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Molecular characterization of microbial populations in a low-grade copper ore bioleaching test heap

Cecilia S. Demergasso [a,*], Pedro A. Galleguillos P. [a], Lorena V. Escudero G. [a], Víctor J. Zepeda A. [a], Danny Castillo [b], Emilio O. Casamayor [c]

[a] *Laboratorio de Microbiología Técnica, Universidad Católica del Norte, Avda. Angamos 0610, Antofagasta, Chile*
[b] *Proyecto Lixiviación de Sulfuros, Minera Escondida Ltda, Chile*
[c] *Limnología-Departamento de Biogeoquímica Acuática, Centro de Estudios Avanzados de Blanes (CSIC), Spain*

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Abstract

A culture-independent approach based on PCR amplification and denaturing gradient gel electrophoresis (DGGE) and sequencing of 16S rRNA gene fragments from both *Bacteria* and *Archaea* were used to analyze the microbial community inhabiting a low-grade copper sulfide run-of-mine (ROM) test heap of a project in Chile. In this paper, we summarize results of a 1-year monitoring study. Phylogenetic analyses of 16S rRNA fragments revealed that the retrieved sequences clustered together with *Acidithiobacillus ferrooxidans*, *Leptospirillum ferriphilum*, *Ferroplasma acidiphilum* and environmental clones related to them. In addition, some sequences were distantly related (<95% similarity in the 16S rRNA gene fragment analyzed) to cultured microorganisms from the *Sulfurisphaera* and *Sulfobacillus* genera. Thus, the prokaryotic assemblage might be mainly composed of sulfur- and iron-oxidizing microorganisms. The remaining sequences were related to uncultured chrenarchaeota clones or had only partial homology with known microorganisms. Attempts were made to estimate the dynamic of phylogenetic microbial groups in different stages of the leaching cycle and to correlate them with chemical and physical parameters in the heap. The temporal distribution of microbial 16S rRNA gene sequences could be divided in three periods. In the bioleaching cycle, first stage *A. ferrooxidans* and *Sulfurisphaera*-like archaea were dominant within each respective phylogenetic domain. In the second stage (from days 255 to 338), *Leptospirillum* and *Ferroplasma* groups were mainly detected, respectively. Finally (the third period from operation days 598 to 749), *Sulfobacillus*-like microorganisms became predominant, while *Ferroplasma* was the only *Archaea* detected. These data are now being used to obtain more detailed and quantitative information on prokaryotic community structure over time and to explore the nature of the community metabolic pathways. These results extend our knowledge on microbial dynamics in bioheaps, a key issue required to improve commercial applications.

Keywords: Bioleaching; Heap; Biodiversity; *Archaea*; Thermophiles

1. Introduction

Heap bioleaching is a rapidly emerging technology for the extraction of base metals from sulfide minerals. Significant attention has been focussed on the development of bioheap leaching in recent years (Brierley and Brierley, 2001). This can be explained by the

* Corresponding author. Fax: +56 55 355423.
E-mail addresses: cdemerga@ucn.cl (C.S. Demergasso), pgallegu@ucn.cl (P.A. Galleguillos P.), lescudero@ucn.cl (L.V. Escudero G.), vzepeda@ucn.cl (V.J. Zepeda A.), Danny.I.Castillo@BHPBilliton.com (D. Castillo), casamayor@ceab.csic.es (E.O. Casamayor).

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enormous natural reservoir of copper as secondary copper sulfide, a substrate traditionally considered waste due to its low grade. Commercial application of bioheap leaching designed to exploit microbial activity, was pioneered in 1980 for copper leaching. The Lo Aguirre mine in Chile processed about 16,000 tonnes of ore/day between 1980 and 1996 using bioleaching (Bustos et al., 1993). Numerous copper heap bioleaching operations have been commissioned since then (Brierley and Brierley, 2001). Overall, Chile produces about 400,000 tonnes of cathode copper by bioleaching process, representing 5% of the total copper production (Informe al Presidente de la República, Comisión Nacional para el desarrollo de la Biotecnología, Gobierno de Chile 2003). Since 1990, Escondida Mine has stockpiled 100 million tonnes of low-grade copper ore. All this material could be considered as waste without an appropriate bioheap leaching technology.

The microbial ecology of full-scale bioheap leaching of secondary copper ores and dump leaching is poorly understood and little effort has been made to handle the microbiological components of these processes (Brierley, 2001). As recently stated, "Understanding the microbiology of heaps is key to advancing commercial bioheap operations" (Brierley, 2001). Direct bacterial counts and indirect measurements such as oxygen uptake in solid and liquid samples, redox potential, pH, ferrous iron concentration and temperature have provided useful information on the bulk activity of microorganisms (Brierley, 2001). In addition, microbial enrichments and pure cultures from solutions and ores of experimental- and industrial-scale processes have provided an initial view of important microorganisms associated with bioleaching. In heap leaching processes running at ambient temperature, bacterial strains related to *Acidithiobacillus thiooxidans*, *Acidithiobacillus ferrooxidans*, *Acidiphilium cryptum* and *Leptospirillum ferrooxidans* have often been cultured (Goebel and Stackebrandt, 1994; Rawlings, 2001). However, it is not known if cultured strains are the key players in the system, and very few culture-independent data are available from commercial processes. Therefore, our present knowledge relies almost exclusively on the culturable community fraction whereas the whole microbial picture of commercial bioleaching processes is still missed.

In microbial molecular ecology it is well established that DGGE offers a rapid culture-independent way for detecting and identifying predominant PCR-targeted populations (Casamayor et al., 2000). DGGE is a powerful and convenient tool for a study like the present one, which requires extensive sampling of several points and at different times. The goal of the present work was to report the results from a 1-year study on the microbial populations inhabiting a low-grade copper sulfide ROM test heap at the Escondida Mine in Northern Chile.

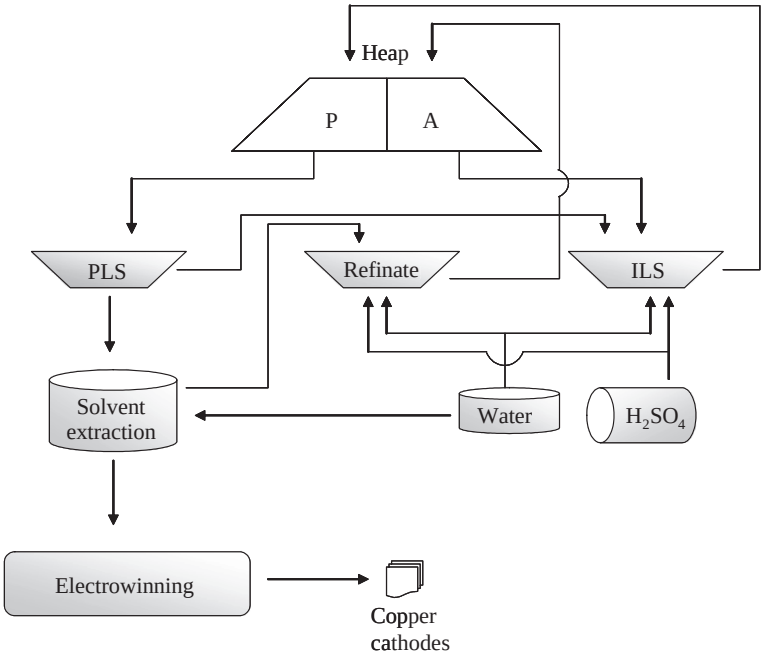

Fig. 1. Solution circulation scheme in between operation days 111 and 233 of the Andesite Module from the low-grade copper ore bioleaching test heap at Escondida Mine. ILS = intermediate leaching solution, PLS = pregnant leaching solution, P = Porphyry module, A = Andesite Module.

2. Materials and methods

2.1. Heap description

Escondida Mine is located 170 km south-east from Antofagasta. To test the potential for bioleaching low-grade sulfide mineralization, two samples of run-of-mine (ROM) marginal ore were obtained from the Escondida pit, and placed in approximately 12-m-high heaps. Sampled material was selected from the mine to represent poorly enriched, low-grade sulfide material hosted by two major material types: Porphyry with quartz–sericite–pyrite (QS) alteration, and Andesite altered to sericite–chlorite–clay (SCC). The samples (average of 200,000 tonnes) were loaded onto two adjacent modules, approximately 80 × 80 m in size each at the heap crest. The test samples were placed on lined pads that contained drainage piping (10.2 cm diameter) every 4 m to collect the solution and a 0.7-m drainage layer. Then an aeration system was buried on top of the

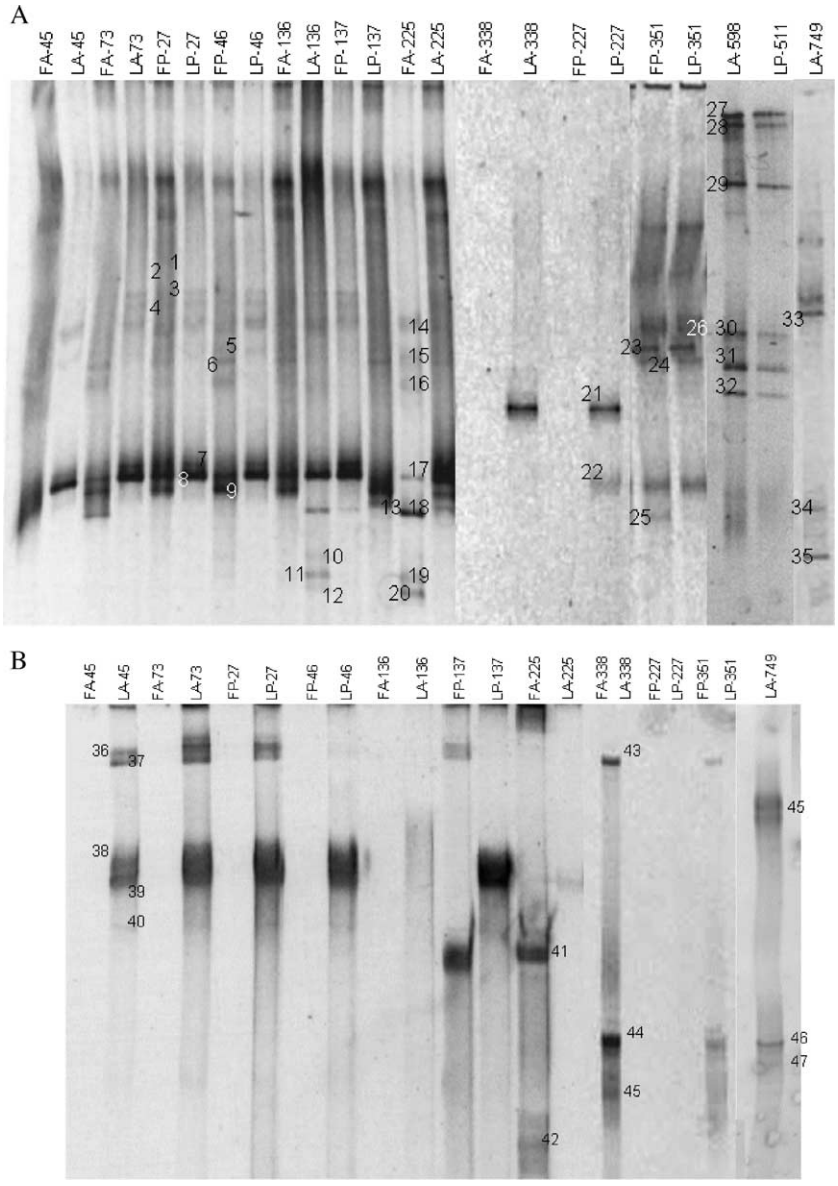

Fig. 2. SYBR-Gold stained DGGE gels of bacterial (A) and archaeal (B) 16S rRNA gene fragments amplified by PCR from the different samples (F = feed solution; L = Leached solution; A = Andesite Module; P = Porphyry module. Numbers represent operation day). Numbered DGGE bands correspond to data presented in Figs. 3–6.

drainage layer. Finally, the aeration system was protected with a 1 m layer of ROM material. The irrigation for the Andesite and Porphyry modules started on May 21 and August 17, 2001, respectively. Solutions were circulated through both modules in different circuit designs during the leaching cycle. For instance, in between operation days 111 and 233 of the Andesite Module, the feeding solution for this module (Fig. 1) was the rafinate from the solvent extraction step. Feed and leach solution samples were taken periodically from both modules for molecular analysis. The pH of feed solutions for both modules was controlled by adding sulfuric acid ranging from pH 1.0 to 1.2 approximately.

2.2. Nucleic acid analyses, DGGE and sequencing

DNA samples were designated using F for feed and L for leach solutions, and A and P for Andesite and Porphyry Modules, respectively. For temporal identification, the module operation day was added next.

Between 800 and 1000 mL of solution (feed or leach solutions) were filtered through 0.2 μm polycarbonate membranes (Nuclepore) and stored at −20 °C in 1 mL of lysis buffer (50 mM Tris–HCl pH = 8.3, 40 mM EDTA and 0.75 M sucrose). Filters were cut and incubated with lysozyme and proteinase K, and genomic DNA was purified by using phenol–chloroform–isoamyl alcohol (25:24:1, vol/vol/vol) as previously described (González-Toril et al., 2003). Purified genomic DNA was quantified using a Perkin Elmer spectrophotometer model MBA 2000. The genomic DNA was used as target in the PCR to amplify the 16S rRNA gene using the bacterial universal primer set 358F-GC (5′–CCTACGGGAGGCAGCAG–3′) and 907R (5′–CCGTCAATTCMTTTGAGTTT–3′) and for Archaea 344F-GC (5′–ACGGGGYGCAGCAGGCGCGA–3′) and 915R (5′–GTGCTCCCCCGCCAATTCCT–3′). Forward-GC primers had a high GC content clamp (CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG) bound to 5′end. The 16S rRNA gene fragments suitable for subsequent denaturing gradient gel electrophoresis (DGGE) analyses were obtained by PCR as previously described (Muyzer et al., 1996; Casamayor et al., 2002). DGGE, excision of bands and reamplification were

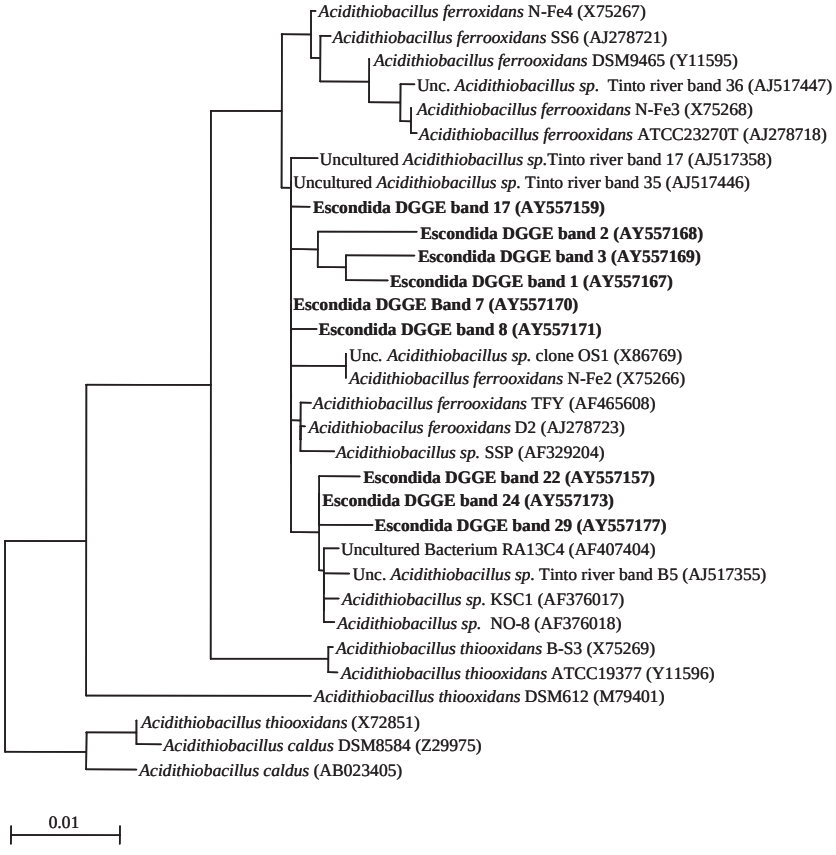

Fig. 3. Phylogenetic affiliation of 16S rRNA gene sequences from DGGE bands (in bold) closely related to the *Acidithiobacillus* spp. cluster. Database accession numbers are indicated between brackets. The scale bar corresponds to 0.01 mutations per nucleotide position.

performed as previously described (Casamayor et al., 2002). A photograph of the SYBR-Gold stained gel was taken using a Polaroid camera model GelCam under UV transillumination. When bands were found in the same gel position along different lanes, it was assumed that these belonged to the same sequence. Partial sequences obtained (around 500 bp) were first evaluated using the basic local alignment search tool (BLAST) on the internet (http://www.ncbi.nlm.nih.gov). Alignments and phylogenetic analyses of the partial 16S rRNA gene sequences were carried out by ARB software (http://www.arb-home.de). Partial DGGE sequences were inserted without modifying the original topology of an optimized tree based on a parsimony analysis that included only complete or almost-complete 16S rRNA gene sequences of representative bacteria, as previously described (Demergasso et al., 2004).

Nucleic acid extraction yielded between 1.1 and 31.5 ng of DNA per mL of sample. In four of the samples (FA-338, FP-351, LP-351 and LA-598) DNA extraction was unsuccessful. Two samples (FP-351 and LP-351) did not produce direct bacterial PCR amplification product. These were submitted to a nested PCR protocol with primers Eub27f (GAGTTTGATCCTGGCTCAG) and Eub1492R (TACGGYTACCTTGTTACGACTT) for

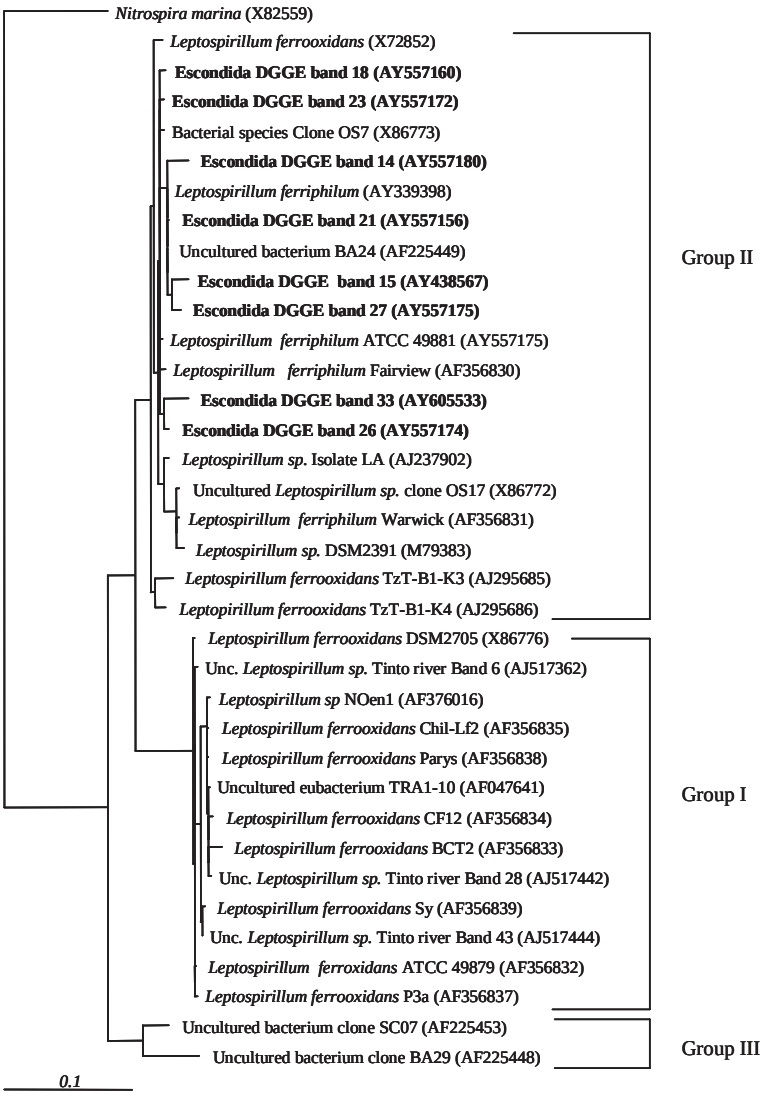

Fig. 4. Phylogenetic affiliation of 16S rRNA gene sequences from DGGE bands (in bold) closely related to *Leptospirillum* spp. cluster. Database accession numbers are indicated between brackets. The scale bar corresponds to 0.1 mutations per nucleotide position.

the complete 16S rRNA gene amplification, followed by a second PCR with the DGGE primers set using 1–2 μL of the first PCR product as template. Two samples (LA-598 and LP-511) did not produce archaeal 16S rRNA fragments, even after nested PCR. The feed solutions LA-338 and LP-227 yielded very few bacterial PCR products. In addition, samples FA-45, FA-73, FP-27, FP-46, FA-136, LA-225, FA-338, FP-227, LP-227, FP-351, LP-351 produced very few archaeal PCR product or none at all, even after using nested PCR.

The relative percentages of the intensity of each DGGE band to the total band signal in the lane were obtained with the software CrossChecker (available on http://www.dpw.wau.nl/pv/pub/CrossCheck/). A total of 40 16S rRNA sequences from both *Bacteria* and *Archaea* have been deposited in GenBank with accession numbers indicated in Figs. 3–6.

3. Results

3.1. DGGE fingerprinting and phylogenetic analyses

Twenty-three samples from the industrial solutions (10 feed- and 13 leach-solutions from the two modules, respectively) were used for genetic analysis. Overall, up to 7 different bands were observed in each bacterial fingerprint, suggesting low species richness (Fig. 2A). Only 1 to 4 different bands were observed in the archaeal fingerprinting (Fig. 2B). Thirty-three predominant bands were excised from the bacterial DGGE gel (Fig. 2A). From these, 27 bands were sequenced and analyzed by ARB software and only 6 failed to produce a useable sequence.

Nine bands (bands 1, 2, 3, 7, 8, 17, 22, 24, and 29) grouped within the *A. ferrooxidans* cluster of the *gamma-Proteobacteria* group (Fig. 3). These sequences

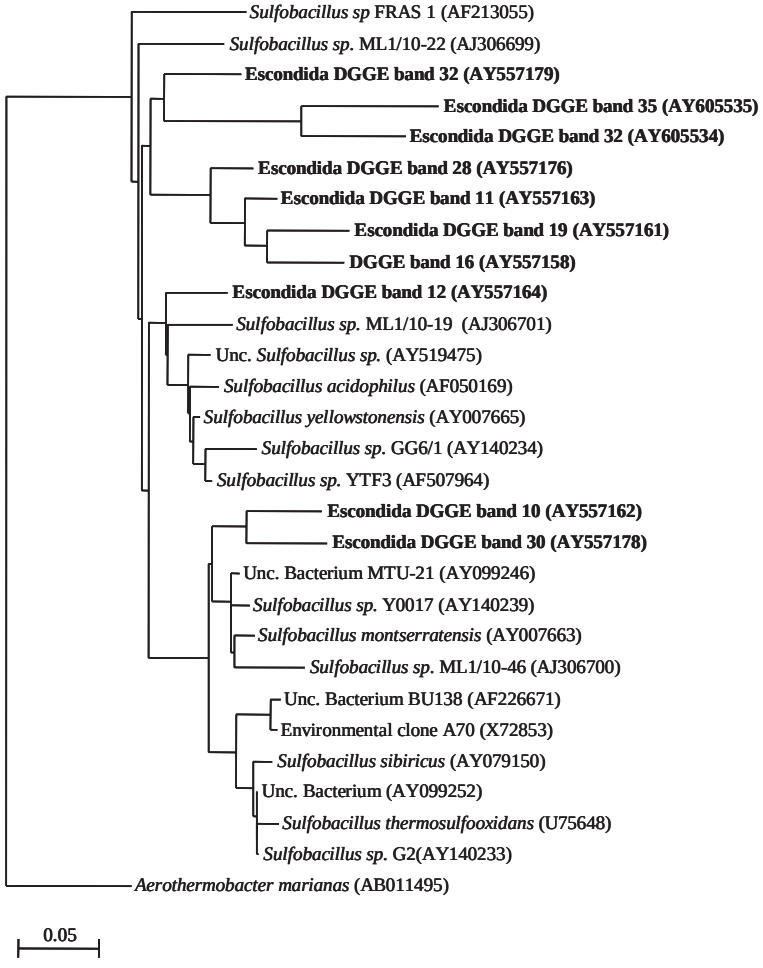

Fig. 5. Phylogenetic affiliation of 16S rRNA gene sequences from DGGE bands (in bold) closely related to *Sulfobacillus* spp. cluster. Database accession numbers are indicated between brackets. The scale bar corresponds to 0.05 mutations per nucleotide position.

had a high similarity to each other (97.3% to 99.9%). DGGE bands 1, 2 and 3 from sample FP-27 affiliated with *A. ferrooxidans* group III (Bond et al., 2000a; Karavaiko et al., 2003). Meanwhile, bands 22, 24 and 29 affiliated with *Acidithiobacillus* group IV.

Eight bands (bands 18, 21, 23, 26, 27, 15, 14 and 33) grouped with *Leptospirillum ferriphilum* (Fig. 4). The similarity of these sequences ranged between 95.4% and 99.6% among them. In the same cluster, we found sequences obtained from a ferric sulfate fluidized-bed reactor (Kinnunen and Puhakka, 2004), and clones from an abandoned copper mine in Australia (Goebel and Stackebrandt, 1994). Ten sequences were related to *Sulfobacillus* within the *Firmicutes* group (low GC Gram Positive) and all these sequences showed similarity lower than 97% to each other (Fig. 5).

For *Archaea*, 13 bands were excised from the DGGE gel and the 16S rRNA gene sequences analyzed with the ARB software (Fig. 6). Five sequences were related to the crenarchaeal Sulfolobaceae group (only bands 37 and 39 showed similarity value of 97% to each other, but the remaining sequences had identities lower than 88.5%). Six sequences (DGGE bands 43 to 48, similarity to each other >96.1%) were related to the euryarchaeal

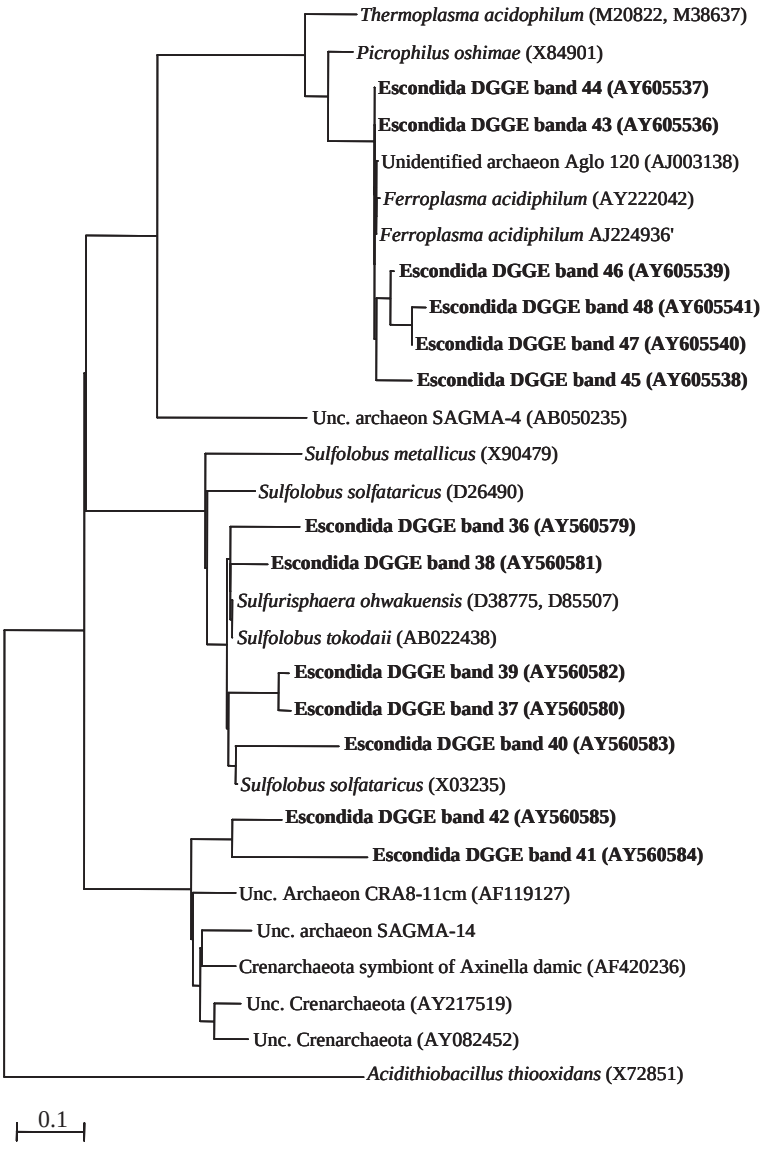

Fig. 6. Phylogenetic affiliation of 16S rRNA gene sequences from DGGE bands (in bold) of Fig. 2 closely related to *Archaea*. Database accession numbers are indicated between brackets. The scale bar corresponds to 0.10 mutations per nucleotide position.

Ferroplasma acidiphilum (Golyshina et al., 2000) of the thermoplasmatales. Two more sequences (DGGE bands 41 and 42, similarity 83% to each other) were distantly related to uncultured Crenarchaeota.

3.2. Temporal dynamics in the bioheaps

In order to understand the dynamics of the microbial populations in the heap, we determined time profiles, using the percent of DGGE band intensity as a surrogate of relative abundance. Fig. 7 shows the temporal distribution of the bacterial assemblages. The percentage of unknown members (i.e., DGGE bands that failed reamplification or sequencing) ranged from 0 to 15%. Sequences related to *Acidithiobacillus*, *Leptospirillum* and *Sulfobacillus* groups were recovered from all the samples from which PCR product was obtained. Temporal distribution of bacterial sequences could be divided in 3 periods. The first period covered from day 0 to operation day 255 for andesite, and from day 0 to operation day 137 for the porphyry modules, respectively. Along this period, the *Acidithiobacillus* sequences clearly predominated. In a second period (from days 255 to 338), *Leptospirillum* was detected as prominent bacterial component in the solutions. Finally, in the last samples (third period from operation days 598 to 749), *Sulfobacillus*-related sequences became predominant.

Fig. 8 shows the temporal distribution of *Archaea* in the solution samples. *Sulfolobales*-related 16S rRNA sequences affiliated with *Sulfurisphaera* and *Sulfolobus* were prominent in Andesite and Porphyry module leach

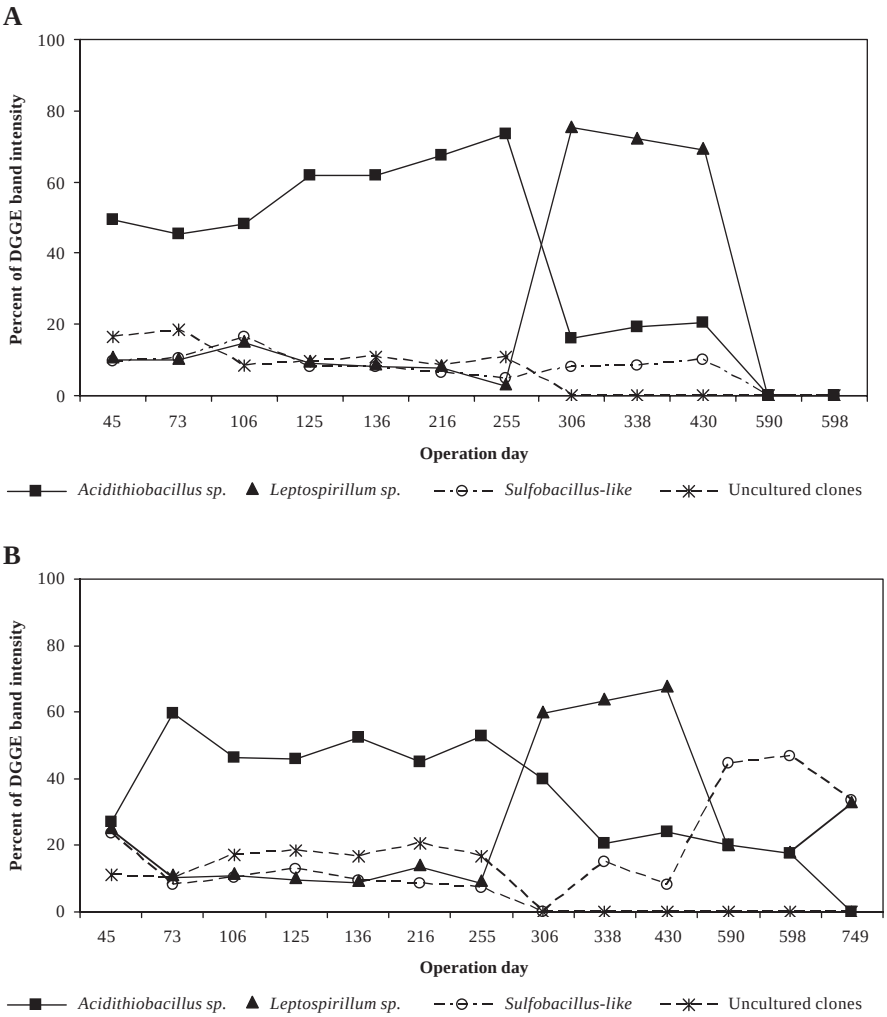

Fig. 7. Temporal distribution of the relative abundance of the 16S rRNA gene from the different bacterial groups (relative DGGE band abundance vs. operation day): (A) feed solutions; (B) leach solutions. The operations days consider the Andesite Module as the operation of the whole system.

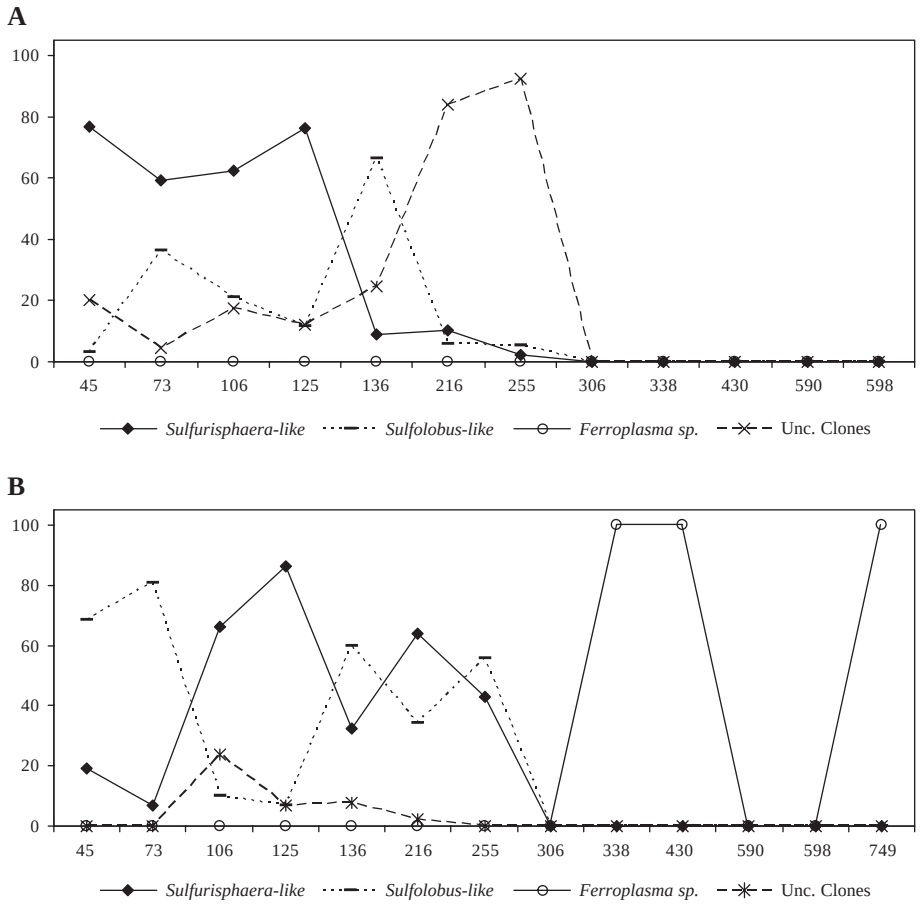

Fig. 8. Temporal distribution of the relative abundance of the 16S rRNA gene from the different archaeal groups (relative DGGE band abundance vs. operation day): (A) feed solutions; (B) leach solutions. The operation days consider the Andesite Module as the operation of the whole system.

solutions during the first period described above. However, with time, *Sulfolobales* sequences were no longer detected. The uncultured Crenarchaeota group was also found only through this period. For the second and third periods, *Ferroplasma* became the only group detected in samples with PCR positive results (LA-338, LA-749 and LP-351).

4. Discussion

The application of biotechnology to extraction and recovery of metals is rapidly growing. Recent reviews indicate the number and sizes of the bioleaching–biooxidation operations (Olson et al., 2003; Rawlings et al., 2003). Another review shows the remarkable adaptability of the population in the biooxidation tanks (Rawlings, 2005) assisted by continuous-flow systems, open and non-sterile processes for the possibility of new genes to be selected from the horizontal gene pool. Previous unreported research experience (Rawlings, 2005) has found that after a period of operation, the metabolic capabilities of a population of biomining organisms may improve from the culture originally inoculated into the tanks.

This paper is the first report of the microbial temporal dynamics in a demonstrative heap using culture-independent techniques. Although not free of limitations, signal intensity of DGGE bands is a useful tool to estimate the relative abundance of the different groups and to have a rough picture of the microbial dynamics in the process (Casamayor et al., 2000). Therefore, the results show general trends over time rather than detailed quantitative estimations of the microbial population structure.

4.1. Relationship between sequences retrieved and cultured strains

Phylogenetic analyses of 16S rRNA fragments revealed that more than a half of the sequences

retrieved were included in clusters containing cultured *A. ferrooxidans*, *L. ferriphilum* and *F. acidiphilum* strains. Thus, the predominant metabolic characteristics in the original assemblage should be sulfur and iron oxidation.

All the *Leptospirillum*-retrieved sequences from the heaps were affiliated with *Leptospirillum* group II (Bond et al., 2000a; Coram and Rawlings, 2002). Recent work has shown group II(b) and III(a) to be closely related to *L. ferriphilum* (Coram and Rawlings, 2002). In commercial bioreactors for metal leaching working up to 40 °C, *L. ferriphilum* is reported to be a predominant member of the community and groups II and III are also found in acid mine drainage biofilms. Conversely, sequences related to *Leptospirillum* found in Tinto River were all affiliated with group I(c) (González-Toril et al., 2003). Thus, culturing-targeted efforts on *Leptospirillum* group II should be carried out bearing in mind its potential key role in the metal sulfide minerals oxidation of the bioheaps.

On the other hand, the *Sulfobacillus*-like sequences were related to strains and clones obtained from Yellowstone National Park (Johnson et al., 2003), bioleaching thermophilic processes of pyrite, arsenopyrite and chalcopyrite (Dopson et al., 2004), and sediments of acid mining lakes (GenBank description). The most closely related cultured microorganism to one more sequence was *Sulfolobus solfataricus*, a hyperthermophile isolated from volcanic springs (Zillig et al., 1980).

Finally, most of the archaeal sequences were only distantly related to cultured microorganisms from the Sulfolobaceae family and with uncultured chrenarchaeotal clones. Inferring phenotypic properties from the recovered sequences was difficult due to limited database on bioleaching microorganisms. This is certainly evident in the case of *Archaea* and suggests that physiology of the microorganism inhabiting the heap can be very different from the cultured strains available in the laboratory. This is the first time that the presence of putative hyperthermophilic archaeal organisms related to *Sulfolobus* has been detected in industrial bioleaching process operating below 60 °C. In a previous work, the *Ferroplasma*-related clone Aglo 120 (AJ003138) was found in laboratory columns inoculated with industrial bioleaching copper process solutions and operating at high sulfate concentration (Vásquez et al., 1999). The existence of sequences related to thermophilic microorganisms suggests the presence of microenvironments with high temperatures in the heap. Therefore, it is possible that *Archaea* play more important roles in the commercial process than previously thought.

4.2. Prokaryotic community structure in the bioheaps

To understand bacterial dynamics in this process, it is necessary to bear in mind that both modules formed a single system where solutions were continuously recirculated through the circuit.

The colonization of the system started with the predominance of *A. ferrooxidans* plus distantly related to *Sulfolobales* sequences (Figs. 7 and 8). We observed that the shift in predominance of *Acidithiobacillus* to *Leptospirillum* (operation day 256, Fig. 7A) coincides with decreasing concentrations of ferrous iron in the feed solutions of Andesite Module (Figs. 7 and 9B). Differences in affinity for ferrous iron have been described by previous reports (Norris et al., 1987; Boon et al., 1995; Scherpenzeel et al., 1998; Rawlings et al., 1999; Battaglia-Brunet et al., 2002), showing that *L. ferrooxidans* posses higher affinity for ferrous iron than *A. ferrooxidans*. Such a difference may explain the shift observed in the Andesite Module. Furthermore, the relative abundance of *Sulfobales*-related sequences in the bioheap is in accordance with the temperature evolution (Figs. 8 and 9B) in the leach solution of both modules (data not shown for porphyry module). The presence of *Sulfolobales*-like microorganisms could be associated with iron precipitation in Andesite Module (data not shown). The SCC material is characterized by the presence of a more reactive gangue compared to the QS material.

Increasing sulfate concentrations during the leaching cycle (Fig. 9B) seems to be another reason for the microbial population predominance changes among *Acidithiobacillus*, *Leptospirillum* and *Sulfobacillus*.

The microbial succession that we observed in the heaps seems to match the molecular data available from Acid Mine Drainage (AMD) systems. The community is dominated by *Leptospirillum* and *Ferroplasma* groups at the most extreme conditions of lower pH and higher ionic strength (Edwards et al., 1999; Bond et al., 2000a; Okibe et al., 2003). Conversely, *Acidithiobacillus* and *Sulfobacillus* are the main components at slightly higher pH and lower conductivity (Bond et al., 2000a). However, the predominance of *Sulfobacillus*-related sequences have only been reported in a column bioreactor (d'Hugues et al., 2003) compared to the mechanically stirred reactor test carried out in parallel with the same inoculum. In those experiences, the proportion of *Sulfobacillus*-related sequences usually increased at the end of the test. They generally occurred more on the solids than in solutions (D'Hugues et al., 2002), and the authors suggest that *Sulfobacillus*-like organisms were more in competition with iron oxidizers

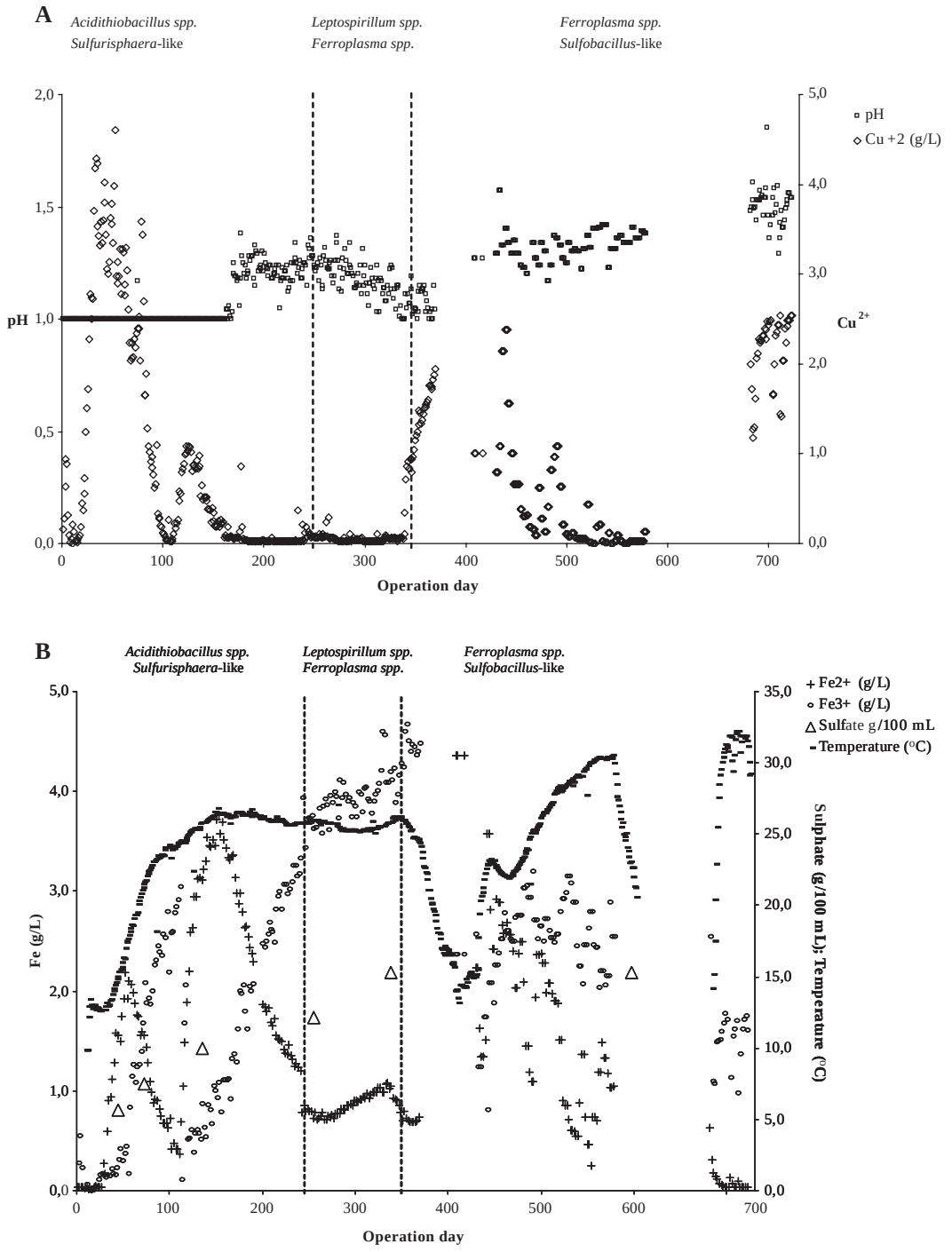

Fig. 9. Predominance in microbial community composition related to chemical data from the circulating solutions: (A) Cu^{2+} (g L^{-1}), pH; (B) Fe^{2+} (g L^{-1}), Fe^{3+} (g L^{-1}), SO_4^{2-} (g L^{-1}), temperature (°C).

than with sulfur oxidizers. In the case of *Archaea*, the presence of *Ferroplasma* as the only nearest relative of the sequences in the leach solutions above 150 g L^{-1} sulfate (Fig. 9B) coincides with previously reported data (Vásquez et al., 1999; Okibe et al., 2003). Highly concentrated acidic solutions clearly enriched these *Archaea*, both in the environment and in some bioleaching processes, although it must be taken into

account that it was not possible to obtain archaeal PCR product from samples L1-598 and L2-511 (those of the highest sulfate concentration). Sulfate concentrations above 150 g L^{-1} could correspond to an extensive killing of the indigenous bacteria and it is possible that growth of *Ferroplasma* was stimulated by the dead biomass (Okibe and Johnson, 2004).

Correlations could not be found with other physicochemical parameters like pH and Cu^{2+} concentrations (Fig. 9A).

After comparing these DGGE results with analysis from similar acidic environments, the lack of sequences related to *A. thiooxidans* (Battaglia-Brunet et al., 2002), *Acidithiobacillus caldus* (Okibe et al., 2003), *Acidophilum* sp. (González-Toril et al., 2003) (α-*Protebacteria*), and *Acidimicrobium* sp. (Bond et al., 2000b) (*Actinobacteria*) was observed. However, it is important to bear in mind that between 0–15% (*Bacteria*) and 2–13% (*Archaea*) of total band intensity in each lane, could not be assigned to a known organism. In addition, DGGE does not detect populations in natural communities whose abundance is less than 0.3% to 1% of the total cell count (Casamayor et al., 2000). This method does not cover the total microbial species richness of the system. Furthermore, the detection of *Sulfolobales*-like strains in acidic industrial and natural environments below 35 °C has not been previously described. Though *Sulfobacillus*-like strain occurrences have been reported in uranium mines (Muhammad et al., 2003). Obviously, absolute quantitative data on microbial abundances requires the use of other techniques such as fluorescent in situ hybridization (FISH).

Finally, we observed that some DGGE bands had only minor differences in their sequences (microdiversity). Some of this microdiversity might be explained by artifacts such as *Taq* polymerase errors during the PCR amplification, formation of heteroduplexes in the DGGE gel, or rRNA multioperons belonging to a single population. For instance, it is known that *L. ferriphilum* has 2 rrn genes, whereas *L. ferrooxidans* has 3 rrn genes. However, in many cases the microdiversity has ecological and physiological significance in natural populations (Casamayor et al., 2002). Frequent recombination phenomena could explain the microdiversity found and mosaic type genomes would give ability to population survival when the system is perturbed (Tyson et al., 2004).

5. Conclusions

DGGE band patterns have proved to be a valuable method to track microbial population in order to understand (and optimize) copper heap-bioleaching process. The main steps of the bioleaching industrial cycle could be correlated to a microbial dominance profile. The presence of sulfur oxidizers *Sulfolobales*-like and *Sulfobacillus*-like microorganisms in the first step of the cycle instead of mesophile microorganisms might reveal favourable conditions for thermophiles in the heap. Community changes seem to be related to dynamics of the main substrates such as ferrous iron availability and increasing levels of inhibitory ions like sulfate. Therefore, a successful inoculation strategy should consider the use of mixed microbial assemblages composed by mesophile and moderate thermophile strains involving the use of closely related populations. Further efforts are also needed to culture microorganisms involved in the bioleaching process that have been unveiled by the use of culture-independent methodologies.

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References

Battaglia-Brunet, F., Clarens, M., d'Hugues, P., Godon, J.J., Foucher, S., Morin, D., 2002. Monitoring of a pyrite-oxidising bacterial population using DNA single-strand conformation polymorphism and microscopic techniques. Applied Microbiology and Biotechnology 60 (1–2), 206–211.

Bond, P.L., Druschel, G.K., Banfield, J.F., 2000a. Comparison of acid mine drainage microbial communities in physically and geochemically distinct ecosystems. Applied and Environmental Microbiology 66 (11), 4962–4971.

Bond, P.L., Smriga, S.P., Banfield, J.F., 2000b. Phylogeny of microorganisms populating a thick, subaerial, predominantly lithotrophic biofilm at an extreme acid mine drainage site. Applied and Environmental Microbiology 66 (9), 3842–3849.

Boon, M., Hansford, G., Heijnen, J., 1995. The role of bacterial ferrous iron oxidation in the bio-oxidation of pyrite. In: Vargas, T., Jerez, C., Wiertz, J., Toledo, H. (Eds.), International Biohydrometallurgy Symposium, Viña del Mar, Chile.

Brierley, C., 2001. Bacterial succession in bioheap leaching. Hydrometallurgy 59, 249–255.

Brierley, J., Brierley, C., 2001. Present and future commercial applications of biohydrometallurgy. Hydrometallurgy 59, 233–239.

Bustos, S., Castro, S., Montealegre, R., 1993. The Sociedad Minera Pudahuel bacterial thin-layer leaching process at Lo Aguirre. FEMS Microbiology Reviews 11 (1–3), 231–236.

Casamayor, E.O., Schafer, H., Baneras, L., Pedros-Alio, C., Muyzer, G., 2000. Identification of and spatio-temporal differences between microbial assemblages from two neighboring sulfurous lakes: comparison by microscopy and denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 66 (2), 499–508.

Casamayor, E.O., Pedros-Alio, C., Muyzer, G., Amann, R., 2002. Microheterogeneity in 16S ribosomal DNA-defined bacterial populations from a stratified planktonic environment is related to temporal changes and to ecological adaptations. Applied and Environmental Microbiology 68 (4), 1706–1714.

Coram, N.J., Rawlings, D.E., 2002. Molecular relationship between two groups of the genus *Leptospirillum* and the finding that *Leptospirillum ferriphilum* sp. nov. dominates South African commercial biooxidation tanks that operate at 40 degrees C. Applied and Environmental Microbiology 68 (2), 838–845.

Demergasso, C., Casamayor, E.O., Chong, G., Galleguillos, P., Escudero, L., Pedrós-Alió, C., 2004. Distribution of prokaryotic genetic diversity in athalassohaline lakes of the Atacama Desert, Northern Chile. FEMS Microbiology Ecology 48 (1), 57–69.

D'Hugues, P., Foucher, S., Galle-Cavalloni, P., Morin, D., 2002. Continuous bioleaching of chalcopyrite using a novel extremely thermophilic mixed culture. International Journal of Mineral Processing 66 (1–4), 107–119.

d'Hugues, P., Battaglia-Brunet, F., Clarens, M., Morin, D., 2003. Microbial diversity of various metal–sulphides bioleaching cultures grown under different operating conditions using 16S-rDNA analysis. International Biohydrometallurgy Symposium, Athens, Greece.

Dopson, M., Baker-Austin, C., Hind, A., Bowman, J.P., Bond, P.L., 2004. Characterization of *Ferroplasma* isolates and *Ferroplasma acidarmanus* sp. nov., extreme acidophiles from acid mine drainage and industrial bioleaching environments. Applied and Environmental Microbiology 70 (4), 2079–2088.

Edwards, K.J., Gihring, T.M., Banfield, J.F., 1999. Seasonal variations in microbial populations and environmental conditions in an extreme acid mine drainage environment. Applied and Environmental Microbiology 65 (8), 3627–3632.

Goebel, B.M., Stackebrandt, E., 1994. Cultural and phylogenetic analysis of mixed microbial populations found in natural and commercial bioleaching environments. Applied and Environmental Microbiology 60 (5), 1614–1621.

Golyshina, O.V., Pivovarova, T.A., Karavaiko, G.I., Kondrateva, T.F., Moore, E.R., Abraham, W.R., Lunsdorf, H., Timmis, K.N., Yakimov, M.M., Golyshin, P.N., 2000. *Ferroplasma acidiphilum* gen. nov., sp. nov., an acidophilic, autotrophic, ferrous-iron-oxidizing, cell-wall-lacking, mesophilic member of the Ferroplasmaceae fam. nov., comprising a distinct lineage of the *Archaea*. International Journal of Systematic and Evolutionary Microbiology 50 (Pt 3), 997–1006.

González-Toril, E., Llobet-Brossa, E., Casamayor, E.O., Amann, R., Amils, R., 2003. Microbial ecology of an extreme acidic environment, the Tinto River. Applied and Environmental Microbiology 69 (11), 4853–4865.

Johnson, D.B., Okibe, N., Roberto, F.F., 2003. Novel thermo-acidophilic bacteria isolated from geothermal sites in Yellowstone National Park: physiological and phylogenetic characteristics. Archives of Microbiology 180 (1), 60–68.

Karavaiko, G.I., Turova, T.P., Kondrat'eva, T.F., Lysenko, A.M., Kolganova, T.V., Ageeva, S.N., Muntyan, L.N., Pivovarova, T.A., 2003. Phylogenetic heterogeneity of the species *Acidithiobacillus ferrooxidans*. International Journal of Systematic and Evolutionary Microbiology 53 (Pt 1), 113–119.

Kinnunen, P.H., Puhakka, J.A., 2004. High-rate ferric sulfate generation by a *Leptospirillum ferriphilum*-dominated biofilm and the role of jarosite in biomass retention in a fluidized-bed reactor. Biotechnology and Bioengineering 85 (7), 697–705.

Muhammad, A.G., Khalid, A.M., Grant, S., Heaphy, S., Grant, W.D., 2003. Phylogenetic analysis of different isolates of *Sulfobacillus* spp. isolated from uranium-rich environments and recovery of genes using integron-specific primers. Extremophiles 7 (5), 341–345.

Muyzer, G., Brinkhoff, T., Nübel, U., Santegoeds, C., Schäfer, H., Wawer, C., 1996. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. In: Akkermans, A., van Elsas, J., de Bruijn, F. (Eds.), Molecular Microbial Ecology Manual. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 1–27.

Norris, P., Barr, D., Hinson, D., 1987. Iron and mineral oxidation by acidophilic bacteria: affinities for iron and attachment to pyrite. In: Norris, P.R., Kelly, D.P. (Eds.), International Symposium Warwick.

Okibe, N., Johnson, D.B., 2004. Biooxidation of pyrite by defined mixed cultures of moderately thermophilic acidophiles in pH-controlled bioreactors: significance of microbial interactions. Biotechnology and Bioengineering 87 (5), 574–583.

Okibe, N., Gericke, M., Hallberg, K.B., Johnson, D.B., 2003. Enumeration and characterization of acidophilic microorganisms isolated from a pilot plant stirred-tank bioleaching operation. Applied and Environmental Microbiology 69 (4), 1936–1943.

Olson, G.J., Brierley, J.A., Brierley, C.L., 2003. Bioleaching review: Part B. Progress in bioleaching: applications of microbial processes by the minerals industries. Applied Microbiology and Biotechnology 63 (3), 249–257.

Rawlings, D., 2001. The molecular genetics of *Thiobacillus ferrooxidans* and other mesopholic, acidophilic, chemolithotrophic, iron- or sulfur-oxidizing bacteria. Hydrometallurgy 59, 187–201.

Rawlings, D.E., 2005. Characteristics and adaptability of iron- and sulfur-oxidizing microorganisms used for the recovery of metals from minerals and their concentrates. Microbial Cell Factories 4 (1), 13.

Rawlings, D.E., Tributsch, H., Hansford, G.S., 1999. Reasons why '*Leptospirillum*'-like species rather than *Thiobacillus ferrooxidans* are the dominant iron-oxidizing bacteria in many commercial processes for the biooxidation of pyrite and related ores. Microbiology 145 (Pt 1), 5–13.

Rawlings, D.E., Dew, D., du Plessis, C., 2003. Biomineralization of metal-containing ores and concentrates. Trends in Biotechnology 21 (1), 38–44.

Scherpenzeel, D., Boon, M., Ras, C., Hansford, G., Heijnen, J., 1998. Kinetics of ferrous iron oxidation by *Leptospirillum* bacteria in continuous cultures. Biotechnology Progress 14, 425–433.

Tyson, G.W., Chapman, J., Hugenholtz, P., Allen, E.E., Ram, R.J., Richardson, P.M., Solovyev, V.V., Rubin, E.M., Rokhsar, D.S., Banfield, J.F., 2004. Community structure and metabolism through reconstruction of microbial genomes from the environment. Nature 428 (6978), 37–43.

Vásquez, M., Moore, E.R.B., Espejo, R.T., 1999. Detection by polymerase chain reaction amplification and sequencing of an archaeon in a commercial-scale copper bioleaching plant. FEMS Microbiology Letters 173 (1), 183–187.

Zillig, W., Stetter, K.O., Wunderl, S., Schulz, W., Priess, H., Scholz, I., 1980. The *Sulfolobus*–"Caldariella" group: taxonomy on the basis of the structure of DNA-dependent RNA polymerases. Archives of Microbiology 125, 259–269.