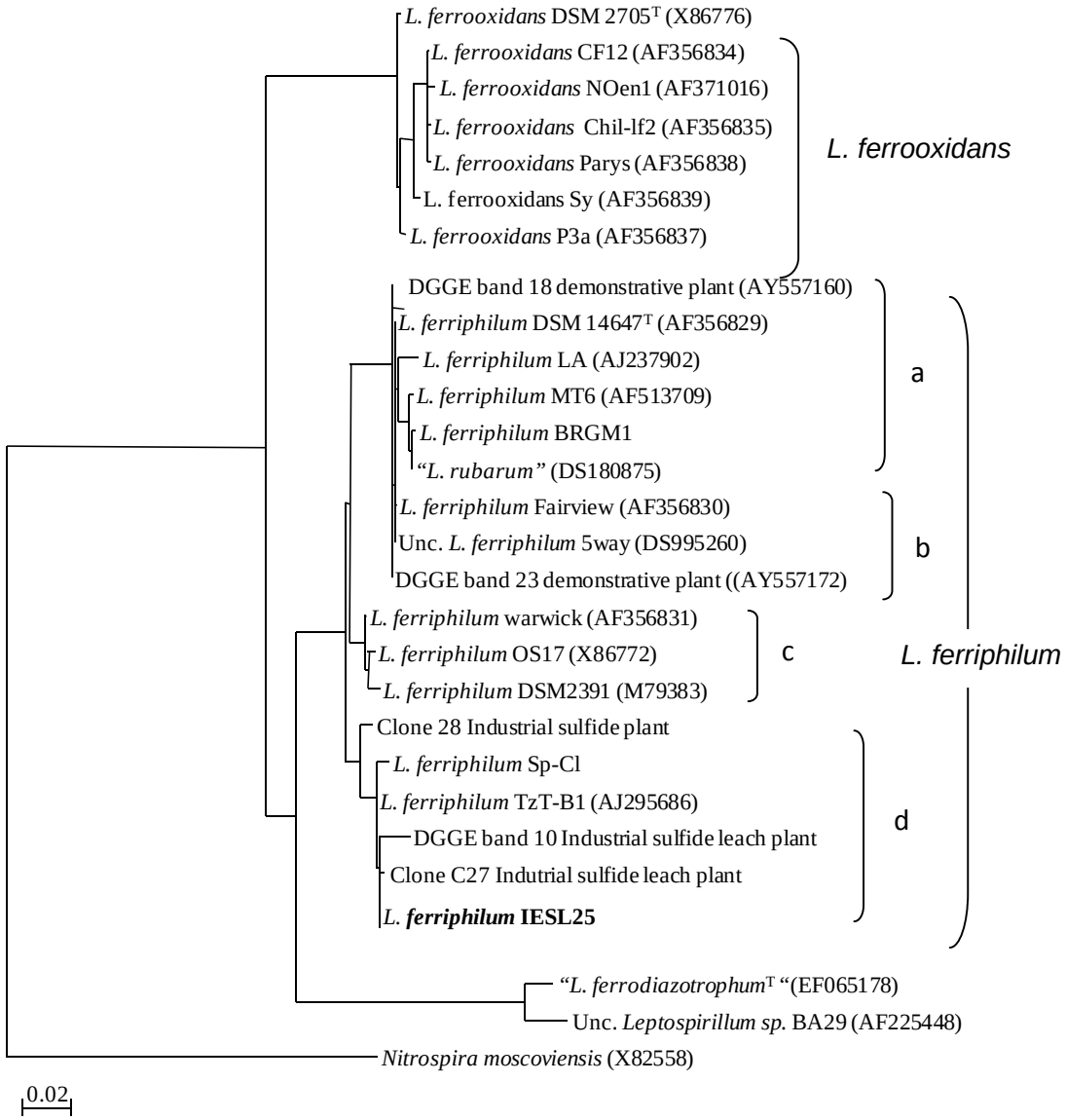


Figure 3.6. Phylogenetic affiliation of 16S rRNA sequence of the isolate (in bold) from the Escondida mine, identified as a strain of *L. ferriphilum*. Four clusters (a, b, c and d) can be distinguished in *L. ferriphilum*.

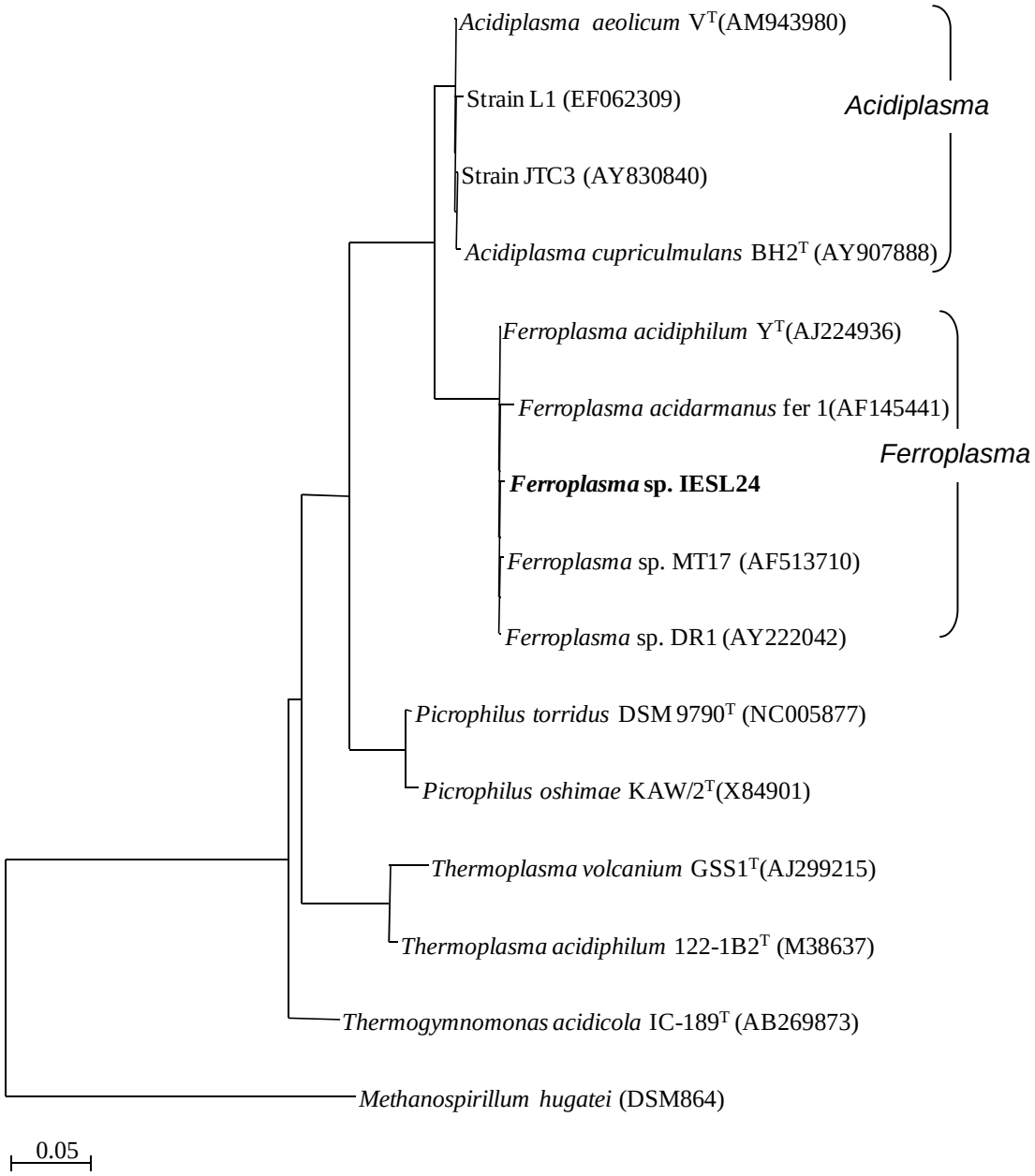


Figure 3.7. Phylogenetic affiliation of the 16S rRNA gene sequence of the isolate (in bold) from the Escondida mine, identified as a strain of *Ferroplasma*.

3.3.5 EFFECT OF TEMPERATURE ON GROWTH OF *ACIDITHIOBACILLUS FERROOXIDANS* IESL32 AND *LEPTOSPIRILLUM FERRIPHILUM* IESL25

The effect of pH and temperature on growth of isolate *At. ferrooxidans* IESL32 are shown in Figure 3.8. The minimum doubling time for this strain was 5.9 hours. Its pH optimum and minimum were 1.9 and 1.0, respectively, and its optimum and maximum temperature were 30 and 45 ℃, respectively. The effect of pH and te mperature on growth of isolate *L. ferriphilum* IESL25 are shown in Figure 3.8. The minimum doubling time for this strain was 5.9 hours. Its pH optimum and minima were 1.5 and 0.8, respectively, and its temperature optimum and maximum were 37 and 47 ℃, respectively .

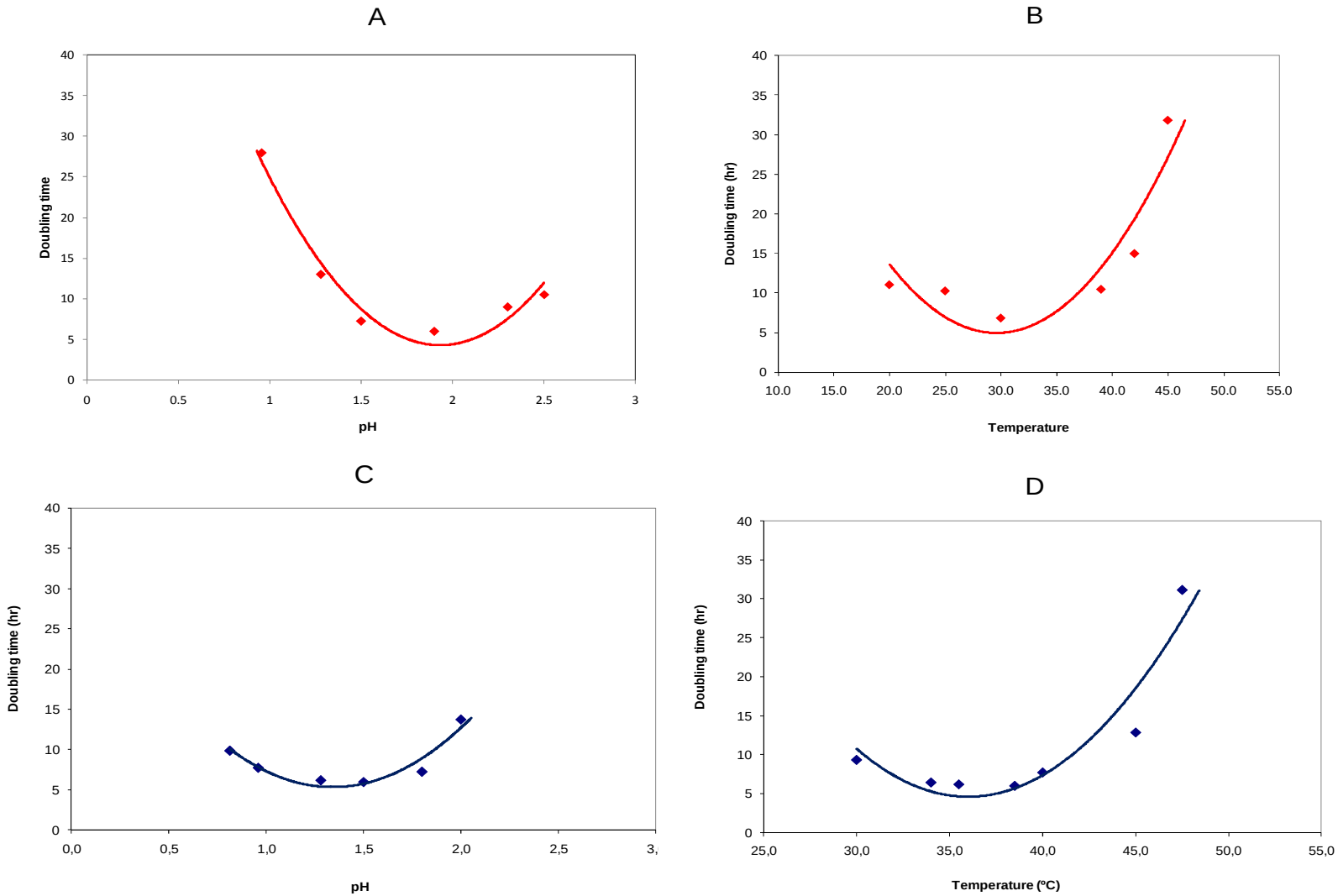


Figure 3.8 Effect of pH and temperature on growth of *At. ferrooxidans* strain IESL32 (A and B) and *L. ferriphilum* strain IESL25 (C and D).

3.4 DISCUSSION

3.4.1 CHEMICAL CHARACTERISTICS OF THE SAMPLES

The design of the industrial heap at Escondida involves the division of the first lift into 21 strips, each 120 m wide, 2000 m long and 18 m high. The PLS samples used for the isolation of the microorganisms were collected in January 2007, when 6 strips had been commissioned, with each having been in operation for different times (Table 3.3).

Although all the strips were irrigated with the same raffinate solution (Fig. 3.2), the physico-chemical parameters of the PLS from each of the strips were clearly different (Table 3.3 and Fig. 3.10). The pH values of the PLS decreased as the operation progressed in time. The lowest and highest pH values were observed in the raffinate solution (pH 1.35 due to the solvent extraction stripping process) and strip 6 PLS (pH 2.15), respectively. The relative high pH values in early stages (compared to pH of raffinate solution) can be attributed to the dissolution of acid-consuming materials, such as gangue and acid-soluble oxides and sulfides. Similarly, copper concentrations in the PLS were observed to decrease as time progressed. The high copper concentration observed during the early stages can also be attributed to the dissolution of chalcocite mediated by either by acid (Eq. 3.1) or ferric iron (Eq. 3.2). The oxidation of ferrous iron, which is catalyzed by acidophilic microorganisms in acidic liquors, is also an acid-consuming reaction (Eq. 3.3), and also probably contributed to the increase in pH values during the early stages of the process.

$$Cu_2S + 0.5O_2 + 2H^+ \rightarrow CuS + Cu^{2+} + H_2O \qquad \text{(Eq. 3.1)}$$

$$Cu_2S + 2Fe^{3+} \rightarrow CuS + Cu^{2+} + 2Fe^{2+} \qquad \text{(Eq. 3.2)}$$

$$2Fe^{2+} + 0.5O_2 + 2H^+ \rightarrow 2Fe^{3+} + H_2O \quad \text{(Eq. 3.3)}$$

Dissolution of covellite

$$2Fe^{3+} + CuS \rightarrow Cu^{2+} + 2Fe^{2+} + S^0 \quad \text{(Eq. 3.4)}$$

Other copper sulfides, such as chalcopyrite, also react with acid, consuming protons and increasing the pH (Eq.. 3.5). However, chalcopyrite is solubilised at about one fifth of the rate of chalcocite (Rear et al., 1994), and therefore chalcopyrite dissolution is likely to have a minor effect on the pH increase observed during first stages of the bioleaching process.

$$CuFeS_2 + O_2 + 4H^+ \rightarrow Cu^{2+} + Fe^{2+} + 2S^0 + 2H_2O \quad \text{(Eq. 3.5)}$$

In addition to its impact on solution pH, the dissolution of the ore and gangue materials generates a higher ionic strength in the PLS solutions.

The E_h in the samples ranged from +775 (PLS strip 6) to +823 mV (PLS strip 1). As with pH and copper concentrations, this parameter changed in the operation time, and a difference of 48 mV was observed between the highest and the lowest value. The E_h in the PLS is mainly determined by the ferric/ferrous iron couple and increased values were due to the oxidation of ferrous iron. Low values of E_h at late stages of bioleaching process can be more readily accounted for by the precipitation of ferric iron as jarosites, rather than being due to greater ferrous iron concentrations.

Pregnant leach solutions (PLS) samples from the six strips also differed in temperature. When the samples were collected, the temperature of strip 6 PLS was the lowest (14.5 ℃) and similar to that of the ambient air temperature (14.7 ℃), while strip 1

PLS was the warmest sample (19.5 ℃). However the PLS of strips 2 and 3 had lower temperatures than PLS of strips that had been in operation for shorter times (Table 3.2). An important parameter affecting the temperature in the process is the amount of pyrite in the ore. When this mineral is completely oxidized to ferric iron and sulfate, it releases a large amount of thermal energy as heat; calorimetric measurements have shown that the complete dissolution of pyrite releases up to 1546 kJ/mol (Rohwerder et al., 1998). As shown in Table 3.3, the difference in operation time and the content of pyrite in strip 2 and 3 could explain, at least in part, the lower temperatures of these strips. The relative high temperature of the raffinate solution (22.6 ℃) can be attributed to its passage through the solvent extraction process (Fig. 3.2).

In general, as the operation time progressed, many of the physico-chemical parameters of the PLS became increasingly extreme, including pH (more acidic), ionic strength (more elevated concentrations of sulfate), and redox potentials (more oxidizing), as shown in Figure 3.10 (data from Table 3.2).

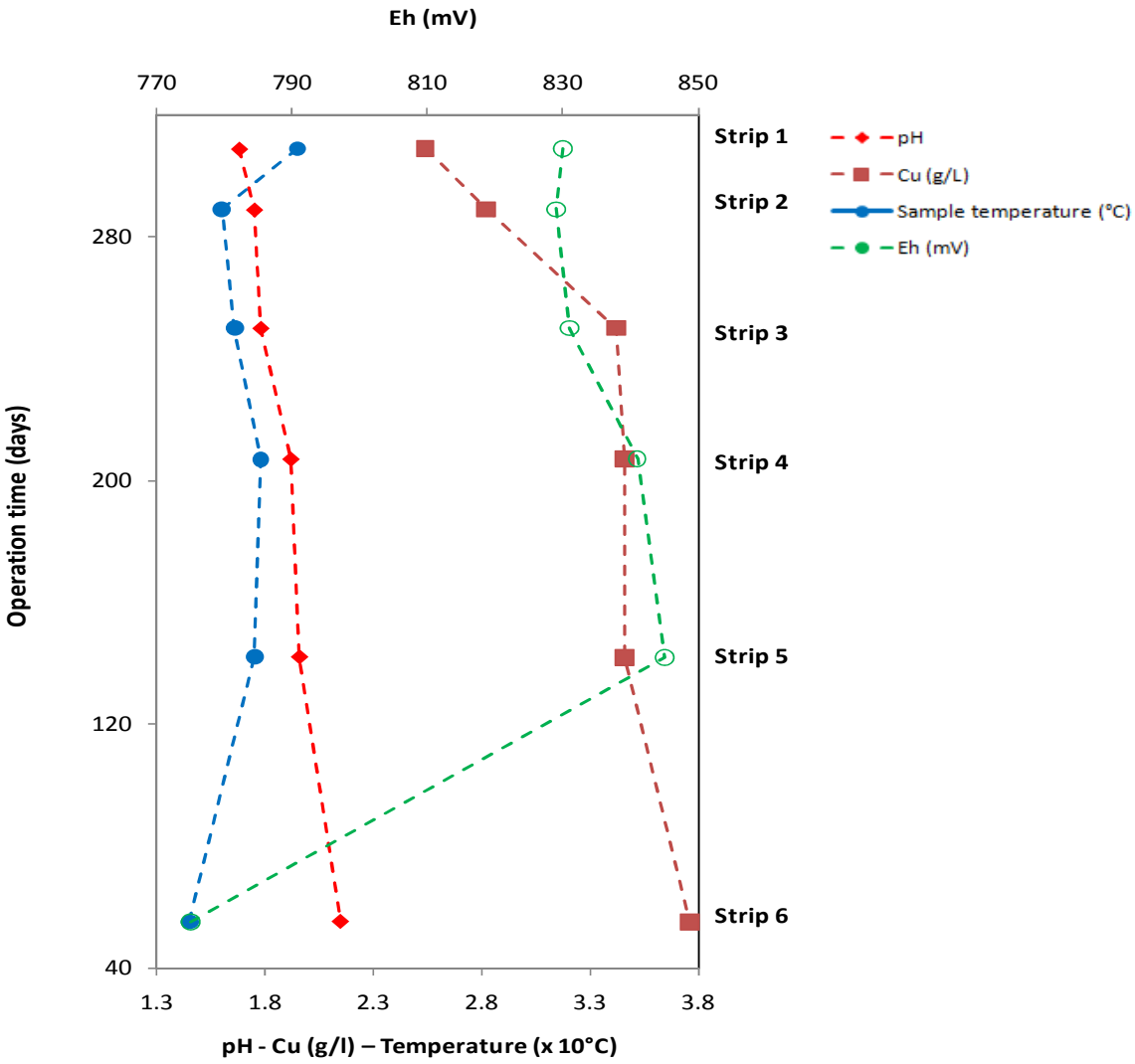


Figure 3.10. Physico-chemical parameters of the samples in relation to heap operation time

An inherent characteristic of heap systems is their spatial and temporal heterogeneity in terms of temperature, humidity, air distribution (O_2 and CO_2 concentrations), pH, redox potential, nutrients and solutes concentrations, among others. This heterogeneity generates a large number of microenvironments, which can allow differences in the abundance and diversity and of acidophilic microorganisms (Rawlings

and Johnson, 2007). The analyses performed on PLS samples have supplied valuable information about the abundance and dynamics of the microbial population in industrial heap processes. However, it is important to bear in mind that the changes observed in PLS samples represent an average of the highly variable conditions occurring in the heap, especially in the case of Escondida mine, where heaps are built with ROM material with no curing or crushing pretreatment.

3.4.2 ANALYSIS OF THE MICROBIAL POPULATION BY CULTURE-INDEPENDENT TECHNIQUES

The microbial components in the bioleaching systems (at demonstration heap and industrial-scale levels) at Escondida had been studied prior to the isolation of the microorganisms. Several culture-independent techniques, based on the amplification of 16S rRNA genes and including PCR-DGGE, PCR-clone library and quantitative PCR (qPCR), were used to reveal the phylogenetic identities and the dynamics of the microbial populations in the process. Several samples, including the raffinate solution and pregnant leach solutions were analysed in order to identify the prokaryotes inhabiting the system. Based on the comprehensive analysis of the 16S rRNA gene sequences obtained from the samples, the presence of at least two distinct strains of *At. ferrooxidans*, two of *L. ferriphilum*, two of *Sulfobacillus* spp., one of *At. thiooxidans* and one of the archaeon *Ferroplasma* were detected. The 16S rRNA gene sequences obtained by the different approaches were used to design specific primers to determine the numbers of each member of the microbial community present in the samples of the demonstration and industrial-scale operations by qPCR (Demergasso et al., 2005; Remonsellez et al., 2007).

Figure 3.3 shows the variation of the populations determined by qPCR in samples used for the isolation of microorganisms from the PLS samples. The population differed in PLS from the different strips, showing different relative abundances of acidophiles in each sample. A clear difference was observed in the population of PLS from strips 1 and 2, compared with those of strips 4, 5 and 6, while strip 3 PLS was unique in being dominated by *At. thiooxidans*. In the first group (PLS strips 1 and 2), *L. ferriphilum* was the dominant organism, while in the PLS from strips 4, 5 and 6 the dominant microorganism was *At. ferrooxidans*. In PLS of heaps with shorter operation times the pH and copper concentrations were higher, and the E_h, ionic strength and temperature were lower (compared with later stages) as noted previously, where *Acidithiobacillus* spp were shown to be the most abundant bacteria present. As time progressed, and PLS chemistry changed, and *L. ferriphilum* was noted to displace *Acidithiobacillus* spp. as the dominant acidophile present.

In general, in the industrial heap bioleaching of run-of-mine ore system at Escondida mine, the microbial population drives physico-chemical changes pushing the system to more extreme conditions (of pH, E_h, ionic strength, metal concentration, substrate (ferrous iron) availability, among others) and at the same time, the communities respond to these conditions by displaying changing relative abundances of acidophilic bacteria in different stages of the process. These results show how in the industrial heap bioleaching systems, the physico-chemical parameters and the microbial populations are closely integrated.

These results can be compared with those previously obtained from the acid mine drainage system in Iron Mountain (California, U.S.A), where researchers reported temperature and conductivity as the parameters that have the greatest impact in the composition of the acidophilic populations. When the conductivity and temperature increased in summer time, the relative abundance of the archaeon *Ferroplasma* also

increased (Edwards et al., 1999). Similarly, at Escondida, as the operation time progressed together with increase of ionic strength in the strips PLS solution, *Ferroplasma* became more abundant (Fig. 3.3). Also, *Leptospirillum* has previously been observed to dominate in systems at low pH and high ionic strength (Bond et al., 2000; Diaby et al., 2007; Okibe et al., 2003).

3.4.3 IDENTIFICATION AND PHYLOGENETIC ANALYSIS OF PROKARYOTIC ISOLATES

With the exception of isolate IESL24 (*Ferroplasma* sp.), which was isolated following enrichment of a stored PLS sample of the demonstration heap, all of the isolates were obtained by direct streaking of PLS samples onto overlay plates, without prior enrichment. Although the on-site temperatures of the samples ranged from 14.5 to 19.5 ℃, biomolecular data from heap bioleaching samples indicated the presence of microorganisms closely related to known species of mesophilic and thermotolerant organisms that have optimal growth temperatures of between 30 and 45 ℃ (Demergasso et al., 2005; Demergasso et al., 2003). Therefore overlay plates inoculated with the industrial samples were incubated at 30, 37 and 45°C. Autotrophic microorganisms were originally retrieved from plates incubated at 30 ℃ (Fig. 3.4), while strain IESL24 (a putative heterotroph) was retrieved from a FeSo overlay plate incubated at 37 ℃, inoculated with an enrichment culture (in "*Ferroplasma* medium"; Section 2.2) of a demonstration heap sample collected in October 2006..

The three that appeared (from their colony morphologies) to oxidize sulfur but not ferrous iron (IESL8, IESL31 and IESL33) were all closely related to *At. thiooxidans*^{T} (16S rRNA gene sequence similarities ranged from 99.7 to 99.9%). *At. thiooxidans* had not previously been detected in liquid samples at the demonstration plant by DGGE analysis. However, in 2009 several 16S rRNA gene sequences of bacteria closely related to *At.*

thiooxidans species were found in clone libraries retrieved from liquid samples from the industrial plant (Remonsellez et al., 2009). This confirmed that the overlay plate technique was an effective method for the detection and isolation of *At. thiooxidans* in liquid samples of the industrial bioleaching process at Escondida mine. The 16S rRNA gene sequences of isolates IESL31 and IESL33 were 100% similar to each other, though the isolates formed distinct and different colony morphologies on FeSo overlay plates (Figure 6). In addition, isolate IESL8 (which had 99% 16S rRNA gene similarity to IESL33, IESL31, and to *At. thiooxidans*^{T}) grew much faster in the presence of tetrathionate rather than in media containing elemental sulfur. Therefore, these three strains appear to differ somewhat in their physiologies, and probably represent different ecotypes in the Escondida bioleaching system.

From a phenotypic point of view, iron-oxidizing bacteria affiliated to the genus *Acidithiobacillus* constitute a heterogeneous cluster, and recently a novel psychrotolerant species, *At. ferrivorans*, has been described (Hallberg et al., 2010). The heterogeneity of iron-oxidizing acidithiobacilli was illustrated by Karavaiko et al. (2003) who applied polyphasic genotypic analysis to twenty-five *At. ferrooxidans* strains isolated from different regions of the world. The twenty-five strains were grouped into four clusters equidistant from *At. thiooxidans*. The authors did not find a geographic correlation among the isolates, but suggested a certain degree of correlation between the similarity of the genomes of *At. ferrooxidans* strains and the mineralogical characteristics of their habitats, suggesting the potential occurrence of micro-evolutionary processes in different econiches, which could promote the growth of strains with different phenotypic and genomic characteristics. In addition, Karavaiko et al. (2003) stressed the importance of taking into account what strains of *At. ferrooxidans* participate in biohydrometallurgical applications. Phenotypic differences observed among iron-oxidizing acidithiobacilli include the presence (or not) of flagella, optimal, maximum and minimum pH values and temperatures for growth, and

suggested different pathways for ferrous iron oxidation in these closely related species of bacteria (Amouric et al., 2011). Based on DNA-DNA hybridization and multi locus sequence analyses, Amouric et al., (2011) also suggested that iron-oxidizing acidithiobailli comprise more than the two species that are currently validated. The latter authors reported a DNA-DNA hybridisation value (DDHV) of 63% between strains ATCC 23270 (type strain, Group I) and ATCC 33020 (Group II). In addition, Tamizuka et al. (1976) reported a 60% DNA-DNA hybridization value between strains ATCC 19859 and 11Fe, affiliated to Groups I and II, respectively (Tomizuka et al., 1976). Since DDHVs of <70% are considered to indicate different species (Wayne et al., 1987), strains affiliated to Group II should not be considered as *At. ferrooxidans*. Isolate IESL32 was affiliated to Group I of iron-oxidizing acidithiobacilli (Fig. 3.5), which includes the type strain (ATCC 23270),

The 16S rRNA gene sequence of isolate IESL32 showed that this isolate was closely related to *At. ferrooxidans*^{T} (99.6%, 16S rRNA gene similarity). As mentioned in section 3.4.3, previous biomolecular data retrieved from the demonstration and industrial heap showed the presence of two different strains affiliated to Groups I and II of iron-oxidizing *Acidithiobacillus* spp. (based on the groupings described by Amoric et al., 2011). Isolate IESL32 was affiliated to Group I strains, and its 16S rRNA gene sequence was highly similar to a DGGE band sequence previously retrieved from liquid samples at the industrial bioleaching plant (Fig. 3.5). The closest related organism to IESL32 (100% 16S rRNA gene similarity) found in NCBI database was strain LMT4. This had been isolated from open-dump acidic mine tailings in China and it was reported to oxidize pyrite very efficiently (Tan et al., 2008).

To date, four species of *Leptospirillum* have been named, including "*L. ferrodiazotrophum*" (Tyson et al., 2005) and three validated species: *L. ferrooxidans* (Markosian, 1972), *L. thermoferrooxidans* (Golovacheva et al., 1993), and *L. ferriphilum* (Coram and Rawlings, 2002). Unfortunately, *L. thermoferrooxidans*, which was reported to

grow at moderately high temperatures (optimum ranging between 45 and 50 ℃) has been lost and is no longer available for further study (Johnson, 2001), though the information published on this bacterium (e.g. G+C, mole percentage of its genomic DNA) suggests that it might have been a strain of *L. ferriphilum*. In addition, a candidate (non-cultivated) species, "*L. rubarum",* has been proposed (Goltsman et al., 2009), though phylogenetic analysis has shown a close affiliation of this organism to *L. ferriphilum* (both are Group II leptospirilli; Fig. 3.6).

Isolate IESL25 was identified as a strain of *L. ferriphilum* (its 16S rRNA gene sequence was 98.2% similar to that of *L. ferriphilum*^{T}). Based on 16S rRNA gene sequences, four phylogenetic clusters can be distinguished among strains of *L. ferriphilum* (Fig. 3.6). Isolate IESL25 was affiliated to Group II *Leptospirillum* (98.2% 16S rRNA gene sequence similarity to type strain). Previous molecular data showed the presence of 16S rRNA gene sequences affiliated to clusters "a", "b" and "d" in bioleach liquors at the Escondida mine. The sequences obtained from the demonstration plant were affiliated to clusters "a" and "b", while sequences obtained from the industrial plant were affiliated to cluster "d". The 16S rRNA sequence gene of isolate IESL25 was also affiliated to cluster "d", together with clones "C27" and "28" obtained from the industrial heap bioleaching plant (Remonsellez et al., 2009).

Culture-independent approaches performed on samples from the industrial and demonstration bioleach heaps showed the presence of 16S rRNA gene sequences related to *Ferroplasma,* and this was the only archaeon detected in both situations (Demergasso et al., 2005; Remonsellez et al., 2009). No *Ferroplasma*-like archaeon was, however, isolated from Escondida samples in the current work.

3.4.4 GROWTH CHARACTERISTICS OF ISOLATES IESL32 AND IESL25

A key metabolic trait, strongly influencing the bioleaching of copper in low grade sulfide ores, is the ability of some acidophilic prokaryotes to oxidize iron, generating ferric iron, which acts as the oxidant for the dissolution of metallic sulfide minerals. Therefore the ability to oxidize ferrous iron at different temperatures and pH values of isolates IESL32 (*At. ferrooxidans*) and IESL25 (*L. ferriphilum*) was studied (Fig. 3.8).

Strain IESL32 showed optimal growth (while oxidizing ferrous iron) at a temperature of ca. 30℃, and a pH of ca.1.9, with a minimum doubling time of 5.9 h recorded. This doubling time was similar to growth rate (equivalent to doubling time 6 h) reported for *At. ferrooxidans*^{T} (Tuovinen and Kelly, 1974). The optimal pH and temperature of IESL32 was similar to that of several other strains of iron-oxidizing acidithiobacilli previously isolated. However, two unusual characteristics of this strain were its ability to oxidize iron at relatively high temperatures (up to 45℃, doubling time 31.8 h) and at low pH (doubling time 28.0 h at pH 1.0). In the first comprehensive study of iron-oxidizing acidithiobacilli based on the DNA homologies, Harrison (1982) identified strains that were, like IESL32, closely related to *At. ferrooxidans*^{T}, and several of these (including strain ATCC 19859, which is phylogenetically closely related to IESL32; Fig. 3.5) were also noted to grow at 40 ℃. The unusual characteristic of IESL32 to grow by ferrous iron oxidation at 45 ℃ could play a key role as the bio heaps mature, as the average temperature of the system has continued to increase (to 27.2 ℃, measured in PLS samples during October, 2010). In addition it will be important to determine the growth temperature optimum and maximum of strain CBAR-D2, which was also isolated from the Escondida mine. While strain IESL32 was able to grow using ferrous iron as electron donor at pH 1.0, the minimum growth pH for *At. ferrooxidans*^{T} has been reported as being

pH 1.3 (Hallberg et al., 2010). These characteristics would confer a competitive advantage to strain IESL32 in warmer and more acidic environments.

L. ferriphilum IESL25 showed optimal pH value and temperature for growth of 1.5 and 37 ℃, respectively, and a minimum doubling time of 5.9 h was recorded at these values. The maximum temperature at which IESL25 was observed to grow was 48 ℃ and no growth was observed at 50℃. *L. ferriphilum*^{T} ATCC 49881 was reported to have optimum growth at pH values between 1.4 to 1.8, and a temperature optimum of between 30 and 37 ℃. *L. ferriphilum* strain MT6, isolated from a commercial pilot plant stirred-tank bioleaching operating at 45℃ and also affiliated to *L. ferriphilum,* was reported to have a minimum culture doubling time of about 2 h, and optimum temperature and pH of 43 ℃ and 1.5, respectively. Strain MT6 was observed to grow up to 50 ℃ and the pH range reported was 0.8 to 2.0; values below and above were not tested (Okibe et al., 2003). Interestingly for strain MT6, the pH of the medium had a relatively minor effect on the culture doubling time (~ 2h between optimum and minimum pH tested). *L. ferriphilum* strains L8 and L6 were isolated from a culture being prepared for a commercial cobaltiferous pyrite ore bioleaching operation in France and firstly described as *Leptospirillum*-like bacteria. These strains showed an optimum pH in the range of 1.3 to 1.8 and an optimum temperature of 37.5℃. Both strains showed similar physiological traits to *L. ferrooxidans*, though the optimum growth temperature (37.5 ℃) for both strains was slightly higher to those reported for *L. ferrooxidans* (Battaglia et al., 1994). In addition the G+C content of L8 strain was 55.6 mol%.

Coram and Rawlings (2002) also noted that the temperature range for growth was a key distinctive characteristic between *L. ferrooxidans* and *L. ferriphilum*, suggesting that strains of *L. ferrooxidans* are non-competitive at temperatures between 35 to 45℃, which is probably why *L. ferriphilum* dominates commercial processes operating between 40 to 45℃. Therefore it would be interesting to determine whether strains of *L. ferrooxidans*

could be found in industrial heap leaching or aeration tank-type processes that operate at temperatures lower than 35℃. Interestingly, only s equences related to *L. ferriphilum* were found in culture-independent studies performed in liquid and solid samples of the industrial heap leaching process at Escondida mine, even though the highest temperature recorded in any of the PLS samples was only 22.6 ℃. The app arent exclusivity of *L. ferriphilum* (of *Leptospirillum* spp.) was supported by the isolation of strain IESL25, affiliated to *L. ferriphilum*, but not of any *L. ferrooxidans* strains. Therefore, some other factor may be restricting colonisation of the heaps by *L. ferrooxidans.* This issue is addressed further in Chapter IV.

CHAPTER 4: TRANSITION METALS AND SALT TOLERANCE IN *LEPTOSPIRILLUM* SPP., AND BIOLEACHING OF A COPPER CONCENTRATE BY PURE AND MIXED CULTURES OF *L. FERRIPHILUM* IN THE PRESENCE OF SODIUM CHLORIDE.

4.1 INTRODUCTION

Natural and industrial acidic environments represent a major challenge to life. Within these environments, strong selective pressures are generated by extreme physico-chemical conditions, such as low pH, elevated concentration of metals and sulfate, and other factors. As a result, the microbial communities that inhabit these environments are composed of relatively few prokaryotic taxa. Moreover, in commercial biomining applications selective pressures conditioned by operational parameters often result in the colonization by only few species of extremophilic microorganisms. Among these are the iron-oxidising chemolithotrophic bacteria, *Leptospirillum* spp..

A key characteristic that differentiates *Leptospirillum ferriphilum* and *Leptospirillum. ferrooxidans* is their temperature ranges for optimum growth, with most strains of *L. ferriphilum* growing optimally at higher temperatures than those of *L. ferrooxidans*. This is the explanation suggested for why, in commercial processes operating to 40 ℃ or higher *L. ferriphilum* has been found as the more dominant species (Coram and Rawlings, 2002; Okibe et al., 2003). The maximum temperatures recorded in the industrial PLS samples and inside the demonstration heap at Escondida at the time of sampling were, however, less than 30℃, but interestingly only *L. ferriphilum* (among *Leptospirillum* spp.) was detected and isolated from samples from this mine (Chapter 3). Therefore, it was postulated that factor(s) other than temperature were more

critical in determining which *Leptospirillum* sp. emerged as the dominant species at the mine site.

In acidic environments, transition metals can also enforce selective pressures on indigenous microbial populations, allowing only tolerant or adapted microorganisms to thrive when these metals are present at elevated concentrations (Duxury and Bicknell, 1983). The production of copper cathodes at Escondida mine uses solvent extraction and electrowinning (SX-EW), and this requires an optimum range of copper in PLS solution of ~ 4.0 to 6.0 g/L. The presence of copper at such high concentrations may be an important parameter producing a strong selective pressure on microbial populations and determining the presence of only adapted or tolerant species or strains of *Leptospirillum*.

Another parameter that can stress not only *Leptospirillum* spp., but the entire microbial community at heap bioleaching operations is the elevated concentration of solutes that often arises. The Escondida mine is located in the Atacama Desert, which is one of the driest regions of the planet with average precipitation of less than 1 mm per annum. Water sources near the mine site are limited to fossil water aquifers that support delicate desert-like ecosystems (salt flats and salt ponds) and are not available for industrial use. In order to alleviate the lack of water, the design of hydrometallurgical processes at the Escondida mine involves a constant recycling of the solutions in the systems, with a consequent increase of the concentration of sulfate and other solutes. A low cost alternative to the use of fresh water, which could also result in beneficial effects on the dissolution of refractory sulfides, would be the use of saline water to irrigate the heaps. However, this would require using salt-tolerant, mineral-oxidising acidophiles (Dutrizac and MacDonald, 1971). In this regard, the recent development of a method for treating sulfide minerals in presence of chloride using an iron-oxidising microorganism isolated from the Spence mine in Chile, was recently patented in the United States by

BHP-Billiton in cooperation with Centro de Biotecnología, Universidad Católica del Norte, Chile (Rauntenbach et al., 2010). This chapter describes the effect of four transition metals (copper, zinc, nickel and silver) on strains of three species of *Leptospirillum* spp., including *L. ferriphilum* strain IESL25 which was isolated from Escondida mine in the course of the present studies. Clear differences in tolerance to two of these metals was found with different *Leptospirillum* spp.. The potential of salt-tolerant acidophiles for bioleaching a copper sulfide concentrate in bench-scale bioreactors containing elevated concentrations of sodium chloride was also assessed.

4.2 MATERIALS AND METHODS

4.2.1 MICROORGANISMS

The strains used in these experiments were originally isolated from different locations worldwide, and include those isolated from two Chilean copper mines (Escondida and Spence; Table 4.1).

4.2.2 TRANSITION METAL TOLERANCE IN *LEPTOSPIRILLUM* SPP.

The tolerance of the different species and strains of *Leptospirillum* spp. listed in Table 4.1 to copper, silver, nickel and zinc was tested in liquid media. Stock solutions of the four metals were prepared in autotrophic basal salts (ABS, section 2.1), adjusted to the same pH as the culture medium used. Ferrous iron medium (50 mM Fe^{2+}, pH 1.5, section 2.2) was used to prepare starter cultures of the *Leptospirillum* strains in universal bottles, and each culture was sub-cultured into increasing concentrations of each of the

four transition metals until bacterial growth was totally inhibited (monitored over a period of 14 days). Culture growth was examined by measuring changes in ferrous iron concentrations during culture incubation.

Table 4.1 Bacteria used in this study

Bacterium	strain	Origin	Reference
L. ferrooxidans	DSM 2705^{T}	Cu mine, Armenia.	(Hippe 2000)
	CF12	Idaho Co mine. U.S.A.	(Johnson 1995)
	SY	Sygun Cu mine, North Wales, U.K.	(Johnson 1995)
L. ferriphilum	ATCC 49881^{T}	Lima, Perú.	(Sand et al. 1992)
	BRGM1	Cobalt-rich pyrite concentrate, Kasese, Uganda.	(d'Hugues et al. 2007)
	MT61	Commercial pilot plant stirred-tank bioleaching operating at 45℃.	(Okibe et al. 2003)
	IESL25	Escondida Bioleaching plant, Chile.	(This work)
	Sp-Cl	Spence mine, Chile.	(Rautenbach et al. 2010)
"L. ferrodiazotrophum"	BA-1181	Acid mine drainage biofilm, Iron Mountain, U.S.A.	(Tyson et al. 2005)
At. thiooxidans	IESL33	Escondida Bioleaching plant, Chile.	(This work)
Sb. thermosulfidooxidans	CBAR13	Escondida Bioleaching plant, Chile.	(Centro de Biotecnología UCN, Chile)
Acidiphilium sp.	SJH	Cae Coch pyrite mine, North Wales, U.K.	(Johnson and McGinness 1991)

4.2.3 TOLERANCE TO SODIUM CHLORIDE BY *LEPTOSPIRILLUM* SPP. AND SOME OTHER ACIDOPHILIC BACTERIA

The tolerance of *Leptospirillum* spp. and the other acidophiles listed in Table 4.1 to sodium chloride was tested in liquid media. Sodium chloride was prepared in MilliQ-grade water as a 5.0 M stock solution, heat-sterilized (121 ℃ for 20 minutes), and added to suitable growth media for the different acidophiles. *Leptospirillum* spp were grown in

ferrous iron medium containing 50 mM of ferrous iron (section 2.2); *At. thiooxidans* was grown in autotrophic sulfur medium (section 2.2); *Sb thermosulfidooxidans* was grown in 0.02% (w/v) yeast extract medium containing 50 mM of ferrous iron (section 2.2); and *Acidiphilium* sp. SJH was grown in 10 mM galactose medium (section 2.2). Bacteria were sub-cultured into increasing concentration of sodium chloride in universal bottles until microbial growth appeared to be completely inhibited. Culture growth was examined by measuring changes in ferrous iron concentrations in cultures of iron-oxidizers, and by direct cell counts for *At. thiooxidans* IESL33 and *Acidiphilium* sp. SJH. Table 4.2 shows the culture conditions for the organisms tested for tolerance to selected metals and salt.

Table 4.2 Culture conditions used to determine the tolerance of selected acidophlic bacteria to some transition metals and sodium chloride.

Bacterium	Liquid medium*	pH	Temp.(℃)
Leptospirillum spp.	50 mM Fe^{2+}, ABS, TE.	1.5	37
At. thiooxidans	S^0 (1%), ABS, TE.	1.8	30
Sb. thermosulfidooxidans	50 mM Fe^{2+}, 0.02% w/v yeast extract , HBS, TE.	1.8	45
Acidiphilium sp. SJH	10 mM galactose , HBS, TE.	2.0	30

*ABS, autotrophic basal salts; HBS, heterotrophic basal salts; TE, trace elements.

4.2.4 EFFECT OF COPPER ON THE GROWTH OF *L. FERRIPHILUM* IESL25

The effect of copper on the growth of *L. ferriphilum* IESL25 was studied in shake flask cultures. Experiments were carried out by duplicate in 250 mL Erlenmeyer flasks, incubated at 37 ℃ in an orbital incubator for 14 d ays. Culture flasks containing 50 mM ferrous iron medium (pH 1.5, section 2.2) were supplemented with either 150 mM of copper or magnesium sulfate. Flasks were inoculated with an active culture of *L. ferriphilum* IESL25 (5% v/v), previously grown in the non-supplemented ferrous iron

medium. Each flask contained 100 mL as final volume and water lost by evaporation was compensated periodically by adding sterile MilliQ-grade water. Total cells were enumerated by direct counts (section 2.5.1) and inorganic iron overlay plates (iFeo) were used periodically to obtain numbers of active bacteria (section 2.4). In addition, ferrous iron oxidation by the cultures was monitored (section 2.6.1). Growth rates were calculated for cultures from semi-logarithmic plots of ferrous iron oxidized against time. Similarly, following completion of iron oxidation, bacterial mortality rates were calculated from semi-logarithmic plots of viable cells against time.

4.2.5 EFFECT OF SODIUM CHLORIDE ON THE GROWTH OF *L. FERRIPHILUM* SP-CL

The effect of sodium chloride on the growth of *L. ferriphilum* Sp-Cl was tested in a 2 L bench-scale bioreactor using liquid medium containing 25 mM Fe^{2+} and ABS(pH 1.5; section 2.2). The working volume of the reactor was 1.5 L, and temperature and pH were set at 37℃ and 1.5, respectively. Strain Sp-Cl was first grown in absence of NaCl and a minimum of two experimental runs were carried out for each NaCl concentration tested. When ferrous iron was completely oxidized the spent media was removed, leaving ~100 mL of culture as the inoculum for the following run. For sequential experiments, fresh medium containing increasing concentrations of NaCl was added to the reactor. Growth was monitored by measuring ferrous iron oxidation (section 2.6.1) periodically in each experimental run. Culture doubling times were calculated for each experimental run from semi-logarithmic plots of ferrous iron oxidized against time.

4.2.6 THE COMBINED EFFECT OF CHLORIDE AND COPPER ON THE GROWTH OF *L. FERRIPHILUM* SP-CL

The effect of increasing copper concentrations in the presence of chloride, and the effect of increasing chloride concentrations in the presence of copper, on the growth of *L. ferriphilum* strain Sp-Cl were tested in shake flask cultures. Experiments were carried out by duplicate in 250 mL Erlenmeyer flasks containing 100 mL of liquid medium and incubated at 37 ℃ in an orbital shaker at 150 rpm for 12 days. To study the effect of increasing concentrations of sodium chloride in the presence of copper, flasks containing 50 mM ferrous iron medium (pH 1.5) and 200 mM of copper sulfate were supplemented with sodium chloride at final concentrations of 50, 100 and 200 mM. Parallel tests were carried out using a single concentration (200 mM) of NaCl and variable concentrations (50, 100 and 200 mM) of copper sulfate. Each flask was inoculated with an active culture of *L. ferriphilum* Sp-Cl previously grown either in the presence of 200 mM of copper sulfate to examine the effect of sodium chloride, or 200 mM of NaCl to examine the effect of copper sulfate. Growth was monitored by measuring ferrous iron oxidation in each culture as described in section 2.6.1. Culture doubling times were calculated from semi-logarithmic plots of ferrous iron oxidized against time. Experiments were performed in duplicate and controls cultures containing no added copper sulfate or sodium chloride were also prepared and evaluated.

4.2.7 BIOLEACHING OF A COPPER CONCENTRATE TESTS IN THE PRESENCE OF SODIUM CHLORIDE

Bioleaching of a copper concentrate by pure and mixed cultures of *L. ferriphilum* Sp-Cl, in the presence and absence of sodium chloride was studied in 2 L bench-scale

bioreactors. The copper concentrate was obtained from former Alliance Copper high temperature bioleaching plant at Chuquicamata, Chile. Table 4.3 shows the mineralogical composition of the copper concentrate determined by Quemscan analysis (Centro de Análisis mineralógico, Universidad Católica del Norte-BHP-Billiton, Antofagasta, Chile).

Table 4.3 Mineral composition of copper concentrate.

Mineral	**Idealised composition**	**(wt. %)**
Chalcocite	Cu_2S	9.40
Chalcopyrite	$CuFeS_2$	5.80
Brochantite	$Cu_4SO_4(OH)_6$	4.43
Covellite	CuS	2.30
Chrysocolla	$(Cu,Al)_2H_2Si2O_5(OH)_4 \cdot nH2O$	0.25
Atacamite	$Cu_2Cl(OH)_3$	0.13
Other copper sulfides including enargite, tennantite, bornite.	Cu_3AsS_4; $Cu_{12}As_4S_{13}$ and others	10.60
Pyrite	FeS_2	30.47
Copper oxides		0.01
Others (gangue)		36.61

In order to remove acid-soluble copper minerals and other acid-soluble components, 350 g of concentrate was washed at room temperature for 6 hours in 2.0 L of MilliQ-grade water acidified with H_2SO_4, at a constant pH of 1.7. After acid washing, the concentrate was filtered and washed again using Milli-Q grade water. The concentrate was then dried (37℃) overnight and 75 g were added to each reactor for the bioleaching test.

The temperature (37 ℃), pH (1.7) and working volum e (1.5 L) were the same for all four bioreactors which were run in parallel. Approximately 9 $x10^9$ cells of each bacterial species were inoculated in reactors 2, 3 and 4, as shown in Table 4.4. All four

reactors contained heterotrophic basal salts (section 2.1.1), trace elements (section 2.1.3) and 25 mM ferrous iron. Reactor 1 was used as an abiotic control and, like reactors 3 and 4, was supplemented with 200 mM NaCl. Reactors 2 and 3 were inoculated with pure cultures of *L. ferriphilum* Sp-Cl, while reactor 4 was inoculated with a mixed culture of acidophilic bacteria (Table 4.4).

Table 4.4 Bacteria and NaCl concentrations used in the reactor bioleaching experiment.

Reactor	NaCl (mM)	Inoculum
1	200	None
2	0	*L. ferriphilum* Sp-Cl
3	200	*L. ferriphilum* Sp-Cl
4	200	*L. ferriphilum* Sp-Cl; *At. thiooxidans* IESL33; *Acidiphilium* sp. SJH; *Sb. thermosulfidooxidans* CBAR-13;

The four bioreactors were run in parallel for 35 days, and samples were taken every 5 days to measure redox potentials (E_h) and the concentrations of soluble copper, iron and sulfate (section 2.6.1). Arsenic was determined by hydride-generation atomic absorption spectrometry using a Varian spectrometer model SpectrAA-50 (Agilent technologies, U.S.A.).

To monitor the microbial populations in the bioreactors, 100 mL aliquots were removed from each bioreactor at days 5 and 35, and filtered through cellulose nitrate membranes (0.2 µm pore diameter). DNA was extracted from each membrane as described in section 2.7.1, and was used for DGGE and qPCR analyses. DGGE and qPCR were performed as described in sections 2.8.4 and 2.10, respectively.

After 35 days of leaching, the concentrate residues were collected from each reactor by filtration through filter papers (Whatman N° 2). The filters containing the residues were dried overnight (37 ℃) and each resi due in the filter was weighed at room temperature several times until constant weight was recorded. The mass of concentrate residue was calculated by subtracting the weight of each pre-weighed filter from the final weight recorded after drying. The copper content in residues and initial concentrate were determined by dissolving 1.0 gram sub-samples of concentrate in *aqua regia* (3 volumes of hydrochloric acid and 1 volume of nitric acid) and determining the concentration of copper by atomic absorption spectrometry.

The relative abundance of mineral species in the concentrate residues of each reactor and the acid-washed concentrate were determined by Quemscan analysis at the Centro de análisis mineralógico UCN-BHP Billiton (Chile).

4.3 RESULTS

4.3.1 TOLERANCE OF *LEPTOSPIRILLUM* SPP. TO CU, AG, NI AND ZN

Overall, the four strains of *Leptospirillum ferriphilum* tested displayed greater tolerance to three of the four transition metals than did the three strains of *Leptospirillum ferrooxidans* and the single available strain of *"L. ferrodiazotrophum"* (Fig. 4.1). This was particularly evident in the case of copper. The maximum copper concentration tolerated by *L. ferrooxidans* and *"L. ferrodiazotrophum"* ranged between 10 and 20 mM, while that tolerated by *L. ferriphilum* strains ranged between 200 to 800 mM (Fig. 4.2). Silver was also inhibited the growth of *"L. ferrodiazotrophum"* and *L. ferrooxidans* strains at lower concentrations than the *L. ferriphilum* strains. The concentration of silver tolerated by *L.*

ferriphilum was between 100 and 200 µM, while for *"L. ferrodiazotrophum"* and *L. ferrooxidans* it ranged between 30 and 50 µM. In contrast, nickel tolerance to was similar in all three *Leptospirillum* species, ranging between 10 to 100 mM, with the most tolerant strain identified being the type strain of *L. ferrooxidans*. The maximum tolerance to zinc observed for *L. ferriphilum* zinc ranged between 200 to 500 mM, while for *L. ferrooxidans* and *"L. ferrodiazotrophum"* the range was between 30 and 250 mM (Fig. 4.1).

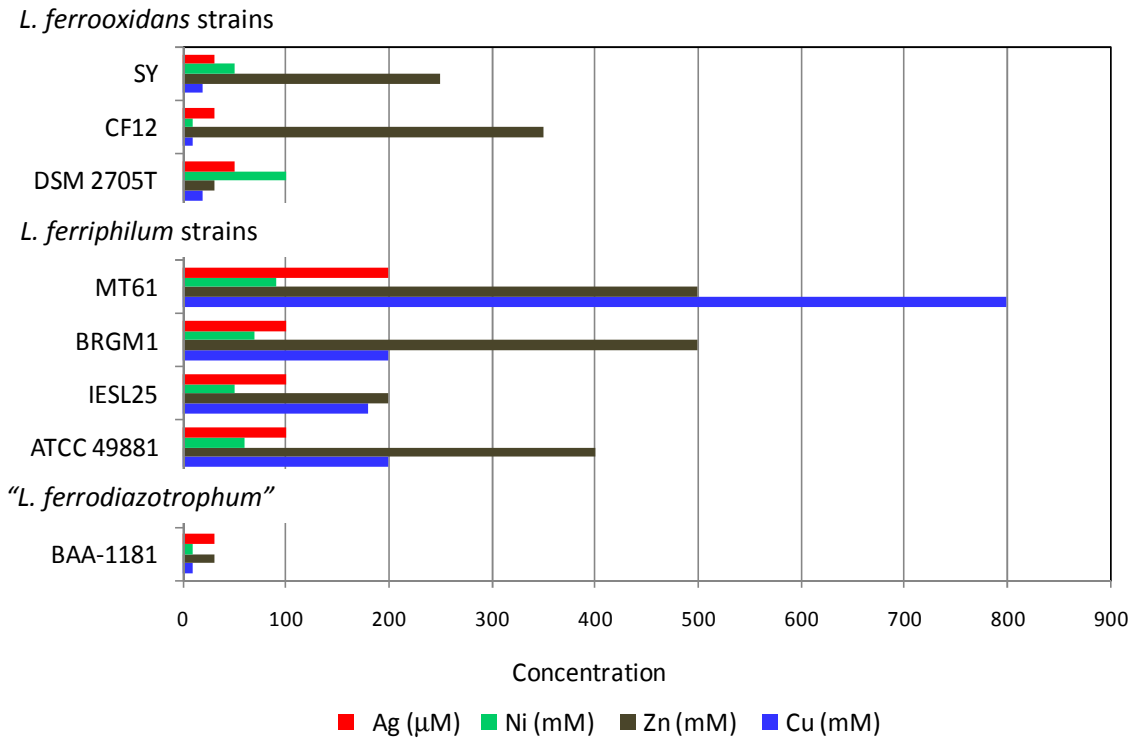


Figure 4.1 Maximum concentrations of four transition metals tolerated by different strains of *Leptospirillum* spp.

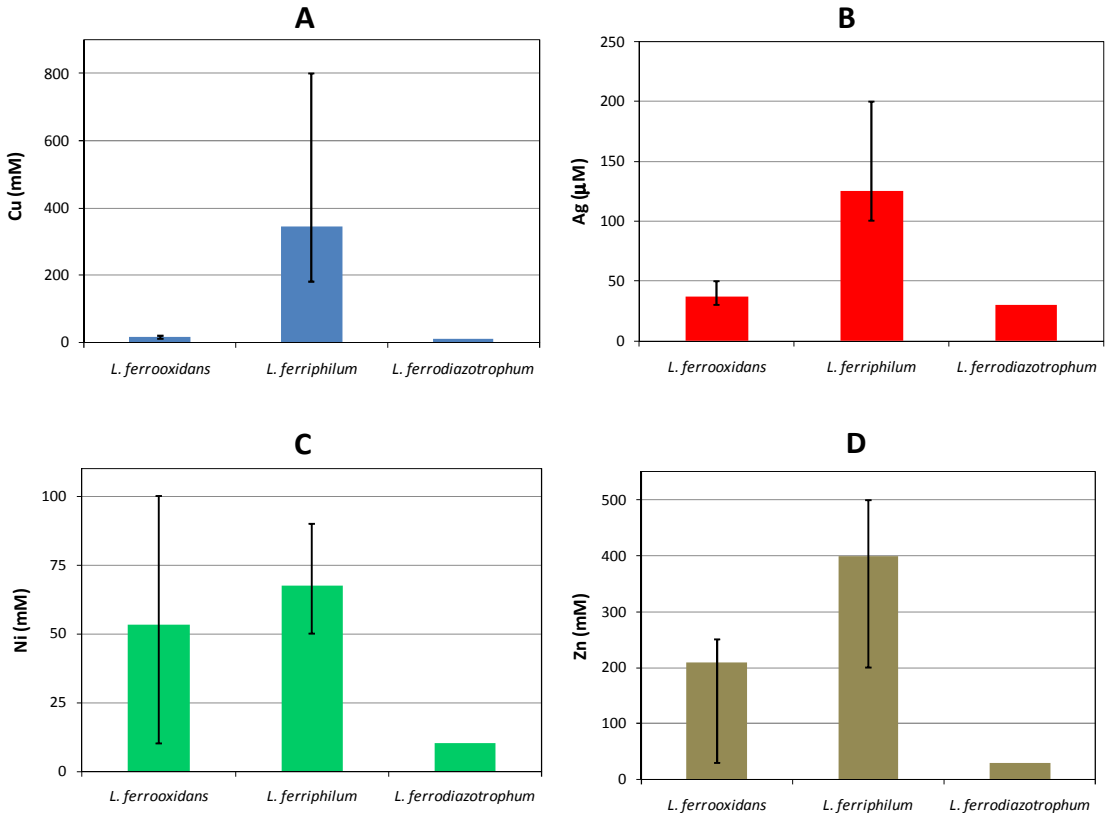


Figure 4.2 Comparison of the tolerance of strains of *Leptospirillum* spp. to four transition metals; copper (A), silver (B), nickel (C) and zinc (D). The bars show the maximum mean concentration tolerated by the different strains of each species, and the error bars represent the highest and lowest concentration tolerated by different strains. Only a single strain of *"L. ferrodiazotrophum"* was available for testing.

4.3.2 Effect of sodium chloride on the growth of *L. ferriphilum* Sp-Cl and some other acidophiles

Culture doubling times of *L. ferriphilum* Sp-Cl increased with increasing concentrations of NaCl, reaching ~17 h in the presence of 500 mM NaCl (Fig. 4.3). Higher NaCl concentrations than 500 mM resulted in a sharp decreases in iron oxidation. There appeared to be a strong polynomial relationship (r^2= 0.99) between culture

doubling times and concentrations of sodium chloride in the growth medium for this acidophile.

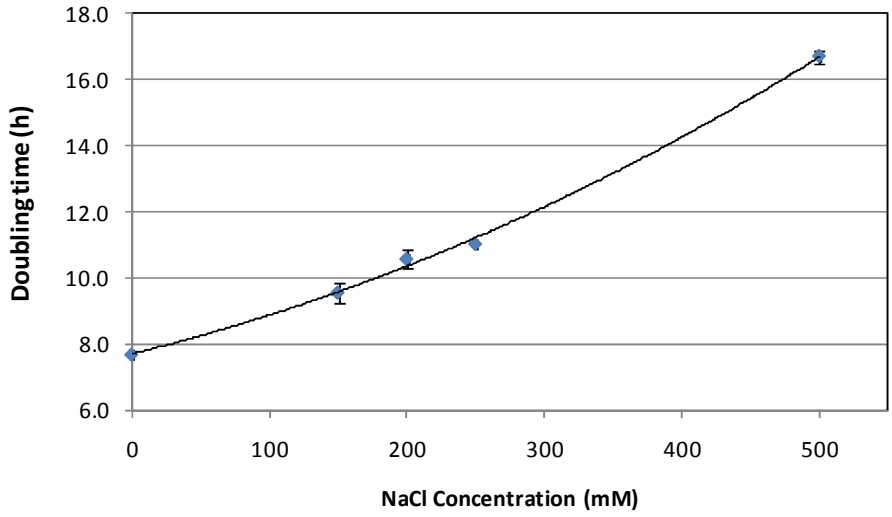


Figure 4.3 Effect of different concentrations of sodium chloride on culture doubling times of *L. ferriphilum* Sp-Cl.

Table 4.2 lists the highest concentration of NaCl at which growth was observed in pure cultures of *L. ferriphilum* and some other acidophilic bacteria commonly found in mineral leaching environments. The maximum concentration of NaCl tolerated was observed in *At. thiooxidans* (1.2 M), while the lowest was observed in *Sb. thermodisulfooxidans* (300 mM).

Table 4.2 Highest concentrations of chloride tolerated by selected acidophilic bacteria.

Bacteria	Maximum of concentration NaCl tolerated (mM)
L. ferriphilum Sp-Cl	500
Sb. thermodisulfooxidans CBAR-13	300 (350)*
At. thiooxidans IESL33	1200 (1300)*
Acidiphilium sp. SJH	500 (550)*

*Concentrations shown in brackets are the next highest concentration at which the bacteria were tested and no growth was observed.

4.3.2 COMPARISON OF THE EFFECTS OF COPPER SULFATE AND MAGNESIUM SULFATE ON THE GROWTH OF *L. FERRIPHILUM* IESL25

Figure 4.2 shows the iron oxidation kinetics, and total and viable cell counts, during the growth of *L. ferriphilum* IESL25 in liquid cultures supplemented with 150 mM of either $CuSO_4$ or $MgSO_4$. In cultures supplemented with 150 mM $MgSO_4$, ferrous iron was completely oxidized in approximately 2 days, while in cultures supplemented with 150 mM of $CuSO_4$, ferrous iron oxidation proceeded was much more slowly, with between 6 to 8 days required for complete oxidation. Following completion of iron oxidation, viable cells numbers declined similarly in replicate cultures of each set-up, falling to <10 cells/mL within 6 to 8 days in cultures supplemented with 150 mM of $MgSO_4$, but within 4 days for cultures supplemented with 150 mM of $CuSO_4$,. Bacterial growth rates (calculated as before from rates of ferrous iron oxidation) for cultures grown in presence of 150 mM $MgSO_4$ were faster (average of 0.084 h^{-1}) than in the presence of 150 mM $CuSO_4$ (average of 0.018 h^{-1}). Bacterial mortality rates (calculated from changes in viable counts), were significantly greater (0.372 h^{-1}) in presence of copper sulfate than with magnesium sulfate (0.115 h^{-1}, Fig. 4.3).

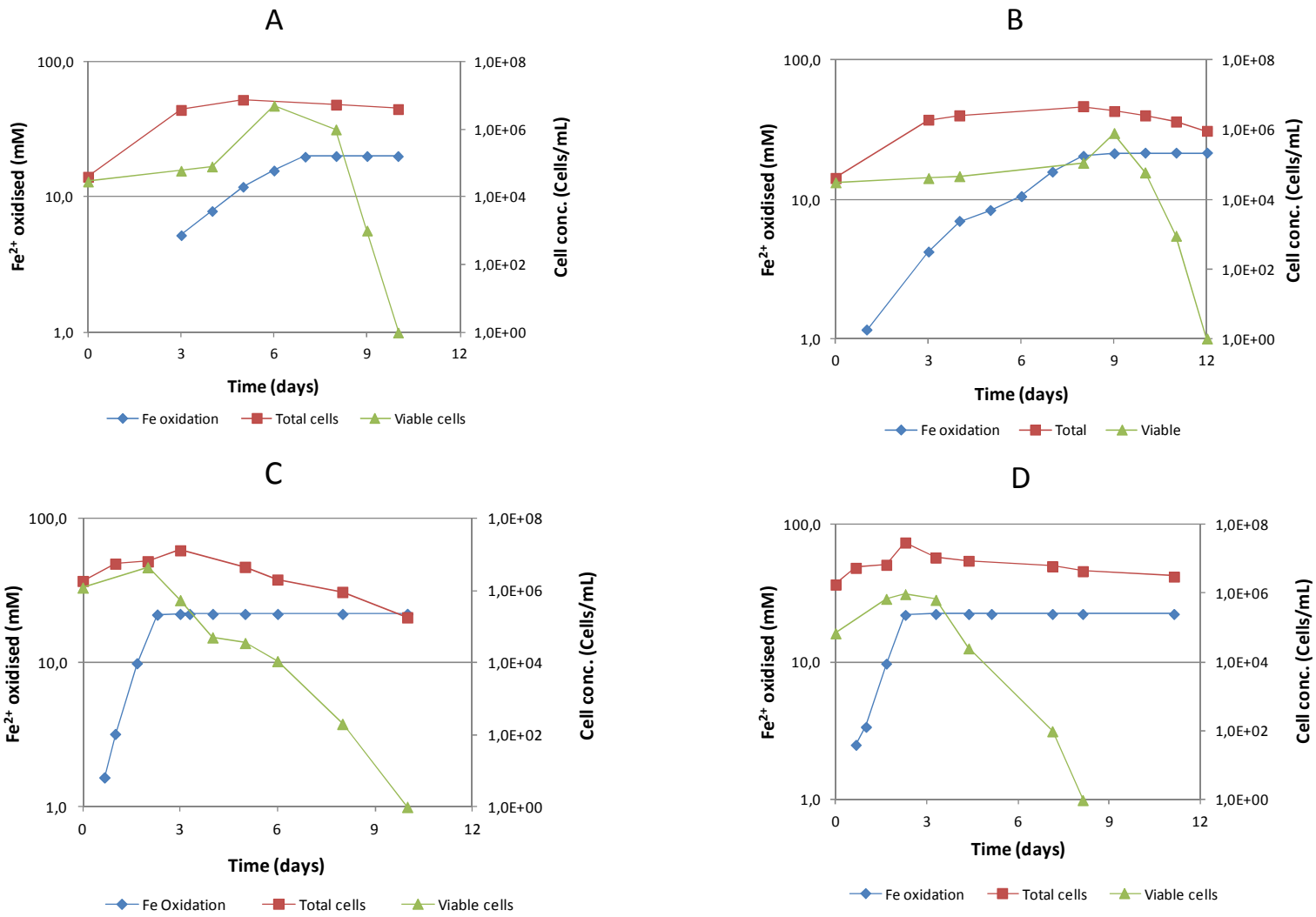


Figure 4.2 Effect of $CuSO_4$ (A and B) and $MgSO_4$ (C and D) on growth and mortality rates of *L. ferriphilum* IESL25.

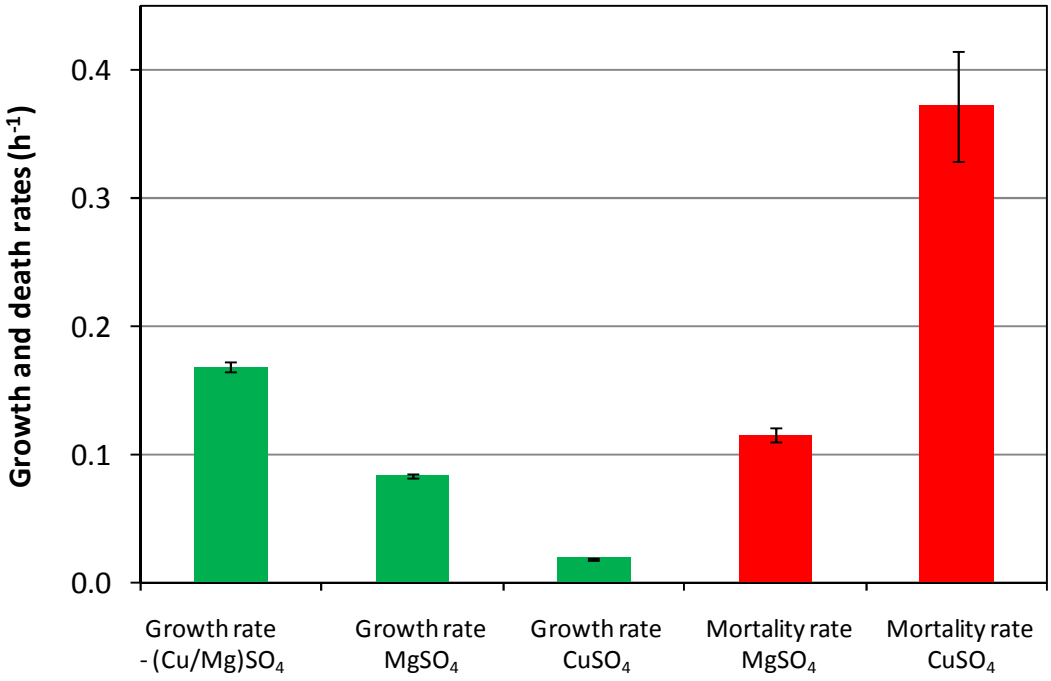


Figure 4.3 Growth and mortality rates determined for cultures of *L. ferriphilum* IESL25 growing in presence of 150 mM magnesium sulfate or 150 mM copper sulfate.

4.3.4 COMBINED EFFECTS OF SODIUM CHLORIDE AND COPPER SULFATE ON THE GROWTH OF *L. FERRIPHILUM* STRAIN SP-CL

The combined effects of copper sulfate and sodium chloride on the growth of *L. ferriphilum* Sp-Cl was studied. Adding either of these reagents (at final concentrations of 200 mM) increased the culture doubling time to similar extents, and only marginally (7.9 h) compared to control cultures (7.7 h, Fig. 4.4). However, addition of 50 and 100 mM copper to cultures containing 200 mM of chloride was found to have much more severe inhibitory effect on bacterial growth than when 50 or 100 mM chloride was added to cultures containing 200 mM of copper. Mean culture doubling times in cultures containing 200 mM of both copper and chloride were about 180 h, and differences between replicates were statistically not significant (assessed by T-test: $t_{calculated}$ (1.7) < $t_{critical}$(4.3) within 95% confidence).

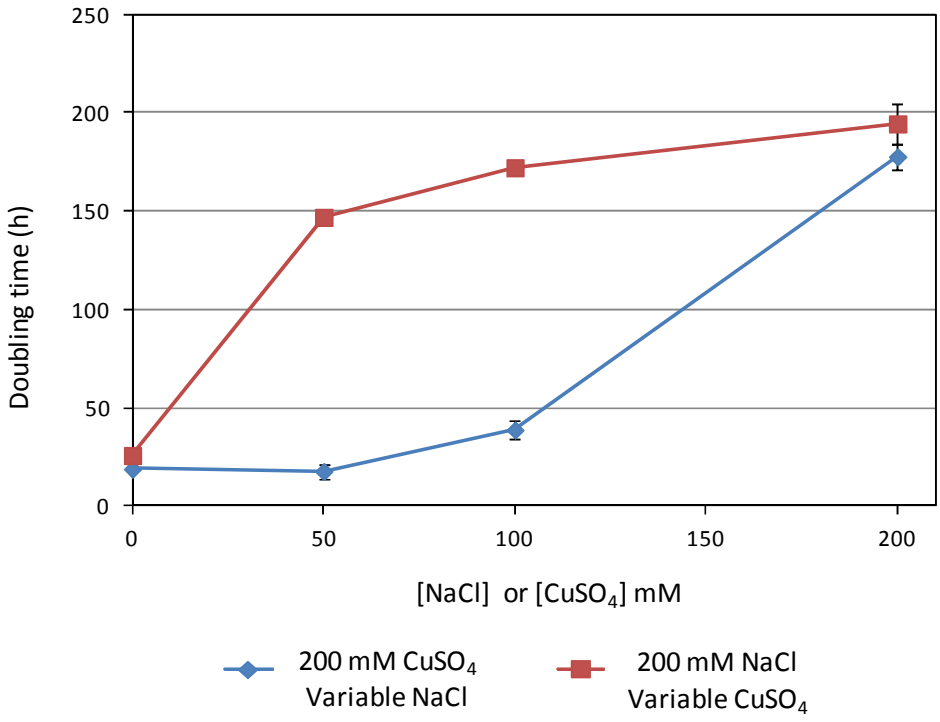


Figure 4.4 Combined effects of sodium chloride and copper sulfate on the growth of *L. ferriphilum* Sp-Cl. Control cultures (not supplemented with copper sulfate or sodium chloride) had a mean culture doubling time of 7.8 h.

4.3.5 BIOLEACHING OF A COPPER CONCENTRATE BY PURE AND MIXED CULTURES OF *L. FERRIPHILUM* SP-CL

4.3.5.1 Physico-chemical changes in bioreactor cultures

Figure 4.5 shows the evolution of concentrations of copper, sulfate, iron, arsenic and changes in redox potential during the bioleaching of copper concentrate in bench-scale bioreactors maintained at pH 1.7 and 35℃ by *L. ferriphilum* Sp-Cl grown in pure and mixed cultures. The highest copper concentration reached after 35 days of leaching

was 2.4 g/L in reactors 3 and 4 (pure culture of *L. ferriphilum* Sp-Cl and the consortium, both with added salt) while in reactor 2 (pure culture of *L. ferriphilum* Sp-Cl but with no added salt) the maximum concentration of copper was 1.8 g/L, and in reactor 1 (the abiotic control) it was 1.3 g/L (Fig. 4.5-A). Interestingly, reactor 2 (salt-free, pure culture) showed a marked inflection in the rate of increase of copper concentrations from day 20 onwards, correlating with increasing redox potentials. On day 35, soluble iron concentration were greater in reactors 3 and 4 (both ~0.6 g/L) than in reactors 1 and 2 (between 0.3 and 0.4 g/L). After day 20, the concentration of total iron in reactors 1 and 2 only increased by 0.2 g/L (Fig 4.5-B). Redox potentials increased with time in all four reactors, but remained relatively low (between +614 and +628 mV vs. SHE) for three of them. The only exception to this trend was the only "salt-free" reactor (number 2), where redox potentials increased rapidly after day 20, reaching a maximum value of +730 mV (Fig. 4.5-C).. Sulfate concentrations displayed similar trends to those of copper. The maximum concentrations for this anion were reached on day 35 in reactors 3 and 4 (4.4 and 4.0 g/L, respectively), while at this time in reactors 1 and 2 the maximum concentrations of sulfate were 1.8 and 1.1 g/L, respectively (Fig. 4.5-D). Arsenic concentrations in the four bioreactors showed similar patterns to those of redox potentials, displaying greatest levels in reactor 2 (10.7 mg/L on days 35) compared to 5.5, 5.9 and 6.1 mg/L in reactors 1, 2 and 3 respectively (Figure 4.5-E).

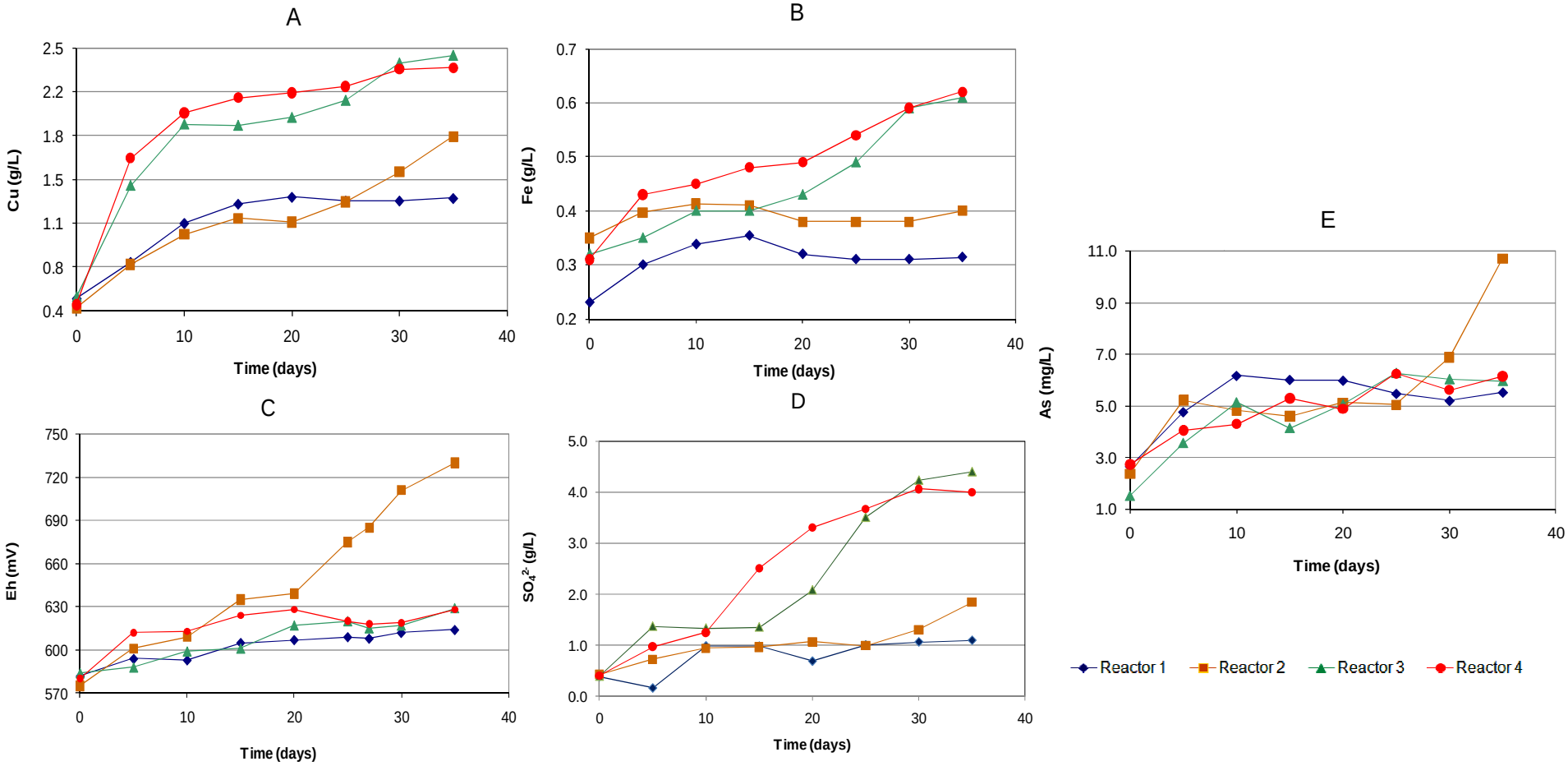


Figure 4.5 Parameters monitored during copper bioleaching of copper concentrate by *L. ferriphilum* Sp-Cl grown in pure and mixed cultures in bench-scale reactors: soluble copper (A), soluble iron (B), redox potentials (C), sulfate (D), soluble arsenic (E).

4.3.5.2 Copper contents of concentrate and leach residues and mass balance calculations

Figure 4.6 shows the copper contents in leach residues and acid-washed concentrate samples. The copper contents of the raw concentrate and that of the acid-washed concentrate were 33.2 and 32.5 % (w/w), respectively. Leach residue of reactor 1 had the highest copper content (32.3 % w/w), while leach residue of reactor 3 had the lowest (30.6% w/w). Leach residues of reactors 2 and 4 were similar (31.8% and 31.7% w/w, respectively). Table 4.3 shows the mass balance for copper in each of the four bioreactors.

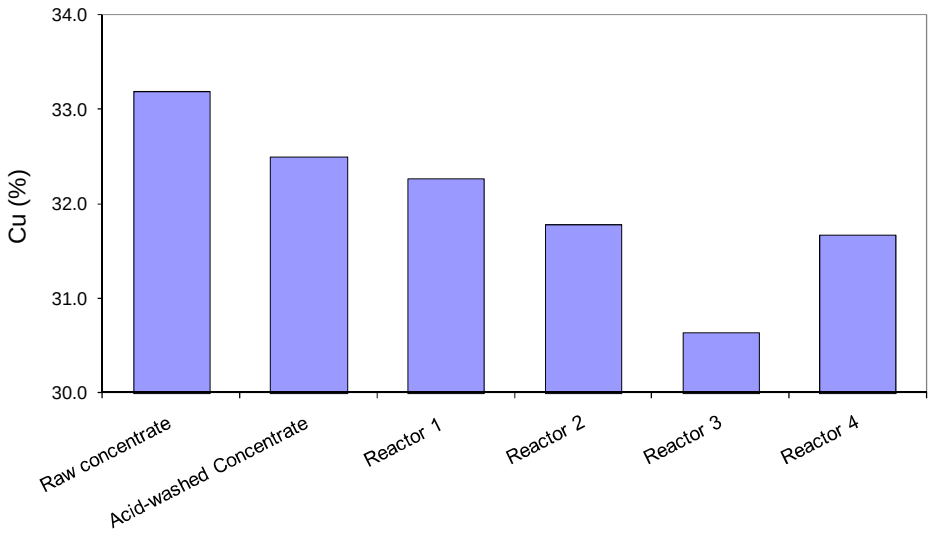


Figure 4.6 Copper contents of the raw, acid-washed, and bioleached copper concentrates.

Table 4.3 Mass balance for Cu in bioleaching tests.

Reactor	Cu in reactor (g/L)	Cu solubilised (g)	Residue weight (g)	Cu in residue (%)	Cu in residue (g)	Cu solubilised + Cu in residue (g)	Cu accounted for (%)
1	1.30	1.95	65.7	32.3	21.2	23.2	95.1
2	1.79	2.69	65.0	31.8	20.7	23.4	95.9
3	2.44	3.66	61.5	30.6	18.8	22.5	92.3
4	2.34	3.51	61.6	31.7	19.5	23.0	94.6

4.3.5.3 Mineralogical analysis

Mineralogical analysis of the concentrate and residues were carried out by Quemscan (section 4.2.7). The distribution of mineral species in the raw and acid-washed concentrate, and in the residues recovered from each bioreactor, is shown in Figure 4.7. The profiles showed different mineralogical abundance, though those of some minor minerals (atacamite and chrysocolla, both present at 0.13% in the raw concentrate and <0.02% in the acid-washed concentrate) are not shown. As anticipated, the copper sulfate mineral bronchantite, which was present at 4.4% in the raw concentrate, was mostly removed by acid-washing. The concentrate that was subjected to bioleaching therefore contained chalocite, chalcopyrite and covellite as the major copper-containing minerals. Bioleaching resulted in the residual solid mass containing smaller relative amounts of chalcocite, greater relative amounts of covellite, and similar relative amounts of chalcopyrite.

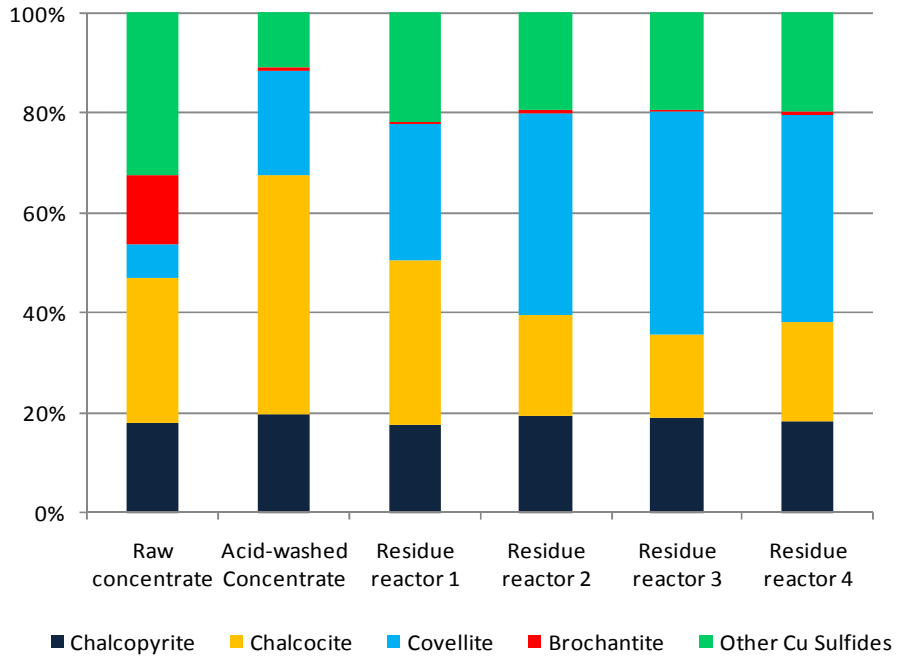


Figure 4.7 Distribution of mineral species in the copper concentrate samples.

4.3.5.4 Bio-molecular analysis of the population in leaching experiments

Amplification of 16S rRNA gene fragments was successful for samples obtained from reactors 3 and 4, but PCR products were not obtained from DNA samples of reactors 1 and 2. DGGE was used to separate PCR fragments and different band profiles were obtained for reactors 3 and 4 on days 5 and 35 (Fig. 4.8). Five bands were excised, re-amplified and submitted for sequencing (section 2.8.4), and the identification of the microbial population based on the DGGE band sequences is shown in Table 4.4. The bands immediately above and below band 1 in lane 1 were not sequenced as experience has shown that such bands appear in gels that have been overloaded with PCR product and that the sequences are always identical to that of the band they sandwich.

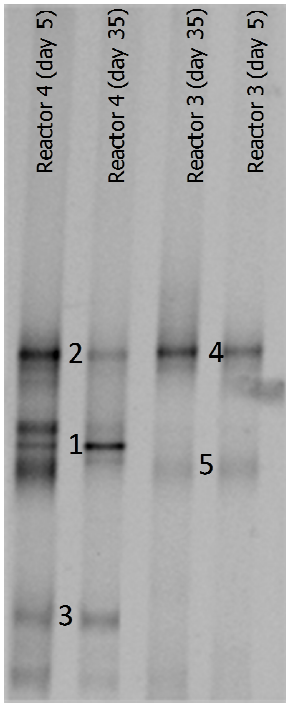


Figure 4.8 SYBR-Gold stained DGGE gel of bacterial 16S rRNA gene fragments amplified by PCR, using degenerate bacterial primers, from DNA obtained from reactors 3 and 4. The indistinct bands that appear above and below band 1 in lane 1 are artifacts of sample overloading, and do not feature in lane 2.

Table 4.4 Identification of band sequences obtained from DGGE analysis. ND = not determined.

Band	Length (pb)	Microorganism	Similarity (%)	Coverage (%)	Accession number NCBI
1	490	*At. thiooxidans* IESL33	99	100	HQ902067
2	486	*L. ferriphilum* Sp-Cl	99	100	ND
3	465	*Sb. thermosulfooxidans*	99	100	ND
4	452	*L. ferriphilum* Sp-Cl	100	100	ND
5	274	Uncultured bacteria clone 3-95	96	100	GU212447

4.3.5.5 Enumeration of bacteria by Quantitative PCR

Specific enumeration of bacteria was carried out by quantitative PCR in samples from reactors 2, 3 and 4. The copy numbers for 16S rRNA gene were quantified using specific forward primers for *At. thiooxidans*, *Sb. thermosulfidooxidans* and *L. ferriphilum* Sp-Cl in DNA samples obtained on days 5 and 35 (section 2.10). In reactor 2, the copy numbers of the 16S rRNA gene determined for *L. ferriphilum* Sp-Cl increased by 2 orders of magnitude from day 5 to day 35, reaching 3.3 $x10^7$ copies/mL, and was the highest increase recorded,. The copy number of 16S rRNA gene of *L. ferriphilum* Sp-Cl also increased in reactor 3 and 4, reaching 3 $x10^6$ and 7 $x10^6$ copies/mL, respectively, on day 35. In reactor 4, while the abundance of *L. ferriphilum* Sp-Cl increased by one order of magnitude (from 6.3 $x10^5$ to 7.0 $x10^6$ copies/mL), *At. thiooxidans* was the most abundant bacterium present, reaching 9.5 $x10^7$ copies/mL on day 35. While *Sb. thermosulfidooxidans* CBAR-13 was detected on days 5 and 35, the copy numbers obtained from these samples were very similar (4.9 x 10^5 and 5.0 x 10^5 copies/mL, respectively; Fig. 4.9). *Acidiphilium* sp. SJH was not detected in bioreactor 4 leach liquors on either day 5 or day 35. Since qPCR results were obtained using species-specific primers, they are more reliably quantitative than DGGE analysis based on degenerate primers.

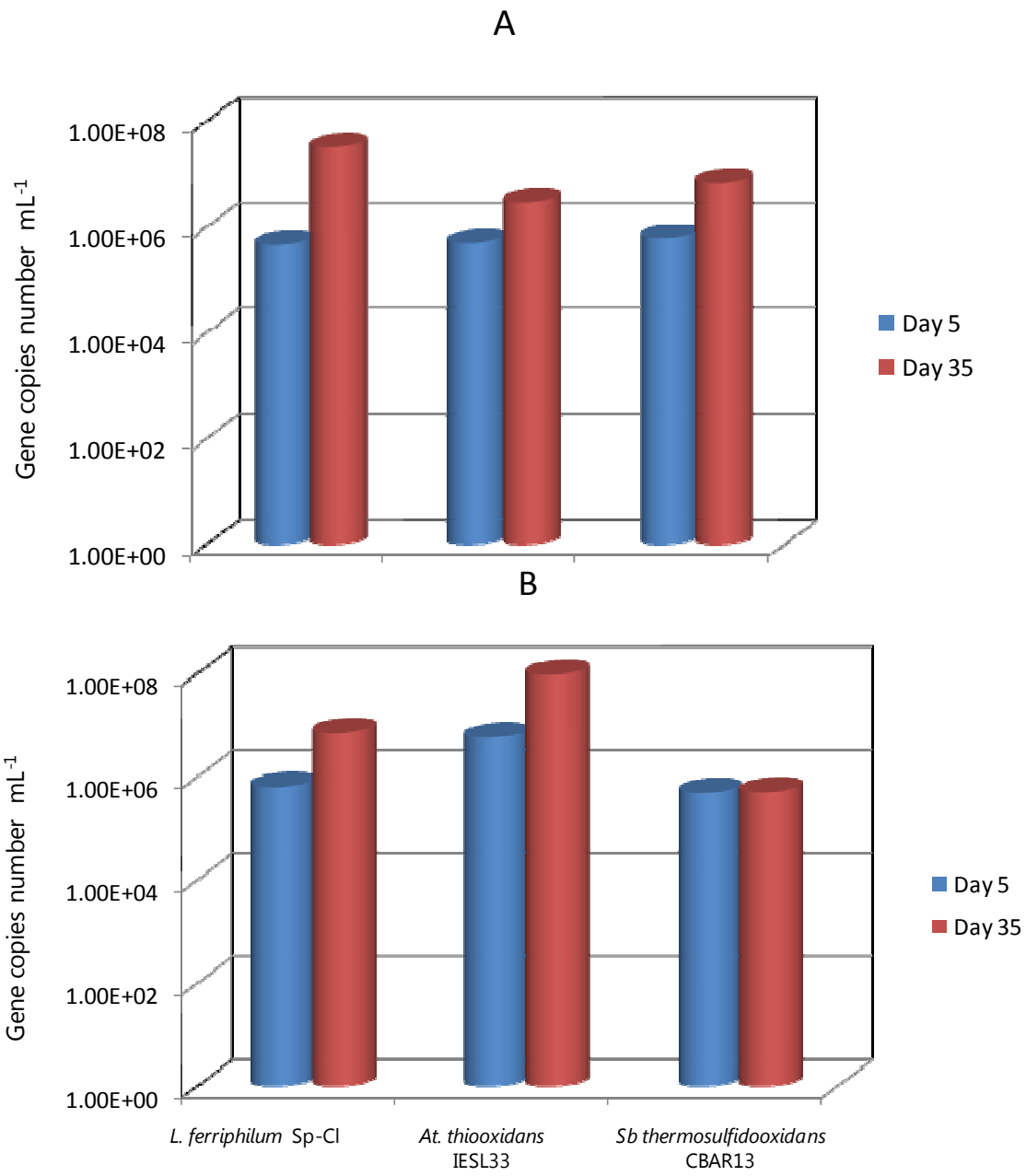


Figure 4.9 Temporal distribution of the microbial population determined by qPCR. A: *L. ferriphilum* Sp-Cl in reactors 2, 3 and 4 and B: *L. ferriphilum* Sp-Cl, *At. thiooxidans* IESL33 and *Sb. thermosulfidooxidans* CBAR13 in reactor 4.

4.4 DISCUSSION

4.4.1 Tolerance of *Leptospirillum* spp. to Cu, Ag, Ni and Zn

Members of the genus *Leptospirillum* are obligately acidophilic, mesophilic, aerobic, (pH <4.0), obligate chemolithoautotrophic bacteria that appear to obtain energy only from coupling the oxidation of ferrous iron to the reduction of molecular oxygen (Johnson, 2001) and carbon by fixing CO_2 via the reductive tricarboxylic acid cycle (Levicán et al., 2008). Though there was evidence that supported the hypothesis that considerable physiological variation existed among different isolates was found in early studies (e.g. Battaglia et al., 1994; Harrison and Norris, 1985), the limited number of physiological characteristics complicated the isolation of strains as pure cultures and therefore the study of the diversity among *Leptospirillum* spp.. Thus, to distinguish between *L. ferrooxidans* and *L. ferriphilum* it was necessary to apply a variety of molecular techniques including DNA-DNA hybridization, 16S rRNA gene sequencing, analysis of 16S-23S intergenic regions, DNA G+C content, among others (Coram and Rawlings, 2002). Moreover, the development of high-throughput sequencing technologies together with high performance computational systems for the analysis of whole genome sequence made it possible to detect a *nif* operon on a genome fragment belonging to a member of *Leptospirillum* Group III. This information was used to isolate this bacterium as pure culture in nitrogen-free media. This isolate was able to fix dinitrogen and was proposed as a novel species named "*"L. ferrodiazotrophum"*" (Tyson et al., 2005). *Leptospirillum* spp. are often the predominant iron-oxidisers in environments with high redox potentials and temperatures below 45 ℃ (Coram and Rawlings, 2002; Rawlings et al., 1999). A distinctive characteristic of most strains of *L. ferriphilum* is the ability to grow faster at higher temperatures than other *Leptospirillum* spp. This characteristic was thought to be

the reason why *L. ferriphilum* dominated commercial processes operating between 40 to 45℃ and it was suggested that *L. ferrooxidans* was not competitive in that temperature range (Coram and Rawlings, 2002).

Molecular phylogenetic analysis performed with liquid and solid samples from the industrial bioheap operation at the Escondida mine detected only *L. ferriphilum* (of *Leptospirillum* spp.) and showed this species was dominant in the PLS during the later stages of the process (Demergasso et al., 2005; Remonsellez et al., 2007). The apparent exclusivity of *L. ferriphilum* (of *Leptospirillum* spp.) was confirmed by the isolation of *L. ferriphilum* (strain IESL25; Galleguillos et al., 2009) and no other *Leptospirillum* spp... In common with most other strains of *L. ferriphilum,* strain IESL25 grew faster at temperatures above 35 ℃ (optimum 37 ℃, section 3. 3.8). However, the highest temperature recorded in samples of pregnant leach solutions used for the isolation of this strain was only 22.6 ℃. Therefore, other factors were thought to overriding bearing on the colonisation of the heaps by different *Leptospirillum* spp..

The plant producing copper cathodes at Escondida mine requires a concentration of copper in the PLS ranging between 4.0 to 6.0 g/L (63 to 100 mM), in order to optimise the recovery of copper. The presence of this transition metal at these elevated concentrations, which are very rarely found in natural environments, may be an important factor producing a strong selective pressure on the microbial population and determining the presence of only adapted or tolerant *Leptospirillum* spp.. The results shown in section 4.3.1 revealed greater tolerance to copper, silver and zinc in strains of *L. ferriphilum* than in strains of *L. ferrooxidans* or the single available strain of *"L. ferrodiazotrophum"* (Figure 4.1). A remarkable difference was observed in the tolerance to copper and silver between *L. ferriphilum* and the other two *Leptospirillum* spp. tested. The average tolerance to Cu determined for four strains of *L. ferriphilum* was 345 mM (22 g/L), while the average determined for three strains of *L. ferrooxidans* was 17 mM (1.1 g/L), and *"L.*

ferrodiazotrophum" BAA-1181 tolerated up to 10 mM (Figure 4.2). Thus the average copper concentrations tolerated by strains of *L. ferrooxidans* and *"L. ferrodiazotrophum"* were less than 5 % of the concentration tolerated by tested strains of *L. ferriphilum*. Interestingly, the highest tolerance to copper was recorded for strain MT61, which tolerated as high as 800 mM (50 g/L), at this level of copper concentrations the osmotic stress could also be considered as a factor inhibiting bacterial growth.

Similar results to copper tolerance were obtained for silver. The strains of *L. ferriphilum* tolerated in average 125 µM of silver, while the strains of *L. ferrooxidans* tolerated in average 37 µM and *"L. ferrodiazotrophum"* BA-1181 tolerated up to 30 µM, it means only a 30% of the concentration tolerated by strains of *L. ferriphilum*. The difference in tolerance to nickel and zinc between strains of *Leptospirillum* spp was less marked than those of copper and silver.

The average tolerance to zinc determined in strains of *L. ferriphilum* was 400 mM, while the average tolerance for strains of *L. ferrooxidans* was 210 mM and *"L. ferrodiazotrophum"* BA-1181 tolerated up to 30 mM of this metal.

The lowest tolerance to nickel was observed in *"L. ferrodiazotrophum"* BA-1181 and *L. ferrooxidans* CF12 with a maximum concentration tolerated for both of 10 mM, while the maximum tolerance for this metal was observed in *L. ferrooxidans* DSM 2705^T (100 mM) and the strains of *L. ferriphilum* tolerated as maximum between 50 to 90 mM (Fig. 4.2)

The results obtained from the tolerance to these transition metals strongly suggest that the concentration of copper in solutions of the bioleaching plant at Escondida mine, rather than relatively high temperatures (higher than 35℃) is the main factor selecting for the presence of *L. ferriphilum* rather than other *Leptospirillum* spp.

In natural acidic environments the maximum copper concentrations in solution are relatively low compared to commercial process (using either reactors or heaps). The

highest copper concentrations observed in acidic mine waters have typically been reported as less than ~12 mM (González-Toril et al., 2003; Johnson and Hallberg, 2003), which is lower than the maximum tolerance (20 mM) observed for the strains of *L. ferrooxidans* tested (Fig. 4.1). The three strains of *L. ferrooxidans* included in this study were isolated from acidic mine waters, the strains DSM 2705^T and SY were isolated from water occurring in copper mines from a copper deposit in Armenia and the abandoned Sygun mine in North Wales, respectively, while strain CF12 was isolated from an abandoned cobalt mine in Cobalt, Idaho. These environments are thought to probably contain similar copper concentration to those found typically in mine waters rather than the elevated concentrations that are typical of PLS.

Though only three strains of *L. ferrooxidans* were used in the present study, other strains of *L. ferrooxidans* had been reported as being isolated from environments with relatively low copper concentrations. Strain L3.2 was isolated from the Rio Tinto, where the copper concentrations ranged between 0.3 to 11 mM (González-Toril et al., 2003), and other strains (e.g. strains PT1, P3c and BCT2) have been isolated from abandoned metal and coal mine in the U.K. (D. B. Johnson, Bangor University, unpublished). On the other hand, several other strains of *L. ferriphilum* have been isolated from industrial applications, where copper concentration can reach up to several grams per litre. In this regard, strain MT6 was found as the dominant strain in a pilot plant stirred-tank bioleaching operation (Okibe et al., 2003) and strains IESL25 and Sp-Cl were isolated from industrial heap bioleaching processes in Chile, at Escondida and Spence mines, respectively.

4.4.2 Effect of copper on the growth of IESL25

Copper is classed as a micronutrient for most life forms. Although this transition metal is needed by living organisms in small amounts, it is required for the optimal metabolic functioning of cells. Due to its chemical characteristics Cu^{2+} is able to form complex compounds with proteins, which can play important roles in biochemical reactions. However, at higher concentrations this metal can form unspecific complex compounds in the cell and interacts with radicals, best with molecular oxygen, which leads to toxic effects (Nies, 1999).

In general, acidophilic bacteria are able to tolerate greater concentrations of cationic metals than neutrophilic bacteria. An important contribution to metal resistance in acidophilic bacteria appears to be the inside-positive potential (which is opposite to the inside-negative of neutrophilic bacteria). This adaptation is essential to generate and maintain a large pH gradient between the exterior and cytoplasm. This large pH gradient provides resistance to cations accumulation, since those would be extruded against their concentration and electrogenic gradient (Alexander et al., 1987; Matin, 1990; McLaggan et al., 1987; Orell et al., 2010). In addition to this particular characteristic, DNA sequences coding for copper translocating ATPases, similar to those involved in active copper efflux transport have been found in genomic sequences of *L. ferriphilum* and could contribute to copper tolerance in this species (Goltsman et al., 2009).

The effect of copper on growth and mortality rates of *L. ferriphilum* strain IESL25 exposed to copper concentration (150 mM) close to the maximum tolerated (180 mM) was assessed (Fig. 4.2). Though the copper tolerance observed for *L. ferriphilum* IESL25 (180 mM) was not the highest observed in strains of *L. ferriphilum* (Fig. 4.1), it was approximately 10 times higher than the average observed for strains of *L. ferrooxidans* and *"L. ferrodiazotrophum"* BA-1181, and about double of the concentration necessary (100

mM) in pregnant leach solutions at Escondida for the optimum performance of the bioleaching plant. Differences in the maximum total cell concentration were observed between cultures growing in presence of magnesium or copper sulfate. In cultures supplemented with magnesium sulfate the maximum total cell concentration was around 10^7 cells/mL (1.3 $x10^7$ and 3.7 $x10^7$ cells/mL, Fig. 4.2 C and D, respectively), while in cultures supplemented with copper sulfate the maximum total cells concentration was around 10^6 cells/mL (7.5 $x10^6$ and 4.6 $x10^6$ cells/mL, Fig. 4.2 A and B, respectively). In addition a strong inhibition by copper on the growth kinetics of *L. ferriphilum* IESL25 was indicated by growth rate values. In presence of 150 mM of copper, the complete oxidation of ferrous iron was achieved between 8 to 10 days with a mean growth rate of 0.018 h^{-1} (Fig. 4.3), representing a 94% of inhibition compared to control cultures controls growing in ferrous iron medium with no copper or magnesium sulfate supplement (0.17 h^{-1}). In the presence of 150 mM of magnesium sulfate, the complete oxidation of ferrous iron was achieved between 2-3 days with a growth rate of 0.08 h^{-1}, meaning 47% of inhibition compared with controls cultures. Assuming that magnesium sulfate has no other effect than increase the osmotic strength in the cultures, these results indicate that osmotic stress played an important role in the inhibition produced by elevated concentrations of copper sulfate in cultures of *L. ferriphilum* IESL25 and based on the growth rates a 50% of the total inhibition could be attributed to osmotic stress.

On the other hand, following completion of the iron oxidation, mortality rates were greater in cultures supplemented with 150 mM of copper sulfate, than those supplemented with 150 mM of magnesium sulfate. In cultures growing in presence of 150 mM magnesium sulfate, viable cells were detected (<10 cells/mL) until 6-8 days after completion of the iron oxidation with a mean mortality rate of 0.11 h^{-1}. In the case of cultures growing in presence of copper sulfate, viable cells counts rapidly fell to <10 cells/mL within four days, with a mean mortality rate of 0.37 h^{-1}, indicating that cultures of

L. ferriphilum IESL25 grown in the presence of copper sulfate lost viability at a rate that was about three times faster than in the presence of similar concentrations of magnesium sulfate. Though no viable cell counts were performed in cultures grown with no supplement of either copper or magnesium sulfate, the detection of viable cells up to 45 days after completed the ferrous iron oxidation has been reported in cultures of *L. ferrooxidans* grown in the same medium (Johnson and Hallberg, 2003). These results suggest that the stress produced in cultures of *L. ferriphilum* IESL25 by elevated copper concentrations has an osmotic component in addition to the toxicity produced by copper at concentrations close to its maximum tolerance.

The lower biomass yield and the higher mortality rate observed in cultures of *L. ferriphilum* IESL25 grown in presence of copper sulfate compared to control cultures or cultures growing in presence of similar concentrations of magnesium sulfate indicate that energy-dependent mechanisms were triggered when *L. ferriphilum* IESL25 was exposed to copper concentrations close to its maximum tolerance limit. In this regard, energy-dependent efflux systems that pump other transition metal (nickel) from the cell have been suggested in *L. ferriphilum* (Tian et al., 2007). In addition, *At. caldus* (another acidophilic bacterium) induced an efflux of copper ions by forming an ATPase-dependent pump, which transported copper ions by consuming ATP, in response to elevated concentrations of copper (Xia et al., 2010). Also, an inducible efflux system has been suggested in *At. ferrooxidans* by copper uptake of biomass adapted to elevated copper concentrations (Boyer et al., 1998).

4.4.3 Combined effects of sodium chloride and copper sulfate on the growth of *L. ferriphilum* strain Sp-Cl

Leptospirillum ferriphilum Sp-Cl was originally isolated from PLS samples at the Spence mine supplemented with sodium chloride. As it was shown in section 4.3.2, this strain was able to oxidize ferrous iron in presence of sodium chloride at concentrations as high as 0.5 M. This high tolerance to salt is an unusual characteristic in autotrophic iron oxidisers, as demonstrated for other strains of *Leptospirillum* spp. and *At. ferrooxidans*, which were inhibited at concentrations much lower than that found in seawater (Gahan et al., 2009; Kieft and Spence, 1998; Romero et al., 2003). So far, "*Thiobacillus prosperus*" is the only iron (and sulfur) oxidiser bacterium able to grow at elevated salt concentration (Norris and Simmons, 2004). The application of "*T. prosperus* or "*T. prosperus"*-like strains for bioleaching has not yet proved at industrial level, however one strain identified as "*T. prosperus"*-like was shown to actively participate in the bioleaching of copper in column tests (Davis-Belmar et al., 2008). A potential copper bioleaching technology using saline water will require microorganisms able to tolerate both copper and salt. Therefore, the effect of combinations of copper and salt on the growth of *L. ferriphilum* Sp-Cl was investigated in the present study. The presence of 200 mM of copper sulfate in cultures of *L. ferriphilum* Sp-Cl was found to have only a minor effect and a marginal increase of culture doubling time (7.9 h) was observed compared with culture doubling time (7.7 h) obtained from control cultures (no copper supplemented), suggesting an elevated tolerance to copper for this strain. Similar results were obtained when sodium chloride was added at the same concentration (Fig. 4.4). The addition of 50 mM and 100 mM of sodium chloride to cultures of *L. ferriphilum* Sp-Cl containing 200 mM of copper had a minor impact on the cultures, causing a doubling of culture doubling times when 100 mM sodium chloride was added. However, the addition of 50 mM and 100 mM of copper sulfate to

cultures containing 200 mM of sodium chloride had a major impact on the cultures, increasing culture doubling times by eight- and nine-fold, respectively. Culture doubling times calculated with media containing 200 mM of both copper sulfate and sodium chloride were similar (~180 h) and differences between replicates were statistically not significant.

Though positive membrane potentials can confer higher tolerance to cationic metals in acidophiles (compared to neutrophilic bacteria), this adaptation could also be the reason why acidophiles are sensitive to single charged anions such as chloride, bromide and nitrate. These anions could be accumulated inside the cell, collapsing the membrane potential and allowing the entrance of protons, whit consequent acidification of the cytoplasm (Alexander et al., 1987; McLaggan et al., 1990). *L. ferriphilum* strain Sp-Cl appears to be more tolerant to chloride than other *Leptospirillum* spp. or other autotrophic iron oxidizers (apart from "*T. prosperus*") and this adaptation can be related to through the control of chloride transport into the cell.

4.4.4 Effect of sodium chloride on the growth of *L. ferriphilum* Sp-Cl and some other acidophilic bacteria

Leptospirillum ferriphilum Sp-Cl is able to oxidize ferrous iron in presence of sodium chloride at concentrations as high as 0.5 M. Higher concentrations than 0.5 mM of NaCl resulted in slow growth completing iron oxidation in several days. Culture doubling times in the absence of chloride were similar to those obtained for strain IESL25 also in absence of chloride. Though both of these strains of *L. ferriphilum* were isolated from copper mines in Chile, clear differences were observed in their tolerance to sodium chloride. When cultures of *L. ferriphilum* Sp-Cl were exposed to increasing concentrations of sodium chloride, the culture doubling times were slowly increasing up to approximately

the double recorded at 0.5 M of sodium chloride (Fig. 4.3).. As mentioned in 4.4.3, this strain probably possesses mechanisms to avoid the excessive influx of chloride ions into cells in presence of elevated chloride concentrations, maintaining the stability of the positive membrane potential and avoiding acidification of the cytoplasm.

Chloride tolerance was also tested in other acidophilic bacteria commonly found in mineral leaching environments, including two strains isolated from Escondida mine (*Sb. thermosulfooxidans* CBAR13 and *At. thiooxidans* IESL33) and *Acidiphilium* sp. SJH. *Sb. thermosulfidooxidans* CBAR13 tolerated up to 300 mM sodium chloride but iron oxidation was completely inhibited at 350 mM. Previous work had demonstrated that iron oxidation by the *Sb. thermosulfidooxidans* type strain (DSMZ 9293) was not completely inhibited at concentrations of sodium chloride ~120 mM and cell numbers were reduced at concentrations >60 mM, however the maximum concentration of sodium chloride necessary to completely inhibit ferrous iron oxidation was not determined (Zammit et al., 2009). Only few reported studies have examined on the tolerance of *At. thiooxidans* to chloride. One strain isolated from seawater required sodium chloride at concentration of 2% w/v (~340 mM) for optimal growth in sulfur medium and sulfur oxidizing activity was observed even at concentration of sodium chloride as high as 2 M (Kamimura et al., 2005; Kamimura et al., 2003). Similarly, *At. thiooxidans* IESL33, isolated from Escondida mine, tolerated high sodium chloride concentrations (up to 1.2 M).

Acidiphilium sp. SJH tolerated up to 0.5 M sodium chloride using galactose as energy and carbon source. Previous work have also showed the ability of *Acidiphilium* spp. to thrive in environments containing chloride. One such example was the detection and isolation of one strain of *Acidiphilium cryptum* from acidic hydrothermal waters in White Island (New Zealand) was reported by Donachie et al (2002). Chemical analyses of the water used as isolation source for this strain revealed temporal differences in

concentration of chloride (from 2-4 g/L) in the streams due to the presence of andesite and other chloride containing minerals.

Of the acidophiles screened (Table 4.2) *At. thiooxidans* tolerated the maximum concentration of NaCl (1.2 M), while the lowest tolerance was observed for *Sb. thermodisulfooxidans* CBAR13 (300 mM). These results show that important metabolic activities necessary for bioleaching or bio-oxidation of minerals (iron and sulfur oxidation, organic compound degradation) can prevail in environments containing moderate to elevated concentrations of sodium chloride and potential industrial bioleaching technologies using saline waters can be established using chloride-tolerant bacteria.

4.4.5 Bioleaching of a copper concentrate by pure and mixed cultures of *L. ferriphilum* Sp-Cl

Bioleaching of a copper concentrate containing chalcocite, chalcopyrite and covellite as major copper sulfides in the presence of salt (200 mM sodium chloride) was studied in bench-scale bioreactors at pH 1.7 and 35℃. This study was motivated by the high tolerance to chloride observed in *L. ferriphilum* Sp-Cl and some other acidophilic bacteria, which could, in theory, establish a suitable bioleaching consortium in the presence of salt. Bioleaching of the concentrate by this consortium in presence of 200 mM sodium chloride was compared with those of a pure culture of *L. ferriphilum* Sp-Cl at the same concentration of sodium chloride, and another containing no added salt. Leaching of the concentrate in an abiotic (chemical) control reactor (also containing 200 mM sodium chloride, and maintained at the same pH) was also compared. Greatest copper dissolution after leaching period (35 days) was observed in the inoculated reactors that contained 200 mM sodium chloride, and there was little difference between the pure culture of L. ferriphilum Sp-Cl and the bacterial consortium. Copper concentrations in the *L. ferriphilum*

reactor with no added salt reached ~75% of that found in the corresponding reactor with added salt, though in this case there was a marked inflection in copper concentrations from day 20 onwards. This inflection was paralleled with changes in redox potentials which, after day 20, increased rapidly up to +730 (day 35), while in other three (salt-containing) reactors redox potentials remained relatively low at between +614 and +628 mV. Copper leaching was also observed in the control (abiotic) reactor, and reached the equivalent of ~54% of that seen in the salt-containing bioreactors. Arsenic concentrations in the four bioreactors showed similar patterns to those of redox potentials, and were greater in the salt-free bioreactor than in other reactors. Soluble iron concentrations, in contrast were greater in the salt-amended bioreactors than in the salt-free bioreactor and the abiotic control. Sulfate concentrations displayed similar trends to those of copper in all four reactors. Arsenic concentrations were greater, at the end of the experiment in the bioreactor to which no salt had been added then in the other three reactors. Changes in arsenic concentrations in this reactor paralleled changes in redox potentials, suggesting that increasing redox potential facilitated the dissolution of As-containing minerals such as enargite or tennantite, which were present (at about 6%, by wt.) in the washed concentrate.

The raw concentrate contained a significant amount of brochantite (4.4% by wt.), a soluble copper sulfate mineral. Acid-washing of the concentrate removed most of this (0.17% in the washed concentrate), and also removed much of the chalcocite (from ~ 19 to ~11%). The major copper minerals in the acid-washed concentrate were chalcopyrite, covellite and chalcocite. The relative abundances of enargite/tennantite and bornite were greater in the acid-washed than the raw concentrate, but, the relative abundance of chalcopyrite was similar in both. Comparing the acid-washed concentrate with the residues obtained after bioleaching, the main differences were the smaller relative amounts of

chalcocite, greater relative amounts of covellite, and similar relative amounts of chalcopyrite in the bio-processed material.

Quantitative PCR produced some interesting data in terms of the relative numbers of *L. ferriphilum* Sp-Cl in the different reactors and the composition at the microbial consortium used at the end of the experiment. Numbers of planktonic-phase *L. ferriphilum* Sp-Cl increased in all reactors, but only by a factor of 2 in the salt-containing pure culture compared with a factor of 100 in the corresponding salt-free bioreactor. In the salt-amended mixed culture, numbers of *L. ferriphilum* Sp-Cl were 10-fold greater at the end of the experiment than at the start. These numbers probably underestimate numbers of *L. ferriphilum* Sp-Cl, in the reactors as they do not take into account cells that were attached to minerals. However, the lower numbers found in the presence of salt correlate with observation described elsewhere in this chapter about the suppression of biomass increases in cultures of *L. ferriphilum* grown in the presence of sodium chloride. By the end of the experiment, the numerically-dominant bacterium in the consortium was *At. thiooxidans* IESL33, followed by *L. ferriphilum* Sp-Cl and *Sb. thermosulfidooxidans*. CBAR13. *Acidiphilium* sp. SJH was not detected in the mixed culture at the end of the experiment. It is interesting to note that *At. thiooxidans* IESL33 has been found to be the most salt-tolerant of these four bacteria (Table 4.2).

Chloride ions can accelerate the dissolution of metallic sulfides in acidic solutions, though reasons for this are not well understood. Analysis of a chalcopyrite surface by SEM-EDS (Scanning Electron Microscopy-Energy Dispersive Spectroscopy) after leaching experiments in presence and absence of sodium chloride showed differences in the sulfur formed on the chalcopyrite surface. In the absence of chloride, the sulfur layer was amorphous preventing the contact of ions with chalcopyrite surface, while in presence of chloride the sulfur layer was much more crystalline and porous allowing better transport of ions (Lu et al., 2000). Formation of copper-chloride complexes have also been involved in

the enhanced dissolution rate of chalcopyrite in sulfuric acid solutions containing salt (Lundstrom et al., 2005).

Together, these results showed that the dissolution of copper from this test concentrate was enhanced when both bacteria and salt were present, with less copper being solubilized in the biotic control and the salt-free *L. ferriphilum* Sp-Cl reactor. In addition, the presence of 200 mM sodium chloride maintained relatively low redox potentials in the bioreactors, and minimized the amount of arsenic that was liberated from the concentrate. In terms of a commercial operation, this might also be highly significant.

Chapter 5: Osmo-protectants in acidophilic mineral-degrading bacteria

5.1 INTRODUCTION

Water is the most important molecule for life. The ability of organisms to cope with changes in water availability/activity is important for survival and metabolic activity. In aqueous environments, regions with different solute concentration will produce diffusion of water from lower to higher solute concentrations and, if a semi-permeable membrane interfaces the two solutions, the process is known as osmosis. When the cytoplasm of cells has a higher solute concentration than its bathing solution, water tends to diffuse into the cell, and the cell is then in a positive water balance. However, if water activity decreases in the environment, water will tend to flow out of the cells and, in extreme situations, this loss of water can produce plasmolysis. Most organisms are not able to thrive in environments with very low water activity (i.e. well in excess of sea water) and either die or become dehydrated and dormant. Organisms that can tolerate low water activity (saline environments) but grow at sub-optimal rates are referred to as “halotolerant”. Organisms thriving in environments with low water activity, such as seawater (~3.5% sodium chloride) and hypersaline environments often require sodium ions for optimum growth. These are generically referred to as “halophiles” and depending on the salt concentration tolerated they can be subdivided into: (i) “mild halophiles” (1-6% NaCl), (ii) “moderate halophiles” (6-15% NaCl), and (iii).extreme halophiles >15% salt. Some extreme halophiles can grow in salt-saturated water (~35% NaCl). Reduction in water activity can also be caused by solutes other than salt (e.g. sugars). Organisms able to growth in such conditions are known as “osmophiles”, while

those that are able to grow in very dry (water-stressed) environments are called “xerophiles” (Madigan et al., 1997).

Prokaryotes use two mechanisms to control water homeostasis: active transport of ions (K^+ or Na^+) and accumulation (or excretion) of compatible solutes. Transport of ions produces osmotic gradients, causing water to flow through the cell membrane to the side where concentrations of dissolved solutes are greater. The accumulation of compatible solutes (osmolytes, or osmo-protectants) allows cells to retain water when water activity decreases in the environment, and conversely these compatible solutes can be transported out of the cell when the latter increases. In general, osmolytes are small molecular weight organic molecules with high solubility in water and, importantly do not disrupt or stabilize macromolecules (proteins, nucleic acids etc.). In most cases, osmolytes are polar, uncharged or in a zwitterionic state under conditions that exist within cells, and belong to several classes of compounds including carbohydrates, amino acids, polyols, betaines, *N*-acetylated amino acids, glutamine amide derivatives and ectoine (Grant, 2004). Osmolytes can also have other protective roles, such as increasing thermostability, protecting membranes from freezing and preserving cell viability in dry conditions (Yancey, 2005).

A common threat faced by microorganisms that inhabit industrial bioleaching environments is the loss of internal water, arising from elevated concentrations of dissolved solutes (such as sulfate, base and transition metals, hydronium ions, and others), producing a decrease in water activity. In spite of the importance of mineral-degrading microorganisms in industrial processes, few studies have been focused on determining the mechanisms and molecules involved in their water homeostasis. This chapter describes the identification and quantification of the principal osmo-protectants used by acidophilic mineral-degrading bacteria in response to osmotic stress.

5.2 MATERIALS AND METHODS

5.2.1 MICROORGANISMS AND SAMPLES OF INDUSTRIAL BIOLEACHING PLANT AND LABORATORY-SCALE REACTORS

Microorganisms described in this chapter are various autotrophic, mixotrophic and heterotrophic mineral-degrading bacteria, and include those isolated from industrial bioleaching plants located in northern Chile (Table 5.1).

Table 5.1. Bacteria used in experiments to identify osmo-protectants in acidophiles.

bacterium	Origin
L. ferriphilum^{T}	Lima, Perú.
L. ferriphilum IESL25	Escondida mine, Chile
L. ferriphilum Sp-Cl	Spence mine, Chile
L. ferrodiazotrophum^{T}	Iron mountain, U.S.A.
At. ferrooxidans IESL32	Escondida mine, Chile
At. thiooxidans IESL33	Escondida mine, Chile
Sb. thermosulfidooxidans^{T}	Copper ore, Russia
Acidiphilium sp. SJH	Pyrite mine, North Wales, U.K.

In addition, PLS samples from the low-grade copper ore bioleaching plant at the Escondida mine, and from a laboratory test-scale reactor operating at 12 g/L of of NaCl were also used in experimental work.

5.2.2 CULTURE MEDIA AND GROWTH CONDITIONS USED TO CULTIVATE ACIDOPHILIC BACTERIA *IN VITRO*

Cultures of *Leptospirillum* spp. were grown in 50 mM ferrous iron medium (section 2.2). The pH of the medium was adjusted to 1.5, and cultures were incubated at 30℃ (*L. ferrooxidans*) or 37℃ (*L. ferriphilum* and "*L. ferrodiazotrophum"*). *At. ferrooxidans c*ultures were grown using the same medium as *Leptospirillum* spp,, but the pH was adjusted to 2.0 and cultures were incubated at 30℃. *At. ferrooxidans* IESL32 was also grown in 1% (w/v) sulfur medium (section 2.2) at the same temperature and pH. *Sb. thermosulfidooxidans* was grown in autotrophic basal salts medium containing 50 mM ferrous sulfate, supplemented with 0.02% (w/v) yeast extract. The pH was adjusted to 1.8 and cultures were incubated at 37℃. *Acidiphilium* sp. SJH was grown in 10 mM galactose medium (section 2.2). The pH was adjusted to 2.0 and cultures were incubated at 30℃.

Growth of iron-oxidizers was assessed by periodic determination of ferrous iron (section 2.6.1), while direct cell counts were used to assess the growth of *Acidiphilium* sp. SJH, and also of *At. ferrooxidans* IESL32 when grown in sulfur medium. The osmotic strength of culture media was increased, when required, by supplementing them with magnesium sulfate, sodium chloride or potassium sulfate at different concentrations. Non salt-amended cultures were used as controls, and analysis of biomass was carried out using cells harvested at the late exponential growth phase on each occasion. Culture conditions for each strain are listed in Table 5.2

Table 5.2. Culture conditions used to determine osmo-protectants in mineral-degrading bacteria. All cultures were grown in non-supplemented media for comparative purposes.

Bacteria	Salt added to medium		
	$MgSO_4$ (mM)	NaCl (mM)	K_2SO_4 (mM)
L. ferriphilum^T	300		
L. ferriphilum IESL25	300		300
	600		
L. ferriphilum Sp-Cl	300	200	
"L. ferrodiazotrophum"^{T}	300		
At. ferrooxidans IESL32	300 (Fe*)		
	300 (S*)		
At. thiooxidans IESL33	300	200	
	500		
	700		
	1000		
Sb. thermosulfidooxidans^T	300	200	
Acidiphilium sp. SJH	300	200	

*Fe and S were used as electron donors.

All cultures were grown in 1L medium in 3 L Erlenmeyer flasks, shaken at 120 rpm. Cells were harvested during the late exponential growth phase by centrifugation at 15,000 rcf for 25 minutes at 4℃ in a Beckman J2-21 M/E centrifuge (Beckman, U.S.A). Biomass was estimated by determining protein contents (section 2.5.2).

5.2.3 CULTIVATION OF *AT. THIOOXIDANS* IESL33 IN A BENCH SCALE BIOREACTOR

Acidithiobacillus thiooxidans IESL33 was grown in a 2 L bench-scale bioreactor containing 1L of 1% (w/v) sulfur medium (section 2.2). Temperature and pH were set at pH 2.0 and 30℃, respectively. Osmotic strength was increased in stages by adding

fresh medium containing increasing concentration of magnesium sulfate. Culture growth was assessed by measuring consumption of alkali. Cells from each run were harvested by centrifugation at late exponential growth phase and biomass determined from measuring protein contents (section 2.5.2).

5.2.4 PREPARATION OF EXTRACTS FOR IDENTIFICATION AND DETERMINATION OF OSMO-PROTECTANTS

Osmo-protectants were extracted from cultures grown in media supplemented with different concentration of magnesium sulfate, sodium chloride or potassium sulfate and with no supplemental salts. Extracts were prepared both by ethanol-extraction and by hypo-osmotic shock, as described below.

5.2.4.1 ETHANOL EXTRACTION

This was carried out in three stages:

Stage 1; Cell washing.

Cells pellets from liquid cultures and industrial samples were collected by filtering 1L or 5 L of solution, respectively, through 0.2 µm (pore diameter) cellulose nitrate membrane filters. The cells were rinsed off the filters and resuspended in 10 ml of fresh media containing the same concentration of magnesium sulfate, sodium chloride or potassium sulfate, and centrifuged again at 15,000 rcf for 25 minutes. This step was repeated twice.

Stage 2: Resuspension in ethanol

The washed cell pellets were resuspended in 1 ml of ethanol (70% v/v), transferred to a 1.5 ml Eppendorf tube, and agitated in a vortex mixer at maximum speed for 5 minutes. After agitation, the tubes were centrifuged at maximum speed and the supernatants transferred to 15 ml Falcon tubes. This step was repeated 5 times, collecting, in total, approximately 5 ml of ethanol extract from each cell pellet.

Stage 3: Freeze-drying

Ethanol extracts obtained during the previous step were transferred to 10 ml round bottom glass flasks, frozen by immersion in liquid nitrogen and freeze-dried overnight in a freeze dryer (Edwards Modulyo EF4-174, U.S.A). The solid materials in the round bottom glass flasks were resuspended in 0.5 ml of sterile MilliQ-grade water and transferred to chromatographic vials prior to chromatographic analysis.

5.2.4.2 HYPO-OSMOTIC SHOCK APPROACH

A hypo-osmotic shock approach was also used to identify osmo-protectants in cultures of acidophiles grown in high osmotic strength media. This involved transferring bacteria grown in high osmotic potential media to acidified MilliQ-grade water, and identifying compounds that were exuded by the cells in the response to the sudden fall in external osmotic potential. Cells grown in high osmotic strength media were harvested by centrifugation, resuspended in 5.0 ml of acidified MilliQ-grade water (pH 1.7, adjusted with sulfuric acid) and held at room temperature by 10 minutes. Bacterial cells were separated by centrifugation (16,000 rcf, 5 minutes), and the supernatant was filtered

through cellulose nitrate membranes (0.2 µm pore diameter) and transferred to chromatography vials.

5.2.5 ANALYSIS OF INDUSTRIAL BIOLEACH LIQUORS

Three liquid samples from different scale mineral bioleach operations were analyzed by chromatographic analyses to determine the presence of compatible solutes in microbial community. The samples analyzed and their characteristics are summarized in Table 5.3. Samples were processed as described in section 5.2.4.1.

Table 5.3. Industrial samples analyzed for the detection of compatible solutes present in their indigenous microflora.

Sample	Description	Origin	Microbial community	Substrate	Concentration of main ionic compound
1	PLS from industrial sulfide heap leach process	Escondida sulfide leach plant.	native acidophilic community	copper sulfide run-of-mine ore	SO_4^{2-}; 80 g/L
2	Culture of reactor running in presence of chloride	pilot-scale reactor BioChlor process, BHP-BIlliton	*At. thiooxidans* and *L. ferriphilum* Sp-Cl.	copper concentrate	Cl^-; 12 g/L
3	PLS from a column test for a bioleaching in presence of chloride	pilot-scale column tests of BioChlor process, BHP-Billiton	Initial inoculation with culture of sample 2	crushed ore	Cl^-; 12 g/L

5.2.6 IDENTIFICATION AND QUANTIFICATION OF COMPOUNDS DETECTED BY CHROMATOGRAPHIC ANALYSIS

Different analytical-grade compounds were prepared in MilliQ-grade water as 50 mM stock solutions and used as standards for the identification and quantification of compounds in ethanol extracts or hypo-osmotic shock solutions. Retention times of peaks observed in chromatograms of extracts were compared with those of standard compounds for preliminary identification. Compounds used as standards are listed in Table 5.4. Mass spectrometry analysis of extracts was used to support chromatographic identification. Compounds were analyzed using a Dionex ICS 3000 system equipped with a CarboPac^{TM} MA1 separation column, including guard column, and an ED40 amperometric detector. The separation column was maintained at 30℃ during the analyses. The flow rate used was 0.4 ml/minute and 50 µL of sample were injected. Column pressure was 1,600 p.s.i. with a lower limit of 200 p.s.i. and a upper limit of 3,000 p.s.i.. A 0.25 M solution of sodium hydroxide was used as eluent and the pH was maintained between 10 and 13 during the analysis. Alternatively, an AminoPac^{TM} PA10 column was used using the same conditions as with the CarboPac^{TM} MA1 column, except that 0.1 M sodium hydroxide was used as eluent. Signals were visualized and analyzed using Chromeleon software ®, and both columns were able to resolve most of the compounds used as standards. The concentration of compounds (identified by coincidence in retention times and m/z values) in samples was determined using the Chromeleon software ®. This software calculates automatically the concentration of each compound in the samples based on retention time (to assume the identification of compound) and the comparison of the peak area detected in samples with peak areas of a standard compound included in the chromatographic run, at least at three different concentrations. The concentrations of compounds determined by chromatographic

analyses were then normalized against the protein concentrations determined in sub-samples from each experiment.

5.2.7 ACID HYDROLYSIS OF EXTRACTS

Acid hydrolysis of extracts, followed by chromatographic analysis was used to detect sugar dimers and oligomers. Hydrolysis was carried out in heat-resistant screw-cap glass tubes containing 1 ml of extract and 0.25 ml of 4.5 M sulfuric acid. The tubes were heated in a dry heating block system (Grant, Cambridge Instruments, UK) at 100 ℃ for one hour and the hydrolyzed liquids transfer red to chromatographic vials. Chromatographic analysis was carried out as described above.

5.2.8 MASS SPECTROMETRY ANALYSIS

Matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) mass spectrometry was used to analyze the low molecular weight compounds present in liquid samples. A suspension of titanium oxide in water (10 mg/ml) was used as the matrix. Extracts were mixed with the matrix in a 1:1 ratio (using 1 µl of each), spotted onto a target plate MTP384 (Bruker Daltonics, Germany), and allowed to dry at room temperature for 2 hours.

Table 5.4. Compounds used as standards for identification of osmo-protectants.

Sugars	Amino acids	Alcohols	Organic acid	Betaines
Monosaccharides	Proline	Sorbitol	Glycolic acid	Ectoine
Hexoses ($C_6H_{12}O_6$)	Lysine	Glycerol		Betaine
Glucose	Arginine	Mannitol		
Mannose	Glycine			
Fructose				
Galactose				
Deoxy hexoses ($C_6H_{12}O_5$)				
Rhamnose				
Fucose				
Pentoses ($C_5H_{10}O_5$)				
Xylose				
Arabinose				
Disaccharides **($C_{12}H_{22}O_{11}$)**				
Maltose				
Sucrose				
Cellobiose				
Trehalose				

A Reflex IV MALDI-TOF device (Bruker Daltonics, Germany) was used for the analysis. The device was set in a linear mode using a nitrogen laser (λ=337 nm and pulse frequency 9 Hz in the positive ion mode). The analyzer delay time was 200 nano-seconds, accelerating electrode voltage 20.0 kV, accumulating electrode voltage 16.45 kV, and focusing lens voltage 9.7 kV. The mass-spectrometer parameters were optimized for the mass/charge (m/z) range 20-2,000. Each mass spectrum was obtained at 20 accumulations at constant power of laser at the level of threshold value for the resolution increase. The lists of spectral peaks obtained from extracts of cultures grown at elevated salt concentrations (magnesium sulfate and sodium chloride) and in non-amended media were compared to determine differential peaks. Each differential peak identified was identified by comparing with those of known common organic osmolytes produced by prokaryotes (Table 5.5).

Table 5.5 Molecular weight and possible adducts for common organic osmo-protectants synthesized by prokaryotes.

		m/z adducts				
Compound	**Molecular weight**	**H^+**	**Na^+**	**K^+**	**$2Na^+$**	**$2K^+$**
Ectoine	142.160	143.168	165.150	181.124	94.070	110.043
Glucose	180.063	181.071	203.053	219.027	113.021	128.995
Hydroxyectoine	158.160	159.168	181.150	197.124	113.021	128.995
Betaine	117.146	118.154	140.136	156.110	102.070	118.043
Arsenobetaine	178.061	179.069	201.051	217.025	81.562	97.536
Dimethylsulfoniopropionate	134.197	135.205	157.186	173.160	112.020	127.994
β-glutamine	146.610	147.618	169.600	185.574	90.088	106.062
N- ε-acetyl-β-lysine	188.116	189.124	211.106	227.080	96.294	112.268
Trehalose anhydrate	342.296	343.304	365.286	381.260	117.047	133.021
Trehalose dihydrate	378.330	379.338	401.320	417.294	194.138	210.111
Glutamate	147.130	148.138	170.120	186.094	212.155	228.129
Glucosylglycerol	254.100	255.108	277.090	293.064	96.555	112.529
Proline	115.13	116.138	138.120	154.094	150.040	166.014
Glycerol	92.094	93.102	115.084	131.058	80.555	96.529
Mannitol	182.172	183.180	205.162	221.136	69.037	85.011
Arabitol	152.146	153.154	175.136	191.109	114.076	130.050
Sucrose	342.296	343.304	365.286	381.260	99.063	115.037
2-Sulfotrehalose	422.073	423.081	445.063	461.037	194.138	210.112
N-δ-acetylornithine	174.100	175.108	197.090	213.064	234.026	250.000

5.3 RESULTS

5.3.1 CHROMATOGRAPHIC ANALYSIS OF STANDARD COMPOUNDS

Table 5.6 shows the retention times (using chromatographic conditions described in section 5.2.5) obtained with 100 µM of various test compounds, using the CarboPac MA1 and AminoPac PA10 columns.

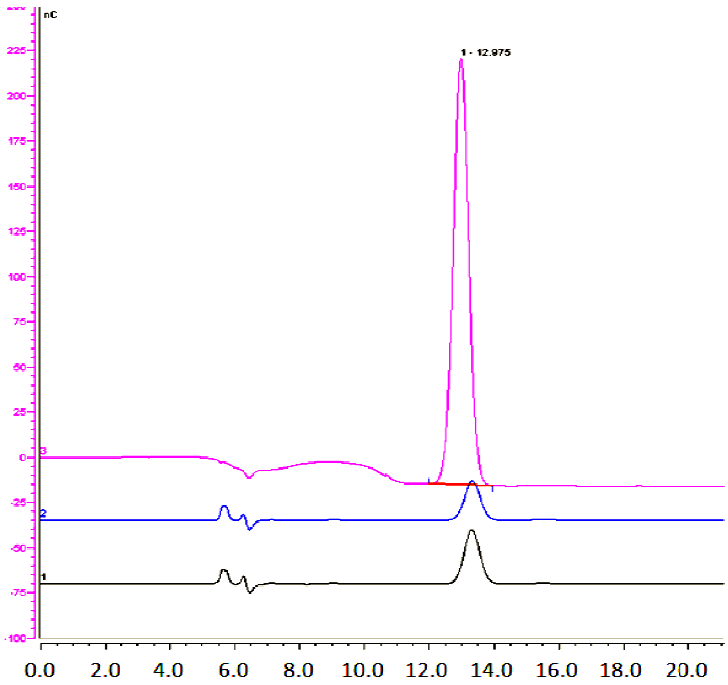
Table 5.6 Retention times obtained for potential osmo-protectants, analyzed by liquid chromatography.

Compound	Retention time (mins)	
	Separation Column	
	MA1	PA10
Glycerol	8.9	-
Fucose	11.2	-
Rhamnose	11.2	-
Trehalose	12.9	2.7
Sorbitol	13.5	-
Mannose	14.1	-
Glucose	15.3	3.2
Galactose	ND	4.0
Arabinose	15.5	-
Mannitol	15.7	2.5
Xylose	16.5	-
Fructose	17.8	4.6
Cellobiose	23.2	10.0
Sucrose	23.5	-
Maltose	28.1	-
Proline	36.7	9.5
Ectoine	-	NS
Betaine	-	2.2
Lysine	NS	3.6
Arginine	NS	
Glycine	-	NS
Glycolic acid	-	NS

"-": not determined; "NS": No signal obtained

5.3.2 *LEPTOSPIRILLUM* SPP.

Chromatographic profiles of ethanolic extracts of *L. ferrooxidans*^{T}, *L. ferriphilum*^{T}, *L. ferriphilum* IESL25, and "*L. ferrodiazotrophum*" grown in the presence of different concentration of ionic solutes were compared with those of standard compounds. Ethanol extracts of all three *Leptospirillum* spp. showed only one distinct peak when either the CarboPac MA1 or the AminoPac PA10 columns were used, and in both cases this corresponded to the glucose disaccharide, trehalose (shown for *L. ferriphilum* IESL25 in Figures 5.1 and 5.2).



Time (minutes)

Figure 5.1. Overlay of chromatograms obtained using the CarboPac MA1 column of trehalose (pink line), and extracts obtained from *L. ferriphilum* IESL25 grown in presence of 300 mM magnesium sulfate (black line) or 300 mM of potassium sulfate (blue line).

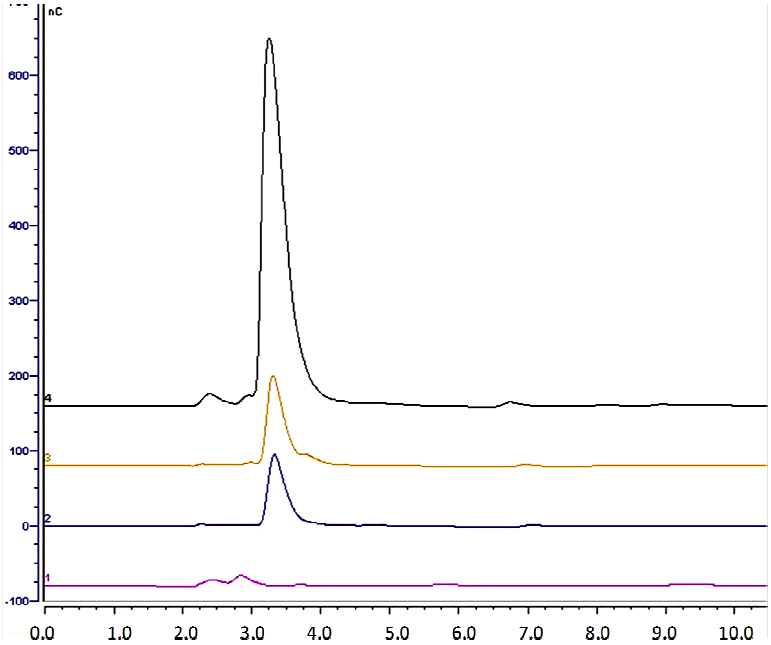


Figure 5.2 Overlay of chromatograms obtained using the AminoPac PA10 column of trehalose (in black) and extracts obtained from *L. ferriphilum* IESL25 grown in the presence of 300 mM potassium sulfate (blue), 300 mM of magnesium sulfate (brown) or non-supplemented medium (pink).

Differences were detected between solutes present in ethanol extracts of *Leptospirillum* spp. and those in the hypo-osmotic shock samples in that, although trehalose was detected in both cases, an additional peak with the same retention time as glucose was detected in hypo-osmotic shock extracts (Fig. 5.3).

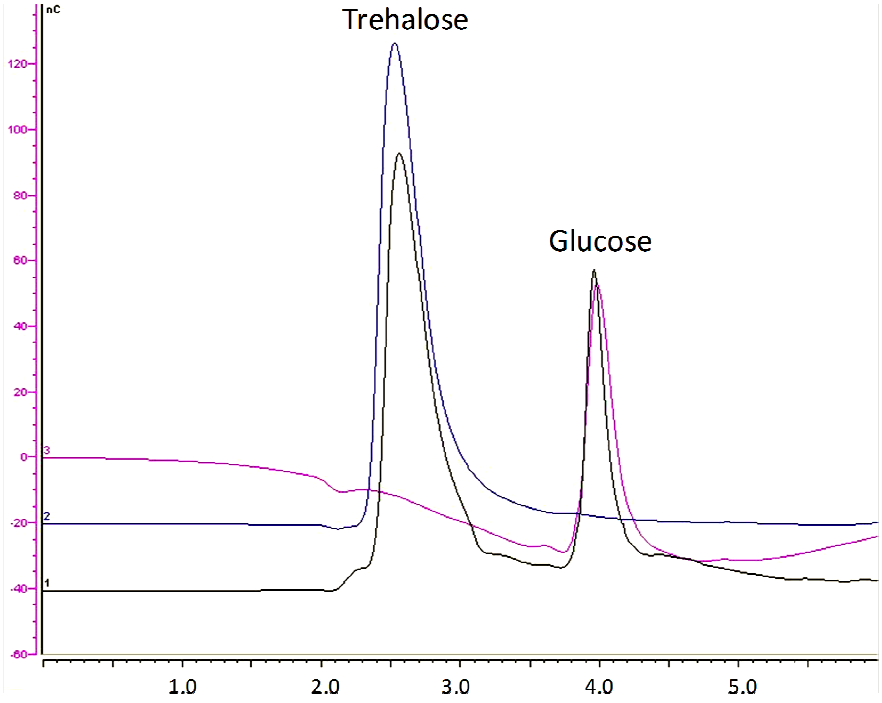


Figure 5.3 Overlay of chromatograms obtained using the AminoPac PA10 column of trehalose (blue line), glucose (pink line) and the hypo-osmotic shock extracts from *L. ferriphilum* IESL25 grown in the presence of 300 mM of magnesium sulfate (black line). The latter shows a peak with the same retention time of glucose, and was not detected in ethanolextract as shown in Figure 5.2.

Chromatographic profiles of ethanol extracts and hypo-osmotic shock samples for cultures of *L. ferriphilum*^{T}, *L. ferriphilum* Sp-Cl and "*L. ferrodiazotrophum*"^{T} were similar to those of *L. ferriphilum* IESL25, showing one peak with the same retention time as trehalose for ethanol extracts and two peaks were observed with retention times corresponding to trehalose and glucose in hypo-osmotic shock solutions.

5.3.3 *ACIDITHIOBACILLUS THIOOXIDANS* IESL33

Ethanol extracts of *At. thiooxidans* IESL33 grown in different concentrations of magnesium sulfate were analyzed by liquid chromatography using the CarboPac MA1 column only. Five distinct peaks were apparent in the profiles, and two of these had retention times corresponding to glucose and proline (Figure 5.4)

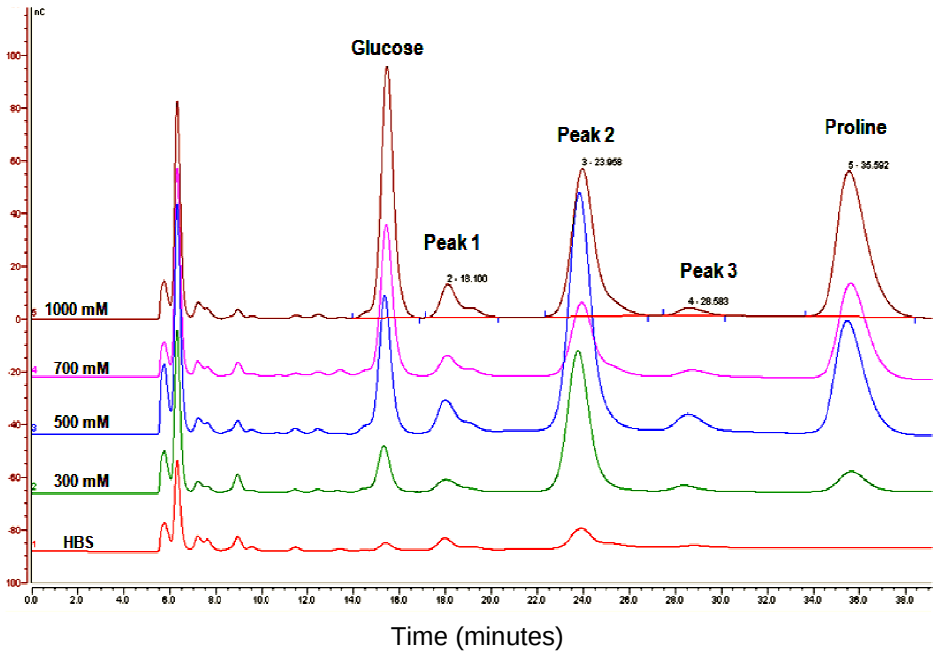


Figure 5.4. Overlay of chromatograms (using the CarboPac MA1 column) of ethanol extracts obtained from *At. thiooxidans* IESL33 cultures grown at increasing concentrations of magnesium sulfate. (HBS indicates non-supplemented medium).

In order to obtain more information about the unidentified peaks in these chromatograms, extracts were hydrolyzed using sulfuric acid, followed by chromatographic analysis. Figure 5.5 shows an overlay of chromatograms of one ethanol extract obtained from a culture of *At. thiooxidans* IESL33 grown in presence of 500 mM magnesium sulfate and a fraction of the same extract after hydrolysis. The increase in the size of the glucose peak indicated that peak obtained at 24 minutes retention time was a glucose oligomer.

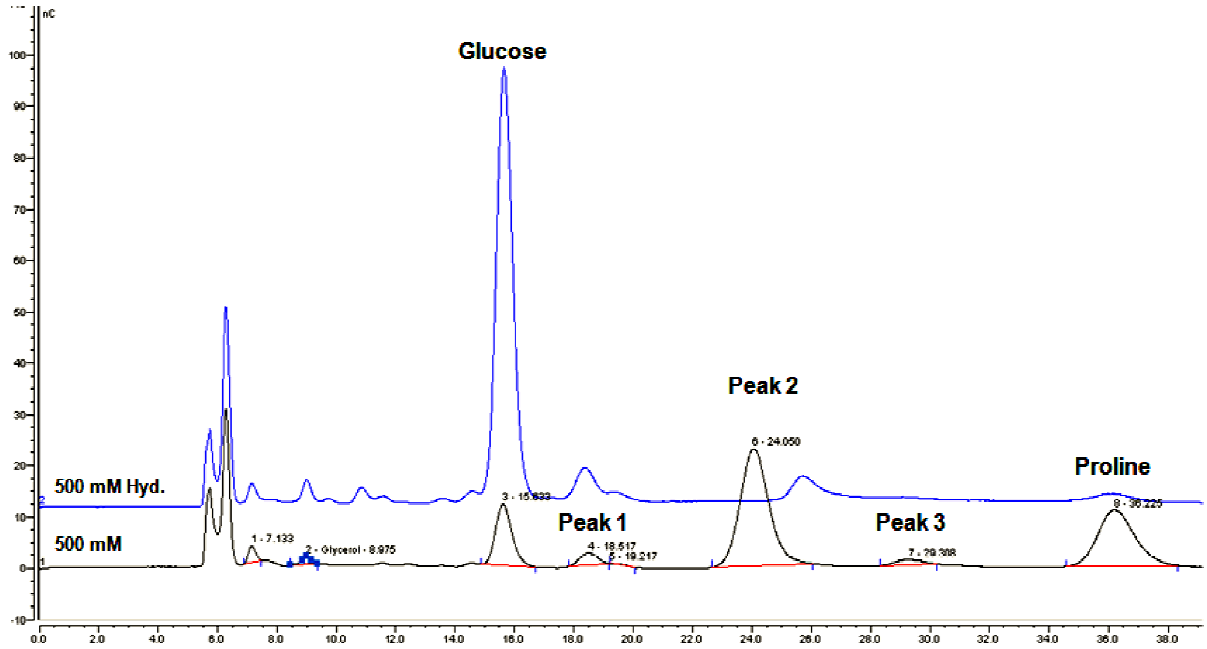


Figure 5.5 Overlay of chromatograms of extract obtained of *At. thiooxidans* IESL33 grown in the presence of 500 mM of magnesium sulfate (black line) and a chromatogram of the same extract after acid hydrolysis (blue line)

5.3.4 *ACIDITHIOBACILLUS FERROOXIDANS* IESL32

Extracts of *At. ferrooxidans* IESL32 were obtained by hypo-osmotic shock of cells cultured in medium containing either ferrous iron or sulfur as electron donor. In both cases two peaks were detected. Figure 5.6 shows the chromatograms obtained of cultures of *At. ferrooxidans* IESL32 grown in the presence of 300 mM of magnesium sulfate and non-amended controls, using ferrous iron or elemental sulfur as growth substrates.

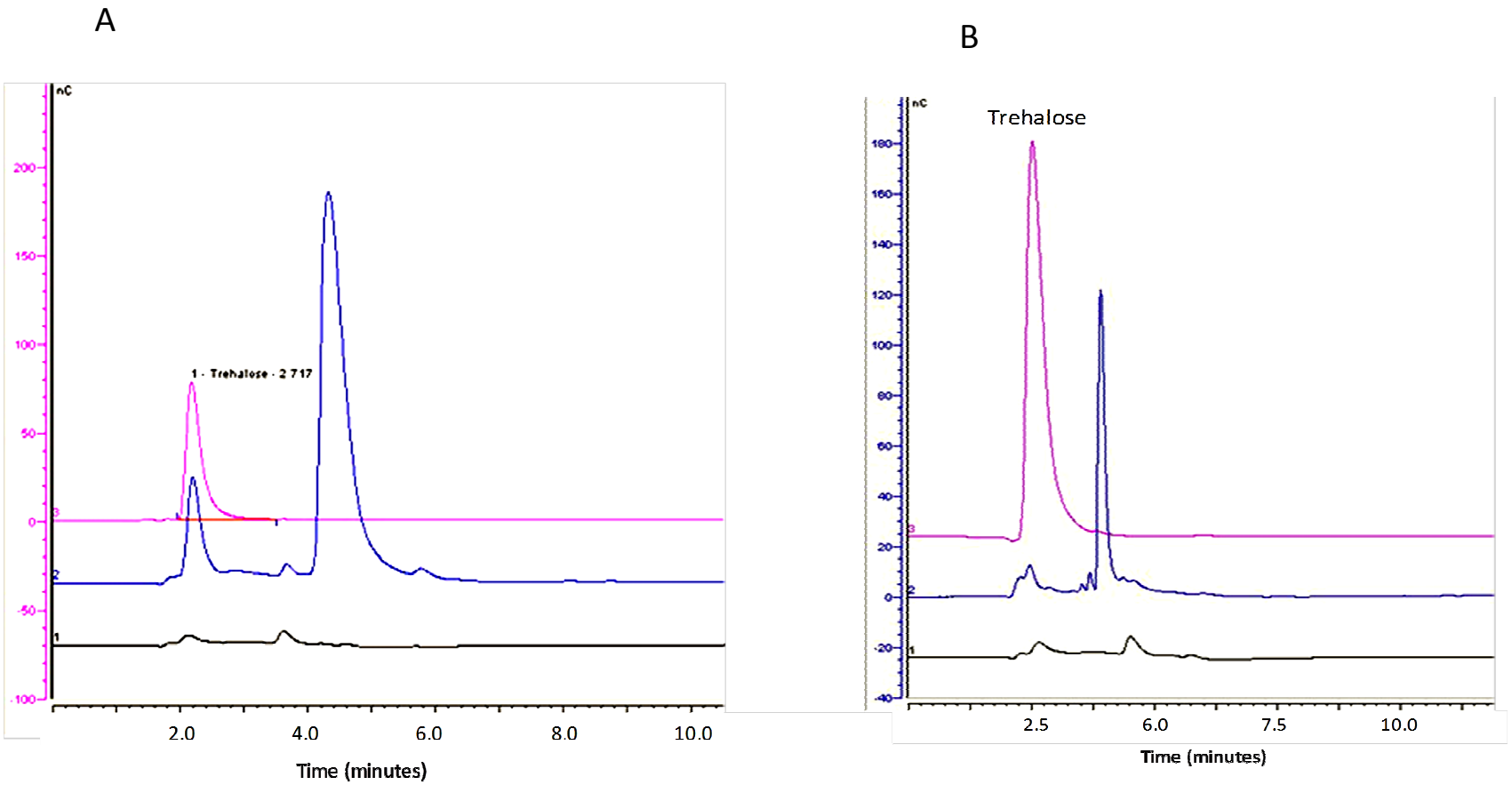


Figure 5.6 Overlay of chromatograms of trehalose standard 0.1 mM (pink line) and hypo-osmotic extracts obtained from cultures of *At. ferrooxidans* IESL32 containing 300 mM magnesium sulfate (blue) and in non-amended medium (black). Chromatograms were obtained from cultures grown with ferrous iron (A) or sulfur (B) as electron donors.

The chromatograms of extracts obtained from cultures of *At. ferrooxidans* IESL32 grown in medium amended with 300 mM of magnesium sulfate showed two distinct peaks with retention times 2.7 and 5.0 minutes. The first peak (2.7 minutes) had the same retention time as that of trehalose, while the second peak did not correspond to any reference compound tested.

5.3.5 *SULFOBACILLUS THERMOSULFIDOOXIDANS*^{T}

Chromatographic analysis of hypo-osmotic shock extracts of *Sb. thermosulfidooxidans*^{T} grown in ferrous iron-yeast extract medium amended with 300 mM magnesium sulfate showed a peak with the same retention time (2.7 minutes, using the AminoPac PA10 column) as that of trehalose (Fig. 5.7).

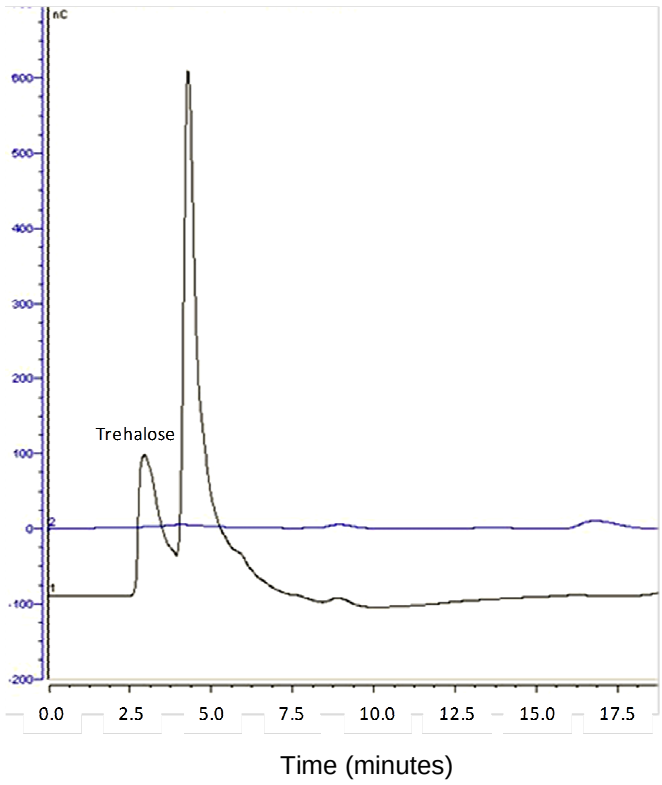
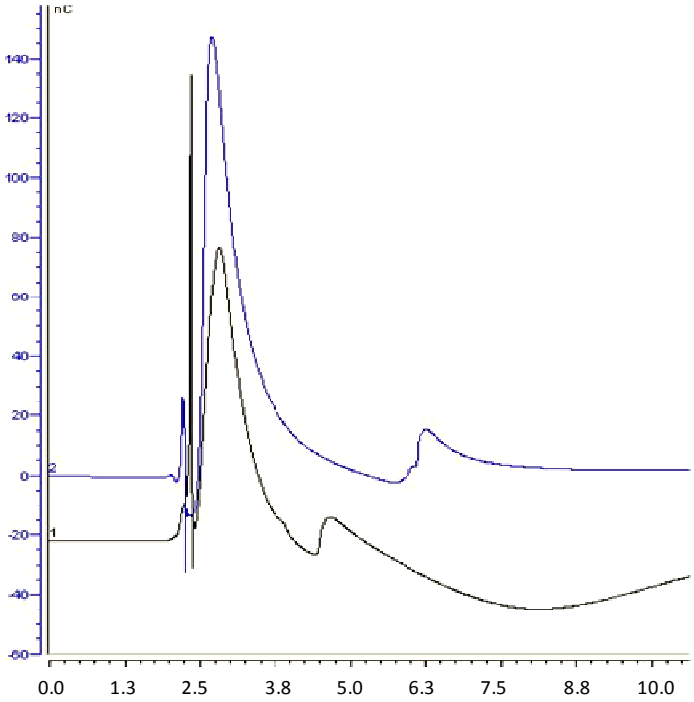


Figure 5.7 Overlay of chromatograms obtained from hypo-osmotic shock extracts of cultures of *Sb. thermosulfidooxidans*^{T} grown in iron-yeast extract medium (blue line), and in the same medium amended with 300 mM of magnesium sulfate (black line).

Acid hydrolysis of ethanol extracts of *At. ferrooxidans* IESL32 and *Sb. thermosulfidooxidans*^{T} were analyzed by liquid chromatography in order to try to identify the nature of the second unidentified peak with retention time of ~5 minutes. In both cases, acid hydrolysis caused the peak to be no longer present but, in contrast to *At. thiooxidans* IESL33, no additional peak corresponding to glucose was produced (Fig. 5.8). It was concluded therefore that the compound represented by this peak was sensitive to acid hydrolysis, but was not a glucose dimer or oligomer.



Time (minutes)

Figure 5.8 Overlay of chromatograms of hydrolyzed extracts obtained from cultures of *At. ferrooxidans* IESL32 (black line) and *Sb. thermosulfidooxidans*^{T} (blue line) grown in media amended with 300 mM magnesium sulfate.

5.3.6 *ACIDIPHILIUM* SP SJH

Chromatographic analysis of water extracts of *Acidiphilium* sp. SJH grown in 300 mM magnesium sulfate or 200 mM sodium chloride obtained using the hypo-osmotic shock approach showed that trehalose was the main osmo-protectant present in both cases. In addition, a peak corresponding to galactose was observed in all the extracts (Fig. 5.7) though this could have been carry over from the growth medium as *Acidiphilium* sp. SJH was grown on galactose in this experiment.

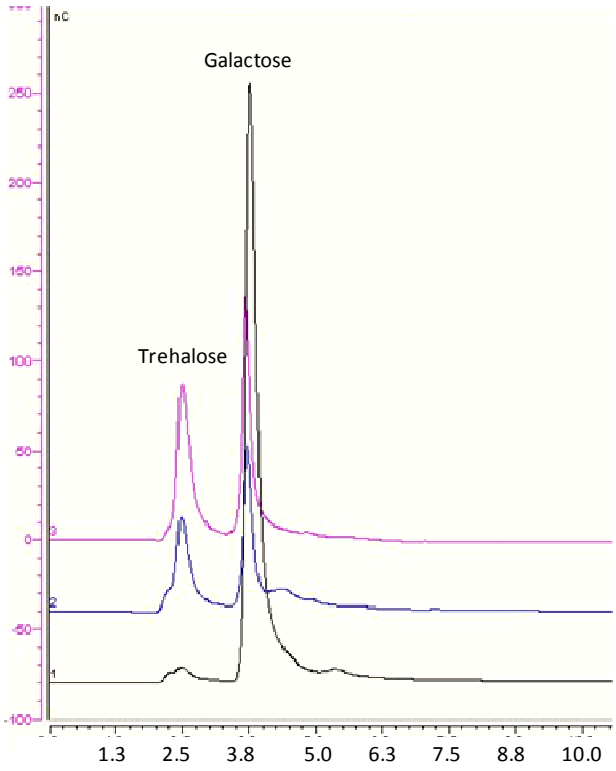


Figure 5.9 Overlay of chromatograms obtained from cultures of *Acidiphilium* sp SJH grown in galactose medium (black line), and the same medium amended with 200 mM sodium chloride (blue line) or 300 mM of magnesium sulfate (pink line)

5.3.7 CHROMATOGRAPHIC ANALYSIS OF INDUSTRIAL SAMPLES

Samples from full-scale and pilot-scale industrial tests at the Escondida mine were analyzed for the presence of the osmo-protectants identified in pure culture studies of bioleaching bacteria. The samples were PLS from the heap leaching operation, and liquid samples from outflow of two column test and from a chloride leaching pilot-scale reactor. A peak with the same retention time as trehalose was detected in all three samples, as was a peak with the same retention time as that of the unknown peak detected in cultures of *At.*

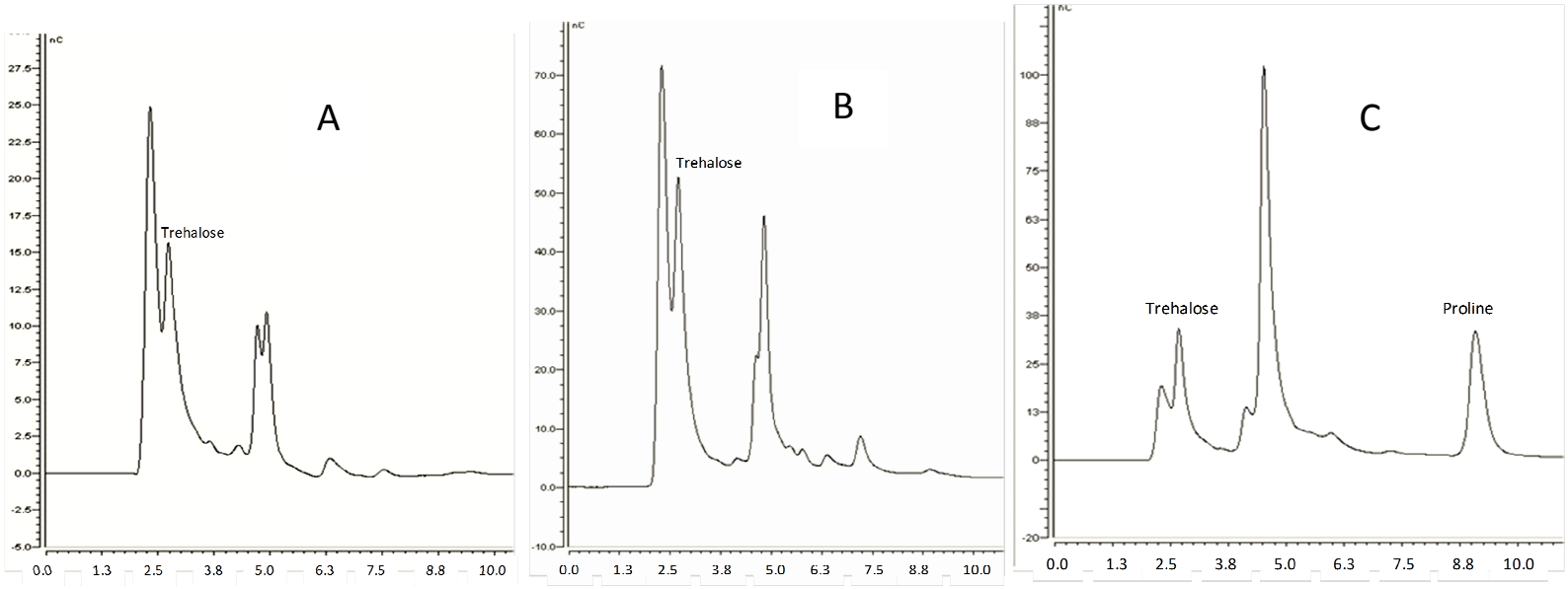


Figure 5.10 Chromatograms obtained of ethanol extracts of cells harvested from samples from: (A) PLS from the full-scale heap bioleaching process of Escondida; (B) PLS from a column leach test; (C) Culture from a 7 L pilot-scale reactor.

ferrooxidans IESL 32 and *Sb. thermosulfidooxidans*^{T}. In the pilot-scale bioheap leach sample, a peak with the same retention time as proline was also detected.

5.3.8 MASS SPECTROMETRY ANALYSES

Mass spectrometry peaks with similar molecular weights to compounds or adducts observed in ethanol extracts of *L. ferriphilum* IESL25 are shown in Table 5.7. Comparison of spectra obtained from extracts of cultures grown in control cultures and those grown in medium amended with 300 mM of magnesium sulfate resulted in the detection of 45 m/z peaks in extracts of cultures grown in medium amended with 300 mM of magnesium sulfate. Eleven of these peaks had m/z close to adducts calculated for common osmo-protectants in other prokaryotes (Table 5.7).

Table 5.7 Similarity of mass spectrometry peaks detected in ethanol extracts of *L. ferriphilum* IESL25 grown in medium amended with 300 mM of $MgSO_4$ to those calculated for adducts of common osmo-protectants synthesized by prokaryotes.

Peaks m/z	Adduct	Theoretical m/z	Δ m/z
112.02	Arsenobetaine 2Na+	112.0203	0.0003
118.12	Betaine H+	118.1538	0.0338
153.08	Arabitol H+	153.1536	0.0736
157.26	Dimethylsulfoniopropionate Na+	157.1865	0.0735
181.13	Ectoine K+	181.1237	0.0063
	Hydroxyectoine Na+	181.1498	0.0198
	Glucose H+	181.0712	0.0588
185.06	β glutamine K+	185.5737	0.5137
211.08	N- ε-acetyl-β-lysine Na+	211.1059	0.0259
227.07	N- ε-acetyl-β-lysine K+	227.0798	0.0098
255.10	Glucosyl-glycerol H+	255.1080	0.0080
293.16	Glucosyl-glycerol K+	293.0639	0.0961
400.99	Trehalose dihydrate Na+	401.3198	0.3298

Extracts of cultures of *L. ferriphilum* IESL25 grown in medium amended with potassium sulfate (300 mM) were also analyzed and 15 peaks out of 91 had m/z similar to common osmo-protectant adducts produced by other prokaryotes (Table 5.8).

Table 5.8 Similarity of mass spectrometry peaks detected in ethanol extracts of *L. ferriphilum* IESL25 grown in medium amended with 300 mM of K_2SO_4 to those calculated for adducts of common osmo-protectants in prokaryotes.

Peaks m/z	Compound or Adduct	Theoretical m/z	Δ m/z
212.95	Trehalose dihydrate 2Na+	212.1548	0.7952
140.85	Betaine Na+	140.1358	0.7142
156.12	Betaine K+	156.1097	0.0103
97.74	Betaine 2K+	97.5367	0.2033
143.09	Ectoine H+	143.1678	0.0778
165.11	Ectoine Na+	165.1498	0.0398
181.09	Ectoine K+	181.1237	0.0337
	Hydroxyectoine Na+	181.1498	0.0598
	Glucose H+	181.0712	0.0188
93.90	Ectoine 2Na+	94.0698	0.1698
201.06	Arsenobetaine Na+	201.0510	0.0090
157.12	Dimethylsulfoniopropionate Na+	157.1865	0.0665
147.04	β glutamine H+	147.6178	0.5778
365.44	Trehalose anhydrate Na+	365.2858	0.1542
381.42	Trehalose anhydrate K+	381.2597	0.1603
197.07	N-δ-acetylornithine Na+	197.0902	0.0202
212.95	N-δ-acetylornithine K+	213.0641	0.1141

Mass spectrometry analysis of extracts of *L. ferriphilum* Sp-Cl grown in liquid medium amended with 200 mM of sodium chloride resulted in 14 (of 98) m/z peaks identified as being close to those calculated for adducts of common osmo-protectants produced by other prokaryotes (Table 5.9).

Table 5.9 Similarity of mass spectrometry peaks detected in ethanol extracts of *L. ferriphilum* Sp-Cl grown in medium amended with 200 mM of NaCl to those calculated for adducts of common osmo-protectants in prokaryotes

Peaks m/z	Compound or Adduct	Theoretical m/z	Δ m/z
365.43	Trehalose anhydrate Na+	365.2858	0.1442
381.41	Trehalose anhydrate K+	381.2597	0.1503
143.09	Ectoine H+	143.1678	0.0778
181.08	Ectoine K+	181.1237	0.0437
181.08	Hydroxyectoine Na+	181.1498	0.0698
159.04	Hydroxyectoine H+	159.1678	0.1278
197.06	Hydroxyectoine K+	197.1237	0.0637
201.06	Arsenobetaine Na+	201.0510	0.0090
127.85	Arsenobetaine 2K+	127.9943	0.1443
117.01	N- ε-acetyl-β-lysine 2Na+	117.0478	0.0378
181.08	Glucose H+	181.0712	0.0088
	Ectoine K+	181.1237	0.0437
	Hydroxyectoine Na+	181.1498	0.0698
203.28	Glucose Na+	203.0532	0.2268
219.10	Glucose K+	219.0271	0.0729
128.82	Glucose 2K+	128.9954	0.1754

Differential mass spectrometry analysis of extracts of cultures of *At. ferrooxidans* IESL32 grown in medium amended with 300 mM of magnesium sulfate resulted in 3 (of 31) similar m/z peaks identified as being similar to adducts calculated for common osmo-protectants produced by other prokaryotes (Table 5.10).

Table 5.10 Similarity of mass spectrometry peaks detected in extracts of cultures of *At. ferrooxidans* IESL32 grown in medium amended with 300 mM of $MgSO_4$ to those calculated for adducts of common osmo-protectants in prokaryotes.

Peaks m/z	Compound or Adduct	Theoretical m/z	Δ m/z
157.00	Dimethylsulfoniopropionate Na+	157.1865	0.1865
212.28	Trehalose dihydrate 2Na+	212.1548	0.1252
69.07	Glycerol 2Na+	69.0367	0.0333

Spectrometry mass analysis of extracts of *At. thiooxidans* IESL33 grown in medium amended with 300 mM of magnesium sulfate resulted in 5 out of 114 m/z peaks being identified as similar to those calculated for adducts of common osmo-protectants produced by other prokaryotes (Table 5.11)

Table 5.11 Similarity of mass spectrometry peaks detected in ethanol extracts of cultures of *At. thiooxidans* IESL33 grown in medium amended with 300 mM of $MgSO_4$ to those calculated for adducts of common osmo-protectants in prokaryotes.

Peaks m/z	Compound or Adduct	Theoretical m/z	Δ m/z
116.17	Proline H+	116.1378	
138.29	Proline Na+	138.1198	
153.98	Proline K+	154.0937	
203.12	Glucose Na+	203.0532	
131.03	Glycerol K+	131.0575	

5.3.9 QUANTIFICATION OF OSMO-PROTECTANTS IDENTIFIED IN CULTURES OF ACIDOPHILES

Table 5.12 shows the quantification of osmo-protectants in cultures of different acidophilic bacteria grown at different concentration of ionic solutes.

Table 5.12 Specific amounts of osmo-protectants produced by mineral-degrading bacteria grown at different concentrations of sodium chloride or magnesium sulfate.

		Osmo-protectant		
	Strain (condition)	Trehalose µg/mg protein	Glucose µg/mg protein	Proline µg/mg protein
L. ferriphilum	IESL25	10.0	<0.01	
	IESL25 (300 mM $MgSO_4$)	184.0	1.0	
	IESL25 (600 mM $MgSO_4$)	587.0	2.0	
	IESL25 (300 mM K_2SO_4)	529.0	2.1	
	Sp-Cl	9.0	<0.01	
	Sp-Cl (300 mM $MgSO_4$)	240.0	1.1	
	Sp-Cl (200 mM NaCl)	300.0	1.0	
	Type	7.0	<0.01	
	Type (300 mM $MgSO_4$)	179.0	1.3	
"L. ferrodiazotrophum"	Type	8.0	<0.01	
	Type (300 mM $MgSO_4$)	198.0	1.1	
At. ferrooxidans	IESL32	5.0		
	IESL32 (300 mM $MgSO_4$) in Fe	21.0		
	IESL32 (300 mM $MgSO_4$) in S	18.3		
At. thiooxidans	IESL33		18.6	<0.05
	IESL33 (300 mM $MgSO_4$)		108.1	<0.05
	IESL33 (500 mM $MgSO_4$)		816.9	8.9
	IESL33 (700 mM $MgSO_4$)		836.2	7.49
	IESL33 (1000 mM $MgSO_4$)		1381.0	11.8
	IESL33 (200 mM NaCl)		319.5	1.4
Sb. thermosulfidooxidans	Type	<0.05		
	Type (300 mM $MgSO_4$)	35.0		
	Type (200 mM NaCl)	46.0		
Acidiphilium sp.	SJH	<0.05		
	SJH (300 mM $MgSO_4$)	157.0		
	SJH (200 mM NaCl)	218.0		

5.3.10 QUANTIFICATION OF IDENTIFIED OSMO-PROTECTANTS IN BIOHYDROMETALLURGY PROCESS WATERS

Table 5.13 shows the quantification of osmo-protectants in samples of industrial bioleach liquors from Escondida. The determination of protein was unsuccessful in these samples and therefore the concentrations of the putative osmo-protectants are shown as micrograms per liter of solution (Table 5.12).

Table 5.13 Determination of osmo-protectants in samples from hydrometallurgical processes at different scale.

Sample	Trehalose (µg/L)	Proline (µg/L)
PLS pond Escondida	0.16	<0.05
Column PC54 outflow	0.12	<0.05
Column PC55 outflow	0.43	<0.05
Chloride leaching reactor	48.7	68.8

5.4 DISCUSSION

Despite the biotechnological importance of acidophiles, the mechanisms and molecules involved in water homeostasis in these prokaryotes are poorly understood. One of the main mechanisms for water homeostasis used by cells in single and multicellular organisms is the accumulation and excretion of compatible organic solutes depending on the osmotic gradient produced between cytoplasm and the environment. Compatible solutes are generally small molecular weight organic molecules that are highly water

soluble. Among analytical methodologies used to separate and determine small organic molecules, liquid chromatography is a suitable and convenient technique and, using the appropriate configuration in terms of separation column and detector, carbohydrates and amino acids can be detected and separated in liquid samples. In addition, the linear relationship between the concentration of a particular compound and the area of its chromatographic peak can be used to determine the amount of such compound in a particular sample. Chromatographic analyses using an electrochemical detector and two suitable separation columns to determine carbohydrates and amino acids were used as a first approach to investigate osmotic adaptation to elevated concentrations of ionic solutes in eight strains of mineral-degrading, including *At. ferrooxidans*, *L. ferriphilum*, "*L. ferroodiazotrophum"*, *Sb. thermosulfidooxidans* and *Acidiphilium* sp. SJH. This group of bacteria included four strains isolated from industrial bioleaching operations of northern Chile (Table 5.1).

Chromatographic profiles of extracts obtained from *Leptospirillum* spp. (*L. ferriphilum*^{T}, *L. ferriphilum* IESL25, *L. ferriphilum* Sp-Cl and "*L. ferrodiazotrophum"*^{T}) grown at elevated concentration of ionic solutes showed a peak at 2.7 minutes (using AminoPac PA10, Fig. 5.2) and at 12.9 minutes (using CarboPac MA1, Fig. 5.1), which had the same retention time as the disaccharide trehalose, while in extracts from cultures grown in medium not supplemented with additional ionic solutes, no peaks were detected at 2.7 or 12.9 minutes, using CarboPacMA1 or AminoPac PA10, respectively. Also in extracts obtained by hypo-osmotic shock an additional peak was detected at the same retention time as that of glucose (Fig. 5.3). These results strongly suggest that these compounds were synthesized as an adaptation by *Leptospirillum* spp. growing in presence of elevated concentrations of ionic solutes. In addition, comparison of mass spectra of extracts in presence and absence of elevated concentration of salts showed, in the latter, m/z peaks

near to adducts calculated for several compounds described as osmolytes including trehalose and glucose (Tables 5.7, 5.8, 5.9). The difference between hypothetical m/z calculated for adducts of trehalose and glucose and signals obtained from mass spectra of extracts were less than one mass unit, suggesting the presence of these carbohydrates. Previous genomic and transcriptomic studies performed with *L. ferrooxidans* strain L3.2 (isolated from the Rio Tinto, Spain) have pinpointed genes involved in the synthesis of trehalose, ectoine and systems for the transport of potassium, which were induced in response to the increasing of salt and sulfate in the environment (Parro et al., 2007). In addition, all of the protein products involved in trehalose and ectoine synthesis pathways have been identified in proteomic analysis performed in biofilms populated by *L. ferriphilum* and "*L. ferrodiazotrophum"* (Goltsman et al., 2009). The information obtained by chromatography and mass spectrometry analyses, together with previous genomic reports, strongly suggests that trehalose is used as an osmolyte by the strains of *Leptospirillum* analyzed in this work. Glucose has not previously been reported as an osmo-protectant in *Leptospirillum*, while ectoine, inferred to be a potential osmolyte in the genomic and transcriptomic studies referred to above, was not detected using the experimental and analytical approaches used in the current work. In addition, the possible role of potassium as an osmolyte in *Leptospirillum* spp. was not studied in the current work. The amount of trehalose determined in *L. ferriphilum* IESL25 grown in the presence of 600 mM magnesium sulfate indicated that this disaccharide was present at a concentration equivalent to 59% of the total protein content, while the amount of trehalose determined in *L. ferriphilum* Sp-Cl grown at 200 mM of sodium chloride was equivalent to 30% of the total protein content (Table 5.12). These figures strongly imply that trehalose is synthesized as the main osmo-protectant by *L. ferriphilum* IESL25 (and probably other leptospirilli) and accumulates within cells that are challenged with elevated external concentrations of ionic solutes. Glucose was only detected when hypo-osmotic shock was

used, and was present in small amounts (at concentrations equivalent to between 0.01% and 0.20% of total protein content; Table 5.12), suggesting a minor role of this monosaccharide as an osmo-protectant. Annotation of the genome of *At. ferrooxidans* ATCC 23270^{T} (Valdes et al., 2008) and *At. ferrooxidans* ATCC 53993 (Lucas et al., 2008) have revealed the presence of genes coding for enzymes involved in the synthesis of trehalose in both bacteria. The genetic evidence found in both strains of *At. ferrooxidans* supports the hypothesis that trehalose is synthesized through trehalose bionsynthesis pathway V, which involves the synthesis of trehalose directly from maltodextrins (such as glycogen) rather than via phosphate compounds such as trehalose-6-phosphate and glucose-6-phosphate (Wolf et al., 2003). The results obtained from mass spectrometry and chromatographic analyses performed in extracts from cultures of *At. ferrooxidans* IESL32 grown in liquid medium amended with 300 mM magnesium sulfate also provided evidence that trehalose acts as an osmo-protectant in this strain. A second potential osmo-protectant was not identified, as its chromatographic peak did not correspond to any reference compound tested and acid hydrolysis indicated that it was not a disaccharide or oligosaccharide. A peak found in mass spectrometry analysis possibly corresponded to dimethylsulfoniopropionate in ethanol extracts of this acidophile, though additional evidence is required to confirm that this compound also acts as an osmo-protectant of *At. ferrooxidans*. Concurrent analysis of this unknown compound and dimethylsulfoniopropionate, using the chromatographic protocols described in this chapter, could confirm the identity of the former. Dimethylsulfoniopropionate has been identified as an osmolyte in some eukaryotic micro-organisms (Yancey et al., 2010). Chromatographic analysis also confirmed that *At. ferrooxidans* IESL32 responded to osmosis stress by producing the same osmo-protectants whether either ferrous iron or sulfur was used as electron donor (Fig. 5.6). The amount of trehalose determined in cultures of *At. ferrooxidans* IESL32 grown in the presence of 300 mM magnesium sulfate was equivalent

to 1.8% (in sulfur) and 2.1% (in ferrous iron) of total protein content, suggesting either a greater contribution as osmo-protectant of the second unidentified compound or the activation of other osmo-regulatory mechanisms, such as intracellular accumulation of potassium. Part of the reason for the greater relative amount of trehalose in ferrous iron than in sulfur-grown cultures can also be attributed to the additional osmotic stress in the presence of 50 mM ferrous sulfate than when grown on elemental sulfur, which is an insoluble substrate. Early studies based on the stimulation of ferrous iron oxidation in *At. ferrooxidans* by amending media with several compounds suggested that proline and betaine acted as probable osmo-protectants in cultures grown in the presence of elevated concentrations of sodium chloride (0.25 M), potassium chloride (0.25 M), sodium sulfate (0.2 M) and potassium sulfate (0.21 M) (Kieft and Spence, 1988). However, no evidence of the synthesis and accumulation of these compounds was found in the present study for cultures of *At. ferrooxidans* IESL32 grown in the presence of 300 mM magnesium sulfate.

Analyses carried out with *At. thiooxidans* IESL33 suggested the intracellular accumulation of several organic compounds in response to increasing concentrations of magnesium sulfate. Retention times of chromatographic peaks that showed increased signal strengths that paralleled concentrations of magnesium sulfate increased were coincident with those for glucose and proline. However three other chromatographic peaks showed different retention times to all of the reference compounds tested, and were not identified. *At. thiooxidans* IESL33 grown in the presence of sodium chloride showed similar profiles to those of grown in the presence of magnesium sulfate. In addition m/z peaks very close to glucose and proline adducts (different by less than one mass unit) were detected in mass spectrometry analyses, providing supporting evidence that these compounds act as osmo-protectants in *At. thiooxidans* IESL33. Acid hydrolysis of an ethanol extract obtained from a culture grown in presence of 500 mM of magnesium

sulfate indicated that one of the unidentified peaks was a glucose oligomer (Fig. 5.5). The amount of glucose determined in cultures growing at increasing concentrations of magnesium sulfate reached up to 1.3 mg of glucose per mg of protein, suggesting the that glucose acts as an important osmo-protectant in this strain of *At. thiooxidans*. In contrast, proline concentrations were a maximum of ~11 µg per mg of protein, suggesting a minor osmotic role for this organic solute.

Chromatographic and mass spectrometry analyses of extracts of *Sb. thermosulfidooxidans* suggested that in cultures grown in the presence of elevated concentrations of either magnesium sulfate (300 mM) or sodium chloride (200 mM) responded by synthesizing and accumulating trehalose. A second chromatographic peak was observed in cultures growing in medium amended with magnesium sulfate or sodium chloride with the same retention time (5.0 minutes) as that observed for an unidentified compound found in of *At. ferrooxidans* IESL32. Although acid hydrolysis of extracts resulted in the disappearance of this peak (retention time 5.0 minutes), no new peaks were observed (Fig. 5.8). The genomic sequence of *Sb. thermosulfidooxidans* DSM 9293 is available, and recently the annotation of the genome of this bacterium has been completed by the DOE Joint Genome Institute, though this annotation did not identify proteins involved in trehalose synthesis. However, BLAST analysis of several amino acid sequences of putative trehalose synthase from different organisms matched a putative protein coded in the contig 143 (from bases 990180 to 993435) of *Sb. thermosulfidooxidans* DSM 9293. The BLAST analysis of this putative protein indicated a high similarity to the putative trehalose synthase of several organisms listed in Table 5.14.

Table 5.14 BLAST results for the analysis putative protein coded in contig 143 of *Sb. thermosulfidooxidans* DSM 9293

Putative trehalose synthase	Similarity Score	Coverage (%)	∆E Value	NCBI Accession number
Sphaerobacter thermophilus DSM 20745	1143	98	0.0	YP_003319350.1
Thermomicrobium roseum DSM 5159	1130	98	0.0	YP_002523130.1
Synechococcus sp. JA-3-3Ab	1112	98	0.0	YP_474549.1
Geobacter sp. M18	1077	98	0.0	YP_004196883.1
Desulfovibrio fructosovorans JJ	961	98	0.0	ZP_07334816.1

These proteins contain a Tres-like domain, which is typically found in trehalose synthases (Jarling et al., 2004). The presence of a putative trehalose synthase coded in the genome of *Sb. thermosulfidooxidans* supports the evidence obtained by chromatographic and mass spectrometry analyses in terms that trehalose is synthesized by *Sb. thermosulfidooxidans* in response to osmotic stress. In addition the amount of trehalose in µg per mg of protein determined in cultures of *Sb. thermosulfidooxidans*^{T} reached up to 46 in medium amended with 200 mM of sodium chloride (Table 5.12).

Chromatographic analysis of extracts of *Acidiphilium* sp. SJH grown in the presence of either 300 mM magnesium sulfate or 200 mM sodium chloride showed two peaks with retention times coincident to those of trehalose and galactose. However only trehalose was observed to increase in relative amounts (i.e. per mg of protein) in cultures amended with increasing concentrations of ionic solutes, while the amount galactose decreased (Table 5.12), suggesting that trehalose is the probable osmo-protectant in this acidophiles. Cellular concentrations of trehalose were the equivalent of 218 µg/mg of protein in cultures grown in presence of 200 mM of sodium chloride. In addition, genomic annotation of *Acidiphilium cryptum* JF-5 suggest the presence of genes coding for enzymes involved in at least two metabolic biosynthesis pathways of trehalose: alpha-trehalose-phosphate synthase (UDP-forming, NCBI accession number YP 001234003) and trehalose synthase (NCBI accession number YP 001236044), supporting the experimental evidence found for trehalose being the main osmo-protectant synthesized b *Acidiphilium* SJH.

In summary, the collated evidence of these experiments strongly suggested that trehalose acts as the major osmolyte produced by different species of acidophilic

bacteria, including iron-oxidizers (*Leptospirillum* spp. and *At. ferrooxidans* and *Sb. thermosulfidooxidans*) and the heterotrophic bacterium *Acidiphilium* sp SJH.. In contrast, glucose was the major osmolyte produced by *At. thiooxidans* IESL33. The amount of proline in the cytoplasm of this bacterium also increased in parallel with the degree of osmotic stress imposed, though this organic compound appeared to play only a minor role (<1% of that of glucose) in osmotic balance in this sulfur-oxidizing acidophile. Proline has shown to enhance the stability of proteins or membranes during freezing (Rudolph and Crowe, 1985), dehydration (Carpenter and Crowe, 1988) and elevated temperature (Hottiger et al., 1994), it also lowers the T_m of DNA due to destabilization of the double helix during salinity stress tests (Rajendrakumar et al., 1997), increases the solubility of sparingly soluble proteins (Schobert and Tschesche 1978), inhibits protein aggregation during its refolding (Samuel et al., 2000) and has the ability to scavenge reactive oxygen species, such as hydroxyl radicals (Smirnoff and Cumbes, 1989). Therefore, this amino acid could play other roles in *At. thiooxidans* rather than as a major osmo-protectant.

Organic compounds, identified as potential osmo-protectants in acidophiles were also found in industrial bioleach liquors from Escondida copper mine (PLS, and leachates from column tests). The greater amount of trehalose found in leachate from column PC55 could be due the greater amount of sodium chloride (10 g/L) present in this test solution than that in column PC44 (6 g/L). Interestingly, the liquor from the bioreactor containing 12 g/L sodium chloride, and which was originally inoculated with both *L. ferriphilum* Sp-Cl and a strain of *At. thiooxidans* isolated from an acid salt lake in Chile, contained relatively high concentrations of both trehalose (presumably deriving from *L. ferriphilum*) and proline (presumably deriving from *At. thiooxidans*).

CHAPTER 6: GENETIC RESPONSE OF *LEPTOSPIRILLUM FERRIPHILUM* IESL25 TO STRESS BY ELEVATED CONCENTRATIONS OF COPPER AND SULFATE

6.1 INTRODUCTION

At present, one of the major knowledge bottle necks in industrial process copper bioleaching remains the understanding of process at the microbial level. Research has shown that both the microbial community and the maintenance of conditions appropriate to that community are critical factors influencing the efficiency of industrial process copper bioleaching (Brierley 2001; Demergasso 2009). The microbial component is influenced by various operational factors including; temperature, pH, composition of leach solutions, and the solvent extraction circuit (Demergasso et al., 2005; Rawlings and Johnson, 2007). Among the resulting challenges faced by microbial communities thriving in bioleaching processes are the elevated concentrations of soluble ionic solutes (e.g. sulfate) and elevated concentrations of transition metals (e.g. copper). Therefore, any failure to maintain optimal conditions for microbial metabolism can decrease microbial activity and significantly influence the recovery of marketable metals.

Currently a number of methods are employed to give an indication of the state (size and activity) of the microbial community in such processes. Activity can be determined in liquid samples from the bioleaching process plant by measuring the oxidation rates of ferrous iron or sulfur during controlled incubations, while microbial numbers can be determined by direct enumeration and or determination of the most probable number of iron and sulfur-oxidizing microorganisms. Although these methods offer some valuable monitoring tools for the process, a more complete understanding of the microbial process, and the response of individual members of the community, should offer scope for significant process enhancement.

In this chapter Micro-Representational Difference Analysis (MRDA) was used to elucidate the genetic response of Escondida heap *L. ferriphilum* isolate IESL25. MRDA is a technique that has been developed to detect genes differentially expressed in prokaryotic micro-organisms grown under different growth conditions (Becker et al., 2001). *L. ferriphilum* is one of the main iron-oxidizing acidophiles inhabiting the bioheaps at Escondida, and MRDA was used to find genetic evidence of stress response due to elevated concentrations of either copper sulfate (transition metal toxicity) or magnesium sulfate (osmotic stress).

6.2 MATERIAL AND METHODS

6.2.1 STRESS CONDITIONS FOR MRNA EXTRACTION

The genetic response of *L. ferriphilum* IESL25 when subjected to stress by toxic transition metals or elevated osmolarity was studied by Micro-Representational Difference Analysis (MRDA). To extract mRNA to determine the genetic response of isolate IESL25 to these stresses, the isolate was grown in a 2L bioreactor equipped with temperature, aeration, pH and stirring control (model P350, Electrolab, U.K.). These batch cultures were grown at optimal pH- and temperature conditions (pH 1.5 and 37 ℃, see Chapter 3).

The mRNA extracted from bacteria grown in autotrophic basal salts (section 2.2) supplemented with 25 mM of ferrous iron as energy source was used as the “driver” mRNA (see below), while mRNA extracted from bacteria grown in the same culture medium but supplemented with an elevated concentration of magnesium sulfate (300

mM) or copper sulfate (160 mM) was used as the “tester” mRNA (“$tester_{Mg}$” or “$tester_{Cu}$”, respectively).

The growth rate of the respective cultures was monitored by determining the ferrous iron concentration in solution, and culture doubling times were calculated from semi-logarithmic plots of the iron oxidized versus time.

6.2.2 RNA EXTRACTION AND cDNA SYNTHESIS

For all experiments RNA was extracted and treated with RNase-free DNase, as described in Chapter 2 (section 2.7.1) from cultures when 80 to 90 % of initial ferrous iron was oxidized (late exponential growth phase). Cells were collected from a 1.0 L culture by filtering onto a 0.2 μm pore diameter membrane. After filtration, the membrane with the cells was placed into a Petri dish and cells were resuspended in 1.0 mL of the RNA stabilizing reagent RNAlater® (Cat 76104, Qiagen, Germany). The cell suspension was transferred to a microfuge tube and centrifuged for 20 minutes at 15,000·g at 4℃. The supernatant was removed and cell pellet was stored at -80 ℃ until further processing.

Reverse transcription of the total RNA from the control and tester cultures was performed using random primers for the synthesis of cDNA as described in Chapter 2 (section 2.7.2). The cDNA thus obtained was of randomly synthesized portions of mRNA transcripts expressed under driver and tester conditions, and was stored at -20℃. The cDNA was used for Micro-representational-difference Analysis (MRDA) and Real Time PCR analysis. For Real Time PCR experiments, the maximum volume of cDNA added to reactions was 10% of the final PCR reaction volume.

6.2.3 MICRO-REPRESENTATIONAL DIFFERENCE ANALYSIS (MRDA)

MRDA was used to find potential genes involved in the genetic response of *L. ferriphilum* isolate IESL25 when grown at elevated concentrations of magnesium sulfate and copper sulfate. This method consisted of three major steps following the extraction of total RNA and cDNA synthesis including; i) the generation of cDNA fragments by restriction enzyme and linking of adaptors, ii) subtractive hybridization, and iii) selective amplification of the differentially expressed mRNA transcripts by PCR. Details of these steps can be found in Chapter 2 (section 2.7.4). In this method, systematic hybridization rounds between the tester and the driver cDNAs (using driver in excess) were performed in order to allow only the amplification of differentially expressed mRNA transcript fragments relative to the reference (driver cDNA) condition.

Following the final PCR amplification step, DNA bands were excised from the agarose gel using a clean, sterile razor blade and transferred to a pre-weighted microtube. The microtube was weighed again and the weight of the agarose slice containing the band was calculated from the difference between of both weights. Then, Buffer QG (Cat. 19063, Qiagen, Germany) was added to each tube in a ratio of 3 µL per 1 mg of agarose slice and incubated at 50℃ for ten minutes or longer, until the agarose was completely dissolved. This solution containing the DNA was purified using the QIAquick PCR Purification Kit (Cat. 28104, Qiagen, Germany). Each purified band was cloned as described in section 2.7.3. To confirm the presence of a cDNA insert in the plasmids of cultured transformed cells, a PCR reaction was carried out using 0.5 µL of the culture and “PCR primer 1” included in the PCR-Select cDNA Subtraction Kit (5'-TCGAGCGGCCGCCCGGGCAGGT-3'). Plasmid extraction from those cultures showing positive PCR reaction was carried out using the QIAprep ® Spin Miniprep kit (Cat.

27106, Qiagen, Germany). Finally the purified plasmids were submitted for sequencing to Macrogen Inc. (Korea).

6.2.4 DIFFERENTIALLY EXPRESSED CDNA SEQUENCE ANALYSIS

The sequences of the inserts were obtained from plasmid sequences by searching the sequence for "PCR primer 1" in both senses (5´-3´ and 3´-5´) and fragments flanking the insert (Plasmid) were not considered for further analysis. Sequences were translated to peptides fragments using the Translate tool at Expasy website (http://web.expasy.org/translate/) and BLASTP analysis of deduced peptides was performed at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In addition BLASTP analysis of the deduced peptides from differentially expressed cDNAs was performed using a private database containing the genomic sequence of *L. ferriphilum* strain Sp-Cl, isolated from a bioleaching operation at the Spence Mine in Chile. The presence of putative conserved domains in the deduced peptide was detected by BLASTP and pfam analyses were performed at Sanger institute website (http://pfam.sanger.ac.uk/family/)

6.2.4 REAL TIME PCR (QPCR) TO CONFIRM EXPRESSION OF DETECTED CDNAS

Forward and reverse primers were designed to specifically target and quanitify the transcripts detected by MRDA in cDNA obtained from control and tests cultures (Table 6.1). The primers were designed using the tool "Primer-BLAST" at NCBI website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and were synthesised by Integrated

DNA Technologies Inc (IDT, U.S.A.). Primers were tested by PCR using genomic DNA obtained from strain IESL25. To confirm the specificity of each primer pair, the PCR product was cloned and sequenced. After confirmation of the PCR products obtained with specific primers by sequencing, standard concentration curves were built using plasmids containing target amplicons. Real Time PCR reactions were run on a Corbett Rotor-Gene 6000 Rotorgene (Corbette, Australia), using Quantimix Easy kit (Cat. 10.607, Biotools, Spain), 0.3 µM of each primer and 5 to 10 ng cDNA template. The following thermocycler program was used: 95℃ for 1 0 min, and 40 cycles of 95℃ for 30 s, 60℃ for 30 s and 72℃ for 20 s. The copies numb er determined for each target in different cDNA obtained from different growth conditions were normalized by determining the copy number of 16S rRNA transcripts and the expression level was calculated dividing the normalized copy number expressed in stress (“tester”) conditions by normalized copy number expressed in the control (“driver”) condition.

Table 6.1 Primers designed to quantify sequences fragments obtained by MRDA by Real time PCR and primers for the determination of 16S rRNA gene copy numbers of IESL25.

Target	Forward primer 5´-3´	Reverse primer 5´-3´	Amplicon Size (bp)
c	AAGGCCAAACATTCTGATGG	GCGCTTTGTTCTGACTGACA	208
d	CACGGCCATATAGTTCCACC	TTTCGACATTTCTTCGGGAG	215
e	AGGTACAGCTGTGATGCCCT	CTCTTCCATGGCGACTGTTT	208
b	GTCCCCCGAATTTTCTCAAT	CGAGGTACACTTCCGGAGAA	230
f	GACATCACAACAAATGGGCA	AAAGTCCGAGAACGTGATGG	193
16S	GACTCCTACGGGAGGCAGCA	TTGTGCGGGCCCCCGTCAAT	601

6.3 RESULTS

6.3.1 EFFECT OF MAGNESIUM SULFATE AND COPPER SULFATE ON GROWTH OF IESL25

Figure 6.1 shows the effect of elevated concentrations of copper sulfate or magnesium sulfate on growth doubling times of *L. ferriphilum* IESL25 compared to growth rate in standard culture medium. In standard culture medium the doubling time calculated was 9.4 h^{-1}, while in medium amended with magnesium sulfate or copper sulfate the doubling times increased to 15.7 h^{-1} and 26.0 h^{-1}, respectively.

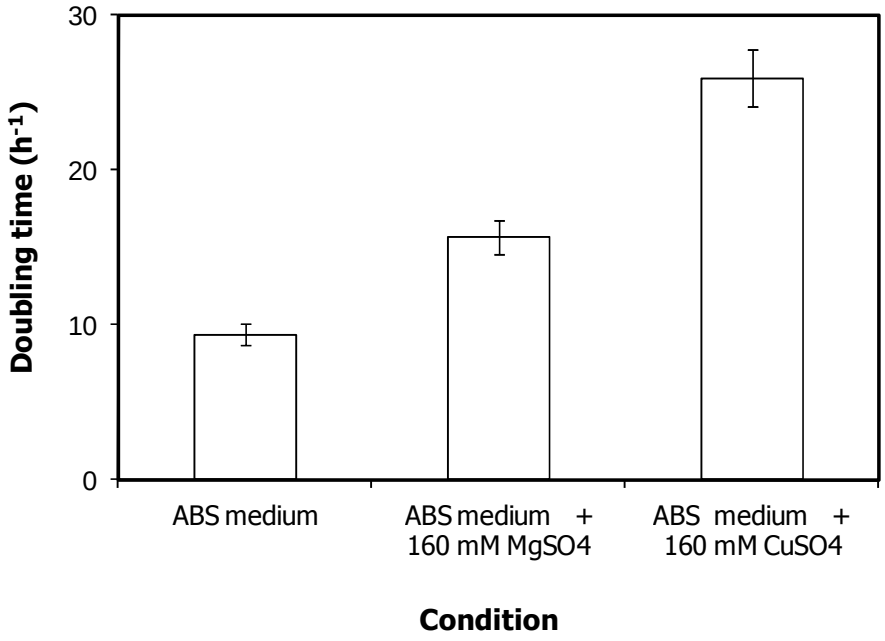


Figure 6.1 Growth doubling times calculated for cultures of *L. ferriphilum* isolate IESL25 in standard laboratory medium and medium amended with either copper sulfate or magnesium sulfate at the same concentrations (160 mM).

6.3.2 BAND PROFILES OBTAINED BY MICRO-REPRESENTATIONAL DIFFERENCE ANALYSIS (MRDA)

Figure 6.2 shows the band profiles obtained in agarose gel electrophoresis stained with ethidium bromide after the application of MRDA protocol. Bands a, b and f were obtained from subtractive hybridization experiments of cDNA of cultures grown in standard medium (Driver) and cDNA obtained from cultures grown in medium amended with 300 mM of magnesium sulfate ($Tester_{Mg}$). In each of the two tester conditions, a total of three dominant cDNAs were detected, and one or two minor cDNAs were also detected. Bands a, b and f were obtained from subtractive hybridization experiments of the driver cDNA described above and cDNA obtained from cultures grown in medium amended with 160 mM of copper sulfate, and bands c, d and e were obtained following subtractive hybridization using the driver cDNA and the $tester_{Mg}$ cDNA. The results obtained after sequencing of the bands are summarized in Table 6.2. In general, the translated gene products of the differentially expressed transcripts were highly similar to those identified in the genome sequences of closely related bacteria in the genus *Leptospirillum*.

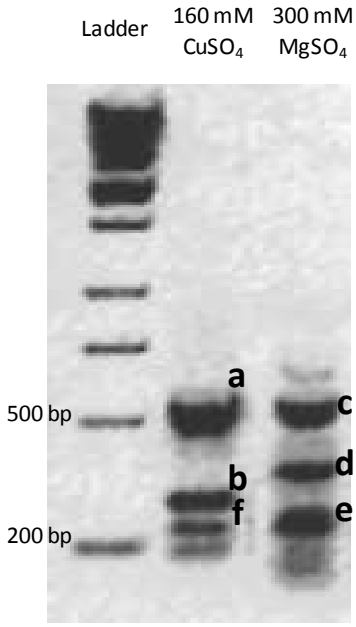


Figure 6.2. Agarose (2% w/v) gel electrophoresis stained with ethidium bromide of nested PCR products obtained after application of MRDA protocol. The tester cDNA used for each subtractive hybridization experiment is indicated above the respective lane. The bands indicated by lettering are described in the text and correspond to the initial amplicons obtained by MRDA, sequences were later confirmed not to be false positives by quantification by qPCR (6.3.3).

Table 6.2. Analysis of the translated gene products of differentially expressed transcripts identified by MRDA analysis using BLASTP at NCBI and BLASTP against a private database of the annotated genome sequence of *L. ferriphilum* isolate Sp-Cl.

		Public database (NCBI)					**Annotated genome of *L. ferriphilum* Sp-CL**		
Growth condition	**Band**	**Similar annotated protein**	**Organism/sequence**	**Similarity %**	**Coverage %**	**NCBI accession number**	**Similar annotated protein**	**Similarity %**	**Coverage %**
In medium amended with 300 mM $MgSo_4$	**c**	Major facilitator superfamily transporter	*"Leptospirillum rubarum"*	94	96	EAY58211	Major facilitator superfamily Transporter	93	93
	d	hypothetical protein MldDRAFT_0058	delta proteobacterium MLMS-1	67	100	ZP_01289064	Transcription-repair coupling factor	53	34
	e	Polyribonucleotide nucleotidyltransferase	*"Leptospirillum ferrodiazotrophum"*	96	90	EES52835	Polyribonucleotide nucleotidyltransferase	96	91
In medium amended with 160 mM $CuSO_4$	**a**	Putative glycosyl transferase, family 2	*Leptospirillum* sp. Group II 5-way CG	91	100	EDZ38475	Rhamnosyl transferase	94	90
	b	Acyl-(Acyl-carrier-protein)-UDP-N-acetylglucosamine O-acyltransferase	*"Leptospirillum rubarum"*	68	100	EAY56285	Acyl-[acyl-carrier-protein]--UDP-N-acetylglucosamine O-acyltransferase	79	68
	f	HNH endonuclease	*Spirosoma linguale* DSM 74	38	98	ADB39101	hypothetical protein	51	34

6.3.3 QUANTIFICATION OF THE DIFFERENTIALLY EXPRESSED TRANSCRIPTS IN *L. FERRIPHILUM* ISOLATE IESL25

Real time PCR analysis was performed for five sequences retrieved by MRDA; for unknown reasons the sequence of band a was difficult to obtain and was only determined late in the project, when quantitative PCR analysis had been already performed for the other sequences. The results indicate that all sequences detected by MRDA were expressed at least 20 times more in the presence of a stress (either osmotic stress or Cu stress) than in standard growth conditions in *L. ferriphilum* IESL25 (Figure 6.3). Even though these mRNA transcripts exhibited higher expression levels under both stress conditions, they all responded somewhat differently to the two stresses.

The expression of some of the transcripts detected by MRDA, e.g. bands b and c, were relatively similar in most of the experiments, and generally exhibtited the lowest levels of expression of the cDNAs detected by MRDA.

In contrast, cDNAs represented by bands e and f seemed to be more highly expressed when *L. ferriphilum* isolate IESL25 was grown in medium containing $CuSO_4$ than in medium amended with $MgSO_4$.

The transcript represented by band d, which was retrieved from RNA extracted from cells grown in presence of elevated concentration of magnesium sulfate, was shown to be highly expressed when cells were grown in media containing an elevated concentration of either salt, and expression of this transcript increased as $CuSO_4$ concentration in medium increased.

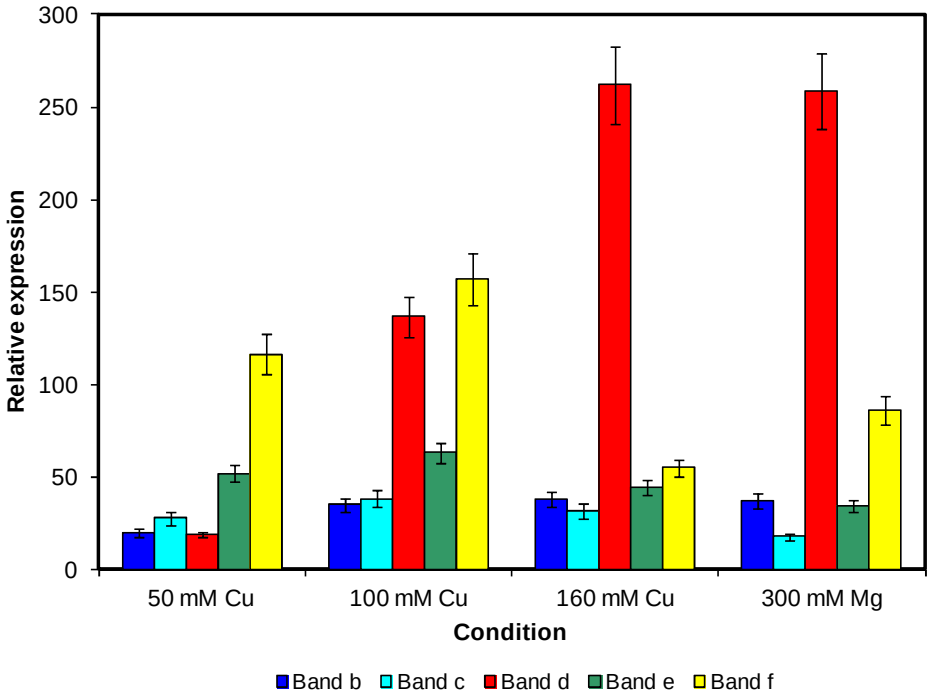


Figure 6.3 Expression of gene transcripts detected by MRDA during growth of *L. ferriphilum* isolate IESL25 under a variety of stresses as indicated relative to expression in cells grown under control conditions (e.g. without Cu or Mg stress). Expression of the transcripts was normalized against the expression of the 16S rRNA in the respective growth condition.

6.4 DISCUSSION

6.4.1 EFFECT OF COPPER SULFATE AND MAGNESIUM SULFATE ON GROWTH OF *L. FERRIPHILUM* IESL25

Magnesium sulfate is a common component in the formulation of liquid culture media for culturing chemolithoautotrophic acidophiles, and given that no toxic effect has been reported, it is assumed that when it is added at elevated concentration in culture medium, its growth inhibitory effect is mainly due to increasing the osmotic pressure of the liquid medium. The addition of elevated concentrations of copper sulfate to medium can

also produce growth inhibition by increasing the osmotic strength of the culture medium due to its ionic solute nature, however, in addition to this, it is known that copper ion can form unspecific complex compounds in the cell and interacts with oxygen radicals, leading to more toxic effects (Nies, 1999).

The toxic effect of copper was confirmed by the differences observed in the maximum tolerances displayed by *L. ferriphilum* IESL25 to magnesium sulfate and copper sulfate. The maximum tolerance to magnesium sulfate observed in *L. ferriphilum* IESL25 was around 900 mM (data not shown), while the maximum tolerance to copper sulfate was 180 mM (see Chapter IV, Figure 4.1). In order to compare the inhibitory effect of magnesium sulfate and copper sulfate on growth of *L. ferriphilum* IESL25, culture experiments were performed adding either copper sulfate or magnesium sulfate to culture medium at a concentration of 160 mM, which is near to the maximum tolerance of strain IESL25 to copper.

Both compounds were able to partially inhibit the growth of *L. ferriphilum* IESL25 when the medium was amended with 160 mM of either magnesium sulfate or copper sulfate (Figure 6.1). The growth doubling time observed for *L. ferriphilum* IESL25 grown in standard cultures media was 9.4 h^{-1}. In comparison with this, the effect of adding 160 mM of either magnesium sulfate or copper sulfate to standard medium produced an increase of 67% and 176% in the growth doubling time, with calculated doubling times of 15.7 h^{-1} and 26.0 h^{-1}, respectively. This is also confirmed by the observation of a higher mortality rate in cultures supplemented with copper sulfate than those supplemented with magnesium sulfate, both supplemented with 150 mM (see chapter IV, section 4.4.2). Therefore, the inhibition produced by the addition of a concentration near to the maximum tolerance (160 mM) of copper sulfate to liquid medium was much larger than the inhibition produced by the addition of magnesium sulfate at the same concentration. The mechanisms involved in the tolerance of *L. ferriphilum* to elevated osmotic pressure or the presence of elevated

concentrations of copper in the environment have been mostly inferred from the information retrieved from genomic sequences (Aliaga et al. 2009), however it is necessary to confirm these responses mechanisms by transcriptional and physiological studies. In order to investigate the genetic response of *L. ferriphilum* IESL25 when grown in presence of elevated concentration of copper sulfate or magnesium sulfate, genetic expression was studied by MRDA.

6.4.2 DETECTION OF TRANSCRIPTS DIFFERENTIALLY EXPRESSED DURING GROWTH UNDER OSMOTIC OR CU STRESS

The electrophoretic band patterns of cDNA amplicons obtained after one round of subtractive hybridization using cDNA driver (obtained from cells grown in standard culture medium at optima pH and temperature) and cDNA testers obtained from cells grown in medium amended with 160 mM of magnesium or copper sulfate are shown in Figure 6.3. The sizes of the amplicons obtained after nested PCR (included in MRDA protocol) were between 537 and 243 pb. Three dominant bands were observed in both experiments. Band “a” was the most intense in experiment when cells were grown in medium amended with 160 mM $MgSO_4$, while three bands obtained from experiment when cells were grown in medium amended with 160 mM $CuSO_4$ showed similar intensities.

6.4.3 ANALYSIS OF THE SEQUENCES RETRIEVED FROM BANDS OF CULTURES GROWN AT 160 MM $MgSO_4$

BLASTP analysis of the peptide deduced from band c (Figure 6.2) indicated high similarity with a putative major facilitator superfamily (MFS) transporter, which has been annotated in genome sequences of *L. ferriphilum* Sp-Cl (a commecial strain held in a

private collection), "*L. rubarum"*, *Leptospirillum* sp "5 way CG" and "*L. ferrodiazotrophum"* (the latter three genome sequences were assembled from metagenome analyses of samples taken from the Richmond Mine at Iron Mountain, California). The similarities of the translated IESL25 gene product to those found in the genome sequences was 93%, 97%, 97% and 90%, respectively (Table 6.2). No putative conserved domains were detected in BLASTP analaysis, but the genomic context including this protein was the same for all four genomic sequences related to *Leptospirillum* species (Figure 6.4). MFS transporters are single-polypeptide carriers capable of transporting small solutes in response to chemiosmotic ion gradients. These single-polypeptide transporters are widespread in nature and participate in the transport of compounds including sugars, protons, sodium cations, among many other metabolites (Pao et al., 1998). A member of this family, ProP, which transports zwiterionic substrates with protons through a symport mechanism (MacMillan et al., 1999) was demonstrated to play an important role as osmoprotectant/proton symporter in *E. coli*, mediating the transport of proline and glycine betain during the adaptation of this bacterium to increased osmotic pressure, revealing its function in water homeostasis in *E. coli*. In addition, an MFS transporter was also found in *L. ferrooxidans* L3.2 and it was found to be over-expressed in cells forming biofilms compared with planktonic cells (Moreno-Paz et al., 2010). The high similarity of the amino acid deduced from the sequence of band c with proteins annotated as MFS transporter in four genomic sequences of *Leptospirillum* spp. suggests that an MFS transporter of *L. ferriphilum* IESL25 could be required for the transport of trehalose or other osmolyte, in a similar fashion to that required by *E. coli* to transport proline and glycine betain growing at elevated osmotic pressure. However more information is required confirm such a mechanism in *L. ferriphilum* IESL25.

The peptide sequence deduced from band d was similar to hypothetical protein annotated in the genome of a delta proteobacterium (Table 6.2), determined by BLASTP

at NCBI website. However, the same analysis, but using the genome of *L. ferriphilum* Sp-Cl as database, showed a 53% of similarity between peptide deduced from band d with a protein annotated as transcription-repair coupling factor. This protein was also detected in the genomic sequences of "*L. rubarum"*, *Leptospirillum* sp. "5 way CG" and "*L. ferrodiazotrophum*". The similarity between the protein annotated as transcription-repair coupling factor of *L. ferriphilum* Sp-Cl and the proteins annotated as the same in first two genome sequences was 100%, but was only 94% similar with that of the latter genome sequence. A conserved putative domain (pfam08747) was detected in the sequences of the protein annotated as transcription-repair coupling factor in genomic sequence of *Leptospirillum* spp.; however no function has been attributed to this domain yet. The genomic context of the gene encoding the transcription-repair coupling factor was similar in the four genomic sequences of *Leptospirillum* spp., but differing in the annotation of two proteins as indicated in Figure 6.5. The sequences annotated up-stream to the sequence exhibiting similarity to the translated product of band d could be involved in the protein folding (PpiC-type peptidyl-prolyl cis-trans isomerase) and maintaining of the cell wall structure during the stationary phase (SurA). The sequences annotated down-stream to this annotated sequence similar to band e could be related to lipopolysaccharide biosynthesis (ADP-heptose synthase and phosphoheptose isomerase) and glycerophospholipid metabolism (cytidylyltransferase family protein).

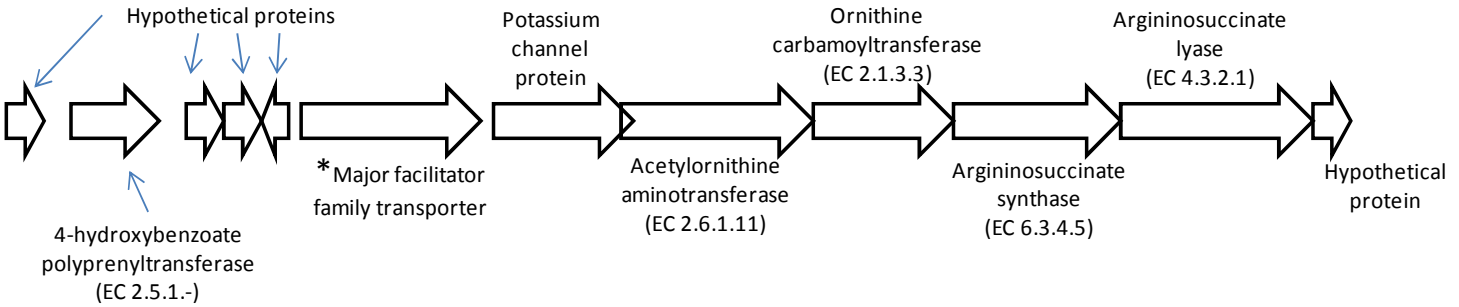


Figure 6.4. Genomic context found in *L. ferriphilum* Sp-Cl, *L. rubarum*, *Leptospirillum* sp. "5 way CG" and *L. ferrodiazotrophum*, including the protein annotated as major facilitator family transporter, similar to peptide deduced from sequence of band c from *L. ferriphilum* IESL25 (*).

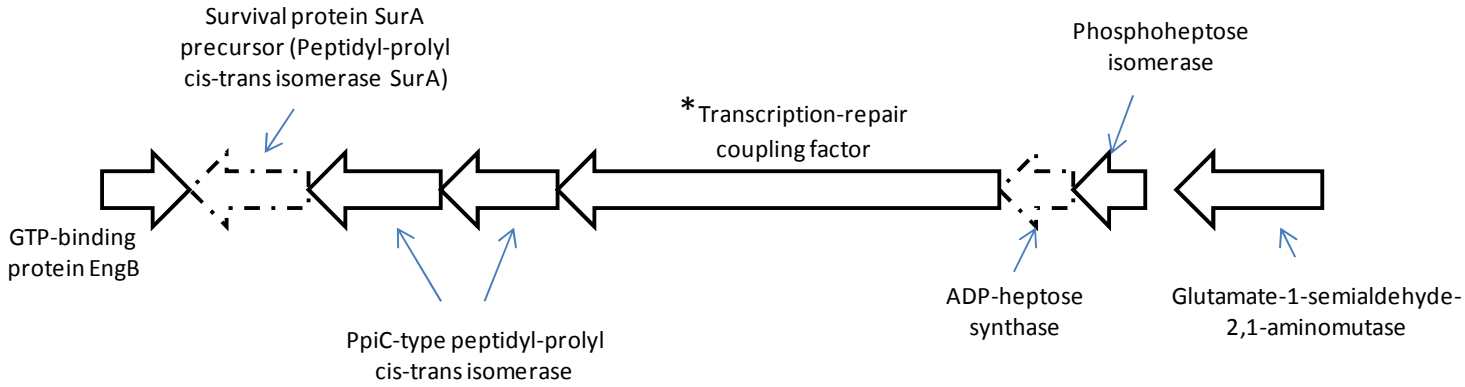


Figure 6.5. Genomic context found in *L. ferriphilum* Sp-Cl, *L. rubarum*, *Leptospirillum* sp. "5 way CG" and *L. ferrodiazotrophum*, including the protein annotated as Transcription-repair coupling factor, similar to peptide deduced from sequence of band d retrieved from *L. ferriphilum* IESL25 (*). Genes indicated with discontinuous arrow lines differed in the annotation of *L. rubarum*, *Leptospirillum* sp. "5 way CG" and *L. ferrodizaotrophum*. In the latter genomes the sequence annotated PpiC-type peptidyl-prolyl cis-trans isomerase and Cytidylyltransferase family protein were annotated instead of SurA and ADP-heptose synthase, respectively.

Altered patterns of genomic DNA, detected by Randomly Amplified Polymorphic DNA Analysis (RAPD) has been described in *E. coli* subjected to osmotic stress (Jolivet-Gougeon et al. 2000). If this effect of osmotic stress is also produced in cells of *L. ferriphilum* IESL25 subjected to elevated concentrations of magnesium sulfate, it could probably trigger a genetic response involving DNA repair systems in cells. However more information is required to confirm the identification of sequence of band d as a protein involved in DNA repair, due to its low similarity found in genomic sequences of *Leptospirillum* spp.

BLASTP analysis of the peptide deduced from band e sequence showed similarity with a polyribonucleotide nucleotidyl transferase annotated in genomes of *L. ferriphilum* Sp-Cl, "*L. rubarum*", *Leptospirillum* sp. "5 way CG" and "*L. ferrodiazotrophum*" with a similarity of 96% in all cases. A putative conserved domain denoted as RNase PH (pfam01138) was detected in the deduced peptide from sequence of band e. This domain is usually found in proteins called 3´ - 5´exoribonuclease. The genomic context of this protein in genome sequences of the leptospirilli contained the same annotated proteins and organization as found in *L. ferriphilum* Sp-Cl (Figure 6.6). The annotated sequences in this genomic context include proteins related to tRNA and protein processing. Polyribonucleotide nucleotidyl transferase is also known as polynucleotide phosphorylase (PNPase) can process (degrade) single-stranded RNA, but is inhibited by double-stranded structures such as stem-loops. In proteomic analysis performed in *Enterobacter sakazakii*, subjected to desiccation, the over-expression of a protein sequence identified as polyribonucleotide nucleotidyl transferase was also detected (Identification of proteins involved in osmotic stress response in Enterobacter sakazakii by proteomics). This information suggests that RNA metabolism in cells of *L. ferriphilum* IESL25 is affected when grown at elevated osmotic pressure and requires the over-expression of polyribonucleotide nucleotidyl transferase.

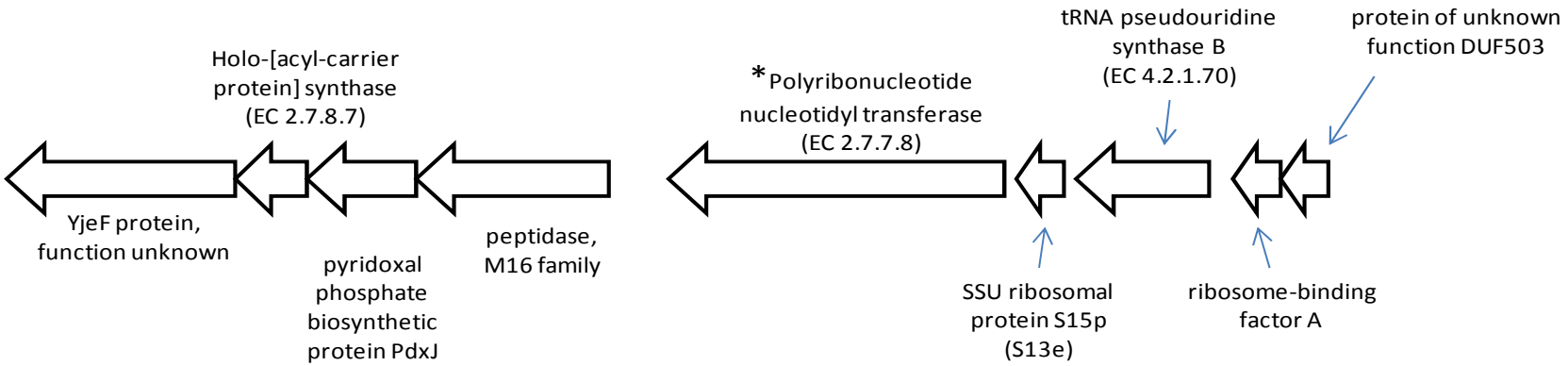


Figure 6.6. Genomic context found in *L. ferriphilum* Sp-Cl, *L. rubarum*, *Leptospirillum* sp. "5 way CG" and *L. ferrodiazotrophum*, including the protein annotated as Polyribonucleotide nucleotidyl transferase, similar to peptide deduced from sequence of band e retrieved from *L. ferriphilum* IESL25 (*).

6.4.4 ANALYSIS OF THE SEQUENCES RETRIEVED FROM BANDS OF CULTURES GROWN AT 160 MM $CuSO_4$

A 95 amino acid sequence was deduced from sequence retrieved from band a. BLAST analysis of this amino acid sequence performed online at the NCBI website did not detect putative conserved domains in the peptide sequence and showed 91% of similarity with a protein annotated as a putative glycosyl transferase (Table 6.2) in *Leptospirillum* sp. "5-way CG" genome sequence. The same analysis of the peptide derived from band a, but using a database of the annotated genome of *L. ferriphilum* Sp-CL showed a similarity of 94% with a protein annotated as rhamnosyl transferase. The complete protein sequence of the glycosyl transferase of *Leptospirillum* sp. Group II 5-way CG had a 96% similarity with rhamnosyl transferase of strain Sp-Cl, as determined by BLAST analysis, using the database including the annotated genome of *L. ferriphilum* Sp-Cl. The high level of similarity showed by the sequence of band a with glycosyl transferase (and rhamnosyl transferase) suggest that a glycosyl transferase could be over-expressed in copper stress cells. The link between copper stress responses through induction of genes coding for glycosyl transferase is still not completely understood. Recently the interaction between protein CopZ (a copper chaperone) with protein Gls24 (stress response regulator) was investigated in *Enterococcus hirae.* The interaction between Gls24 and CopZ was confirmed by using a reporter system in yeast (Yeast two-hybrid analysis). In this bacterium CopZ donates Cu ions to the CopY repressor, releasing its bound zinc and abolishing repressor–DNA interaction. This induces the expression of the *cop* operon, which encodes CopY and CopZ, in addition to two copper ATPases, CopA and CopB. Interestingly, the *gls24* gene appears to be part of an operon containing eight genes that was induced by a range of stress conditions, but most notably by copper. The first two

genes encode proteins similar to glycosyl transferases, followed by a gene encoding a protein of unknown function; *corA* encoding for a predicted Mg^{2+} transporter; *fad* encoding a predicted shortchain fatty acid dehydrogenase, *gapA* a trypsin-like serine protease, *gapB* a protein of unknown function and *gls24* (REF). In available *Leptospirillum* genomes, gls24 was not found annotated.

An important observation in the genomic context of strain Sp-Cl (Figure 6.7) is the annotation of a signal transduction system referred as a two-component system and described for most bacteria. These systems are widely used in response to environmental changes or stresses. In general, two-component systems include a membrane-located sensor kinase and a cytoplasmic transcriptional regulator. The sensor is a protein that is autophosphorylated on a histidine residue once it has bound its ligand. Then this phosphate is transferred to an aspartate residue on the regulator protein that becomes active for the transcriptional activation of target genes (Hoch et al., 1995). The participation of two component systems in response to copper has been demonstrated in several bacteria. A transcriptional study carried out in *Pseudomonas aureginosa* showed that a two-component system was over-expressed when this bacterium was grown at elevated concentration of copper ion (10 mM). The transcriptomic study was performed in cultures adapted to copper, defined as those able to grow exponentially in presence of 10 mM of copper ion and in “copper-shocked” cultures, defined as those active cultures grown in absence of copper and subjected to a pulse of 10 mM copper during 45 minutes. The expression of the two-components systems was over-expressed around ten times more in cultures adapted to grow in copper than those “copper-shocked” cultures. In *Corynebacterium glutamicum* this system is a requirement for adaptation to copper stress. In this bacterium the system is composed by of the histidine kinase CopS and the response regulator CopR. The deletion of CopRS genes increased the sensitivity of *Corynebacterium glutamicum* to copper ions, but not to other heavy metals ions,

suggesting that CopRS is the key regulatory system in this bacterium for the extracytoplasmic sensing of elevated copper ion concentrations and for induction of a set of genes capable of diminishing copper stress (Schelder et al., 2011).

Two-component systems have been described at genomic level in the three known groups of *Lesptosprillum*. In *Leptospirillum* group I (*L. ferrooxidans*), these systems have been demonstrated to participate in the formation of biofilms. Although also present in the genome sequences of *Leptospirillum* groups II and III, several differences have been pointed out regarding the type and a higher representation of these systems in group III. Although these genes have been detected in leptospirilli, their functions remain unknown and no evidence of their participation in the copper stress response has been reported yet.

Figure 6.7-A shows the genomic region containing the annotated rhamnosyl transferase gene in strain Sp-Cl. Two genes sequences are located near to rhamnosyl transferase gene. These two genes have been annotated as a sensory transduction histidine kinase and a response regulator and could be part of a two component system. If the annotation for sensory transduction histidine kinase is correct in strain Sp-Cl, and assuming a similar gene arrangement in strain IESL25, it is likely that the expression of this genomic region could be attributed to the response to copper stress in leptospirilli, however more evidence is necessary for complete confirmation.

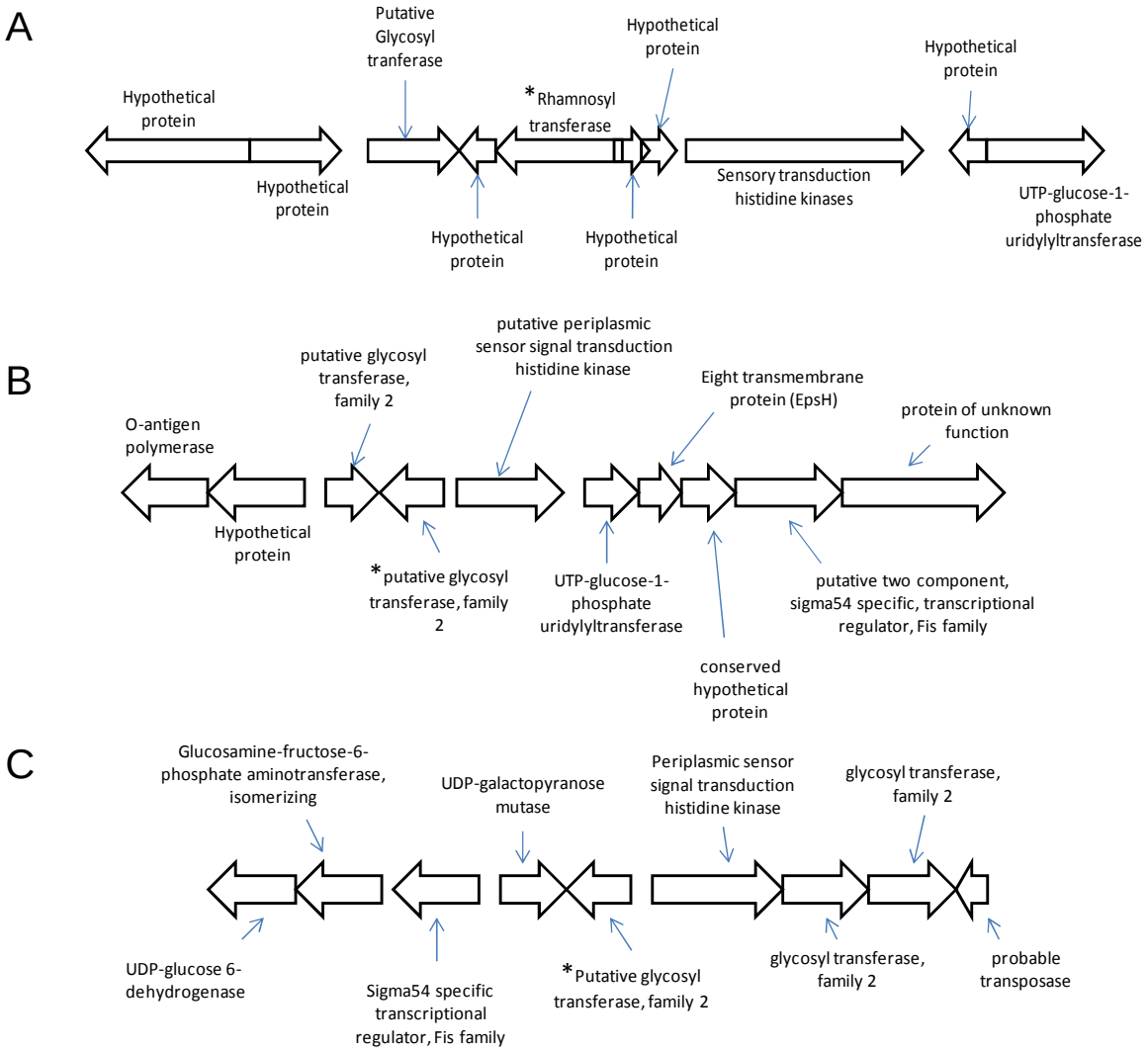


Figure 6.7. Genomic context showing the possible peptide deduced from strain IESL25 band a (*) in *L. ferriphilum* Sp-Cl (A), *L. rubarum* and *Leptospirillum* sp. "5 way CG" (B) and *L. ferrodiazotrophum* (C).

The peptide sequence deduced from sequence retrieved from band b was similar to a sequence annotated as Acyl-(Acyl-carrier-protein)-UDP-N-acetylglucosamine O-acyltransferase in three different genomic sequences of *Leptospirillum* spp.. The similarity of the peptide deduced from sequence of band b, determined by BLASTP analysis was 79%, 68% and 54% similar to the Acyl-(Acyl-carrier-protein)-UDP-N-acetylglucosamine O-acyltransferase annotated in *L. ferriphilum* Sp-Cl, "*L. rubarum*" and *Leptospirillum* sp. Group II '5-way CG', respectively.

The genomic context of the gene annotated as Acyl-(Acyl-carrier-protein)-UDP-N-acetylglucosamine O-acyltransferase was similar in three genomic sequences with characteristics suggesting an operon formed by 7 genes (Figure 6.8-A). A putative homoserine hydrogenase is found in the putative operon. This protein belongs to the family of oxidoreductases that catalyses the oxidation of L-homoserine to L-aspartate-4-semialdehyde using NAD+ or NADP+ as a cofactor and participates in the metabolism of glycine, serine and threonine and lysine biosynthesis. Homoserine hydrogenase is followed by threonine synthase in the putative operon. Threonine synthase is a lyase that catalyses the conversion of O-phospho-L-homoserine to L-threonine and phosphate, using serine and also in the metabolism of vitamin B6. A gene sequence annotated for phosphoglycerate mutase is located next to threonine synthase. This enzyme catalyses the conversion of 3-phosphoglycerate to 2-phosphoglycerate, through a 2,3-bisphosphoglycerate intermediate. Next to this enzyme a gene annotated for aspartokinase is found. This enzyme catalyses the phosphorylation of aspartate. This reaction is the first step in the biosynthesis of methionine, lysine and threonine and also it is the first step in the biosynthesis of ectoine, using aspartate as precursor. Recently a specialized aspartokinase has been demonstrated to enhance the biosynthesis of the osmoprotectants ectoine and hydroxyectoine in *Pseudomonas stutzeri* (Stoveken et al., 2011). In addition to copper toxicity, the addition of 160 mM of copper sulfate to culture

medium produces an elevated osmotic strength and therefore the response to the presence of copper at that concentration level could also involve a response to osmotic stress by producing osmoprotectants, such as ectoine and hydroxiectoine. The next enzyme annotated in *L. ferriphilum* Sp-Cl is citramalate synthase, which catalyses the conversion of acetyl-CoA and pyruvate into R-citramalate. This enzyme is involved in isoleucine biosynthesis. In *L. rubarum* and *Leptospirillum* "5 way CG", 2-isopropylmalate synthase is annotated after aspartokinase (instead of citramalate synthase as that of *L. ferriphilum* Sp-Cl). This enzyme is described to catalyse the conversion of Acetyl-CoA and 3-methyl-2oxobutanoate into 2-isopropylmalate and CoA. In the three genomic sequences the next protein is annotated as Acyl-(Acyl-carrier-protein)-UDP-N-acetylglucosamine O-acyltransferase. This protein is described to participate in the biosynthesis of lipopolysaccharide. After these proteins another annotated as integration host factor alpha subunit is located in this putative operon. This protein is a DNA binding protein and it is related to the architecture of DNA in a sequence-specific manner to induce a large bend. This bending stabilises distinct DNA conformations that are required during several bacterial processes, such as recombination, transposition, replication and transcription.

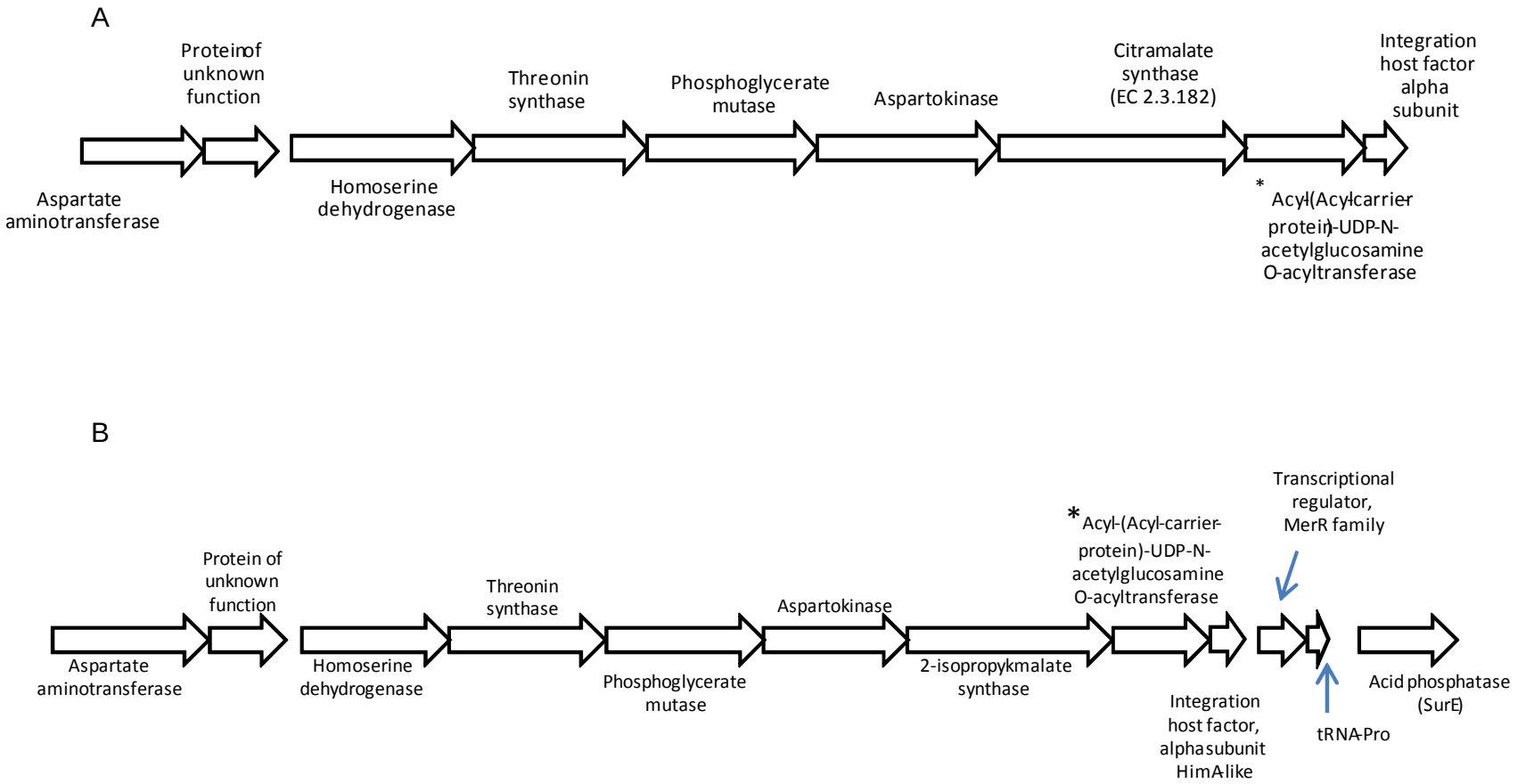


Figure 6.8. Genomic context showing the possible peptide deduced from band b of IESL25 (*) in *L. ferriphilum* Sp-Cl (A), *L. rubarun* and *Leptospirillum* sp. “5 way CG” (B).

The BLASTP analysis performed at NCBI website of the deduced peptide sequence from the IESL25 band f indicated 38% similarty with HNH endonuclease of *Spirosoma lingual*, however the similarity of this peptide determined by BALSTp using *L. ferriphilum* Sp-Cl genome as database showed a 51% of similarity with a protein sequence annotated as hypothetical protein. This hypothetical protein was also found to be annotated in genomic sequences of "*L. rubarum*" (accession number EAY57695), *Leptospirillum* sp. "5 way" (accession number EDZ39466) and "*L. ferrodiazotrophum*" (accssion number EES52707), with similarities of 99%, 78% and 100%, respectively, to the protein annotated as the same in *L. ferriphilum* Sp-Cl.

Interestingly, these proteins presented a conserved domain known as "YHS", as determined by pfam analysis performed at Sanger institute website (http://pfam.sanger.ac.uk/family/). This domain is about 50 amino acid residues long and often contains two cysteines that may be functionally important. This domain is found in copper transporting ATPases, some phenol hydroxylases and in a set of uncharacterised membrane proteins. The domain may be metal binding, possibly copper ions and it is duplicated in some copper transporting ATPases. Figure 6.9 shows the genomic context of the hypothetical protein. According to annotation, the same genomic organization was found in the four genomic sequence of *Leptospirillum* spp.

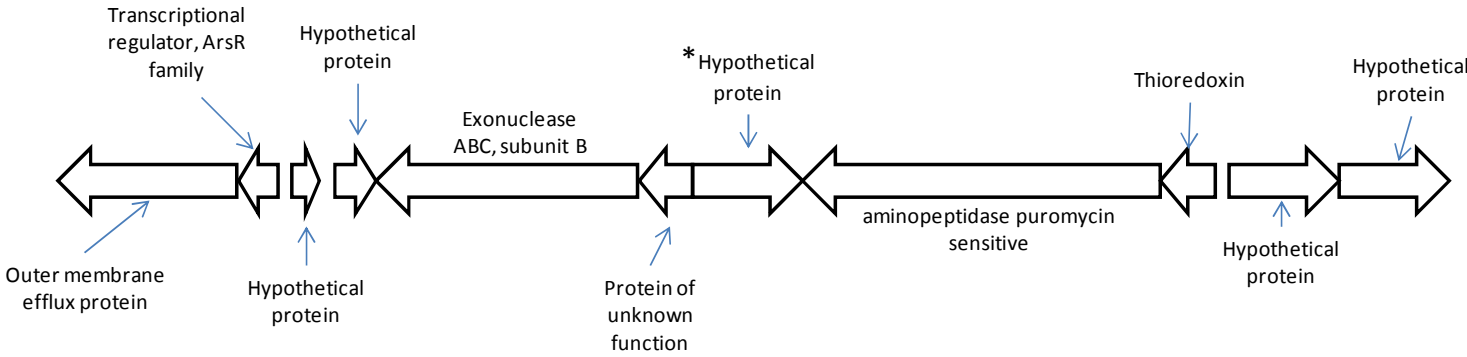


Figure 6.9. Genomic context found in *L. ferriphilum* Sp-Cl, *L. rubarum*, *Leptospirillum* sp. "5 way CG" and *L. ferrodiazotrophum*, including the protein annotated as hypothetical protein, similar to peptide deduced from sequence of band c retrieved from *L. ferriphilum* IESL25. * indicates the protein assumed similar to *L. ferriphilum* IESL25 is indicated.

6.4.5 REAL TIME PCR ANALYSIS OF THE *L. FERRIPHILUM* ISOLATE IESL25 TRANSCRIPTS EXPRESSED UNDER DIFFERENT STRESS CONDITIONS

Real time PCR analysis was performed for five differentially expressed RNA transcripts revealed by MRDA. Such analysis is critical as the various manipulation steps in the MRDA process preclude using it in a quantitative manner, in fact it typically reveals only those transcript that are expressed at the greatest differential level only. In addition, for a better understanding of these results, it is important to bear in mind that these results were obtained from cultures in late exponential phase and expression of the transcripts could vary during the different growth phases.

The results of real time PCR indicate that all sequences detected by MRDA were expressed at least 20 times more in the presence of stressor than in standard growth conditions in *L. ferriphilum* IESL25.

The sequence obtained from band d was retrieved from RNA when *L. ferriphilum* IESL25 was grown in presence of elevated concentration of magnesium sulfate. Real time PCR analysis also indicated that expression of this transcript increased as copper concentration increased in the medium and reached a similar expression level as observed in growth experiments in presence of elevated concentrations of magnesium sulfate, although the concentration of magnesium sulfate was almost double than the maximum copper concentration used in the experiments. Sequence retrieved from band d had low similarity to annotated proteins in genomic sequences of *Leptospirillum* spp. and sequencing of the region encoding this sequence in *L. ferriphilum* IESL, together with the collection of physiological evidence is needed to confirm its identity and its participation as transcription-coupling repair factor in osmotic stress, however these results showed a direct relationship of its expressions with increasing in the osmotic pressure.

The transcript represented by band f was retrieved from MRDA experiments when *L. ferriphilum* IESL25 was grown in the presence of 160 mM of copper sulftate, however its expression level was detected to be higher in experiments carried out in the presence of 50 and 100 mM of copper sulfate, suggesting that probably its expression helped cells to adapt to elevated concentration of copper and that it's function is replaced or inhibited at copper concentrations near to the maximum tolerance. Although the band b transcript was detected in experiments carried out in the presence of elevated concentrations of copper, its expression in presence of 300 mM magnesium sulfate reached similar levels to those observed in the maximum copper concentration tested. Therefore, the function of this transcript is probably to alleviate osmotic stress rather than being specific to copper toxicity stress.

The expression of the transcript represented by band e increased as the copper sulfate concentration increased, suggesting an effect of osmotic pressure, however similar to expression of band f, it decreases at copper concentration near to the maximum tolerated.

The expression of transcripts of bands b and c were relatively similar in most of the experiments, with the lowest expression level detected when *L. ferriphilum* IESL25 was grown at the lowest copper concentration tested.

CHAPTER 7: GENERAL DISCUSSION AND CONCLUSIONS

The Escondida mine in northern Chile operates the biggest heap bioleaching plant in the world. It has been in operation since 2006, producing metallic copper at the lowest cost per unit weight of the metal. In spite of constraints regarding access to sensitive industrial information, this plant is considered as a model of a large-scale copper bioheap operation. Before the start of the current project, several species of microorganisms had been detected and monitored at the Escondida heap bioleaching plant using molecular biology tools (DGGE, qPCR). This study unveiled the identity and dynamics of the microbial population in the process. In addition it showed that physico-chemical changes driven by microbial population pushed the system to more extreme conditions and at the same time the communities responded to these conditions by displaying changes in relative abundances of acidophilic prokaryotes in different stages of the process. Many questions arose from these early observations regarding how microorganisms are able to cope with different conditions imposed by dynamic physico-chemical parameters. In order to answer these questions the first objective proposed in this project (and tackled in chapter III) was the isolation and characterization of indigenous prokaryotes from the bioheaps. Six different strains belonging to three bacterial genera and one archaeal genus were isolated from industrial samples collected in January 2007. Phylogenetic analyses showed that the isolates were closely related to sequences previously identified in molecular analyses. The effect of pH and temperature on the growth two of these isolates, *At. ferrooxidans* IESL32 and *L ferriphilum* IESL25, were studied in detail. These showed that *At. ferrooxidans* IESL32 was able to oxidize iron at more extreme conditions (pH 1.0 and 45 ℃) than several other strains of iron-

oxidizing acidithiobacilli that had been previously described, conferring competitive advantages to strain IESL32 in warmer and more acidic environments. Interestingly, these physiological traits are more similar to strains of *L. ferriphilum* than to iron-oxidizing acidithiobacilli.

Reasons why *L. ferriphilum* rather than other *Leptospirillum* spp. are present in copper heap bioleaching processes were revealed, at least in part, in experimental work described in chapter IV, where the effect of four transition metals (Cu, Ag, Zn and Ni) on the growth of eight different strains of *Leptospirillum* spp., including two strains (IESL25 and Sp-Cl), isolated from bioleaching processes in Chile, was studied. The results showed that the four strains of *L. ferriphilum* tested had greater tolerance to both soluble copper and silver than the three strains of *L. ferrooxidans* and the single (type) strain of "*L. ferrodiazotrophum"* that were examined. This would appear to be the main reason for why *L. ferriphilum* is present in copper heap bioleaching processes, where copper concentrations in PLS solution must be maintained between 2.0 to 6.0 g/L to ensure the efficiency of following solvent extraction and electrowinning steps, even though the relatively low temperature of the bioheap operation at the time of sampling would seemingly favour *L. ferrooxidans*. The effect of elevated concentrations of NaCl on the growth of bioleaching microorganisms was also addressed in chapter IV and the potential of salt-tolerant acidophiles for bioleaching a copper sulfide concentrate was demonstrated in bench-scale bioreactors in the presence of elevated concentrations of sodium chloride. Although, there are no copper bioleaching processes operating with saline water, these results showed that the use of halotolerant microorganisms could be of advantage when brackish waters have to be used in commercial mineral bioprocessing operations. In this regard, BHP Billiton has registered the name BioChlor as a trade mark for a process to extract metals in high saline solutions (Australian

trademark No 1158508), using acidophilic organisms able to tolerate sodium chloride and salt.

The use of solutions with low water activity (elevated osmotic pressure, e.g. saline and brackish raffinates) in bioleaching processes had many advantages in places where water is scarce and the sea is relatively near to mining operations, such as the Atacama desert, which is both the driest region in the world and the location of most of the major copper mining operations in Chile. Water activity is decreased in commercial bioleaching processes mainly due to increasing concentrations of the sulfate anion resulting from recycling of solutions in the biohydrometallurgical circuit.

The ability to maintain metabolic activity in high osmotic strength liquors represents a major challenge for bioleaching acidophiles but, in spite of the importance of these prokaryotes in mineral bio-processing, few studies have been focused on determining the mechanisms and osmolytes involved in their water homeostasis. In the present study, ion chromatography and mass spectrometry were used to identify organic solutes synthesized by several species of acidophilic bacteria (including three strains isolated from the Escondida mine) when grown under conditions of osmotic stress. The two experimental approaches used – comparing concentrations of organic solutes in ethanol extracts of cells grown in the presence of elevated concentrations of inorganic solutes (generally magnesium sulfate or sodium chloride) with those grown in low-salt liquors, and identifying what compounds were excreted by cells when transferred from high to low osmotic strength liquors ("hypo-osmotic shock") – generally gave the same or similar results. The major osmo-protectant identified in different species of iron-oxidizing acidophiles, including *At. ferrooxidans*, *L. ferriphilum*, *L. ferrooxidans*, "*L. ferrodiazotrophum"*, *Sb. thermosulfidooxidans* and the heterotroph *Acidiphilium* sp. SJH in response to elevated salt concentrations was the disaccharide, trehalose. In contrast, the monosaccharide glucose was found to be an important osmo-protectant in the

sulfur-oxidizing acidophile *At. thiooxidans*. Proline was also observed to increase in cells *At. thiooxidans* grown at elevated osmotic pressure, though its relatively low concentration (compared to glucose) suggested that proline might have other functions in salt-stressed *At. thiooxidans*, rather than retaining water within the cytoplasm. While some chromatographic peaks detected in *At. thiooxidans* and *At. ferrooxidans* could not be identified, hydrolysis of cell extracts showed that one of these peaks (found with *At. thiooxidans*) appeared to be an oligosaccharide composed of glucose subunits.

Finally, Micro-Representational Difference Analysis (MRDA) was used as a tool to investigate the genetic response of *L. ferriphilum* IESL25 to copper and sulfate stress. The results give new insights regarding the adaptation of *L. ferriphilum* to elevated concentrations of copper and sulfate and highlighted the activation of certain metabolisms related to lipopolysacharide synthesis, transport systems and nucleic acid processing in response to copper and sulfate stress.

Future research regarding the findings described in this project should include:

- Isolation and characterization of other microorganisms from Escondida, in order to understand community responses to fluctuations of physico-chemical parameters in the bioleaching operation.

- Investigating what osmo-protectants are synthesized by prokaryotic acidophiles (including archaea, such as *Ferroplasma* spp.) that were not included in the present study.

- Application of new approaches, such as NMR, to determine the osmo-protectants that were not identified in the present study and assessment of potential other mechanisms (e.g. the role of inorganic ions, such as K^+) used by acidophiles in response to salt-stress.

- A study the effect of varying physic-chemical parameters (e.g. temperature and pH) on the bioleaching of chalcopyrite concentrate, in the presence of elevated concentrations of salt.

- Identification of the mechanisms involved in the remarkable copper tolerance of *L. ferriphilum* strain MT61.

- To obtain additional physiological data in order to confirm possible genetic responses to stress produced by elevated concentrations of sulfate and copper in *Leptospirillum* spp..

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