Identification of differentially expressed genes in an industrial bioleaching heap processing low-grade copper sulphide ore elucidated by RNA arbitrarily primed polymerase chain reaction

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Keywords:
Bioleaching
Industrial heap
Transcriptome
RNA arbitrarily primed PCR
Real-time PCR

A B S T R A C T

During the past few decades the microbial bioleaching of metallic sulphide ores has become a very important process utilized by the mining industry. The biodiversity studies of industrial systems have revealed the occurrence of acidophilic microorganisms, forming dynamic communities that include bacteria and archaea. A combination of molecular and cultivation-based approaches has been used during the leaching cycle to understand the key microorganisms present and their function in the Escondida Sulphide Leach Plant. The global genomic expression in solution samples has been analyzed by random arbitrary primed polymerase chain reaction (RAP-PCR), to pinpoint those microorganisms with active metabolism in the communities present in regions of the heap leach operation of different ages.

Four random primers were used and one hundred and twenty RAP-PCR products were cloned, sequenced and compared to databases using the BLAST search algorithm. Approximately 48% of the RAP-PCR products were 16S and 23S rRNA genes, and many of these results are consistent with community analysis carried out on the same heap using 16S rRNA gene libraries and real-time PCR. Interestingly, different microorganisms to those previously detected by 16S rRNA PCR-DGGE, represented by sequences distantly related to the archaea *Picrophilus torridus*, and *Thermoplasma acidophilum* sequences, were detected using RNA analysis. Eight different RAP-PCR products shared similarity with functional genes, and sequence analysis revealed the expression of genes associated with transcription (RNA polymerase) and translation (elongation factor and ribosomal protein) components, metabolic function (2-methylisocitrate synthase and pyruvate dehydrogenase), and electron transport (cytochrome). Other genes such as mercuric reductase and ABC transporter ATP-binding proteins were also detected. However, 30% of RAP-PCR products showed no similarity with any sequences currently in the database.

This report represents the first transcriptomic study of the microbial community inhabiting an industrial bioleaching system. The data suggest that the functional spectrum of expressed genes was consistent with genomic and proteomic reports from pure cultures and environmental AMD samples containing microorganisms involved in bioleaching processes. Both studies revealed the expression of genes mainly related to energetic metabolisms and stress responses.

1. Introduction

Heap bioleaching is presently the most successful technology for the extraction of base metals from low-grade sulphide ores. During the last few decades heap bioleaching has become increasingly important due to the depletion of high-grade copper ores and the existence of huge natural reserves of copper in the form of secondary copper sulphides. Several improvements, including heap aeration systems and heap design, have been made to the process since the first full-scale heap bioleaching plant was established in Lo Aguirre mine in Chile in 1980 (Bustos el al. 1993; Watling, 2006).

While design and engineering aspects are thoroughly considered in biomining operations, issues relating to microbiological metabolisms

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have been subjected to a less rigorous scrutiny (Rawlings and Johnson, 2007). An understanding of the microbiological processes in industrial heaps is necessary to commercially improve bioheap technologies (Brierley, 2001; Rawlings, 2002). Rapid culture-independent techniques to detect and quantify PCR-targeted populations (DGGE and real-time PCR) have been applied to describe the dynamics of microbial communities in low-grade copper sulphide heaps on both test- and industrial-scale (Demergasso et al., 2005; Remonsellez et al., 2007). Observed changes in the microbial populations could relate to changes in concentration of the main substrates such as lower levels of ferrous iron, or increased levels of inhibitory ions such as sulphate (upper 50 g/L), as bioleaching progressed (Demergasso et al., 2005). On the other hand, the dynamics of communities depends on the operation time of the process (Remonsellez et al., 2007), although the reasons for the predominance of certain members of the population at specific stages of the bioleaching heap process are still unknown.

Improved knowledge of key capacities of microorganisms, such as iron and/or sulphur oxidation, energy production and conversion, microorganism–mineral interactions and their adaptive response to stress conditions would enable greater improvement of the control and therefore the success of microbial leaching operations.

The uses of genomics, metagenomics and high throughput proteomics to study the global regulatory response of biomining communities represent recent approaches to understand the role and adaptation of these microorganisms in the bioleaching environment (Valenzuela et al., 2006). Gene expression by RNA arbitrarily primed polymerase chain reaction (RAP-PCR) in response to copper, and genome-wide microarrays transcript profiling analysis to elucidate the enzymatic pathways and the electron transfer chains involved in the oxidation of ferrous iron and reduced sulphur compounds have been carried out in *Acidithiobacillus ferrooxidans*, the most well-studied microorganism involved in bioleaching processes (Paulino et al., 2002; Quatrini et al., 2006).

Some proteomics approaches have been performed to analyze proteins that change their expression when *A. ferrooxidans* was grown on different energy sources (Bouchal et al., 2006; Chi et al., 2007). Also, several proteomics analysis have been performed to study electron transport for iron oxidation and resistance to heavy metals in the acidophilic archaeon isolated from acid mine drainage (AMD) *Ferroplasma acidarmanus* Fer1 (Baker-Austin et al., 2005; Dopson et al., 2005; Baker-Austin et al., 2007).

By analyzing the expression of specific genes, it is possible to understand the roles and metabolic functions of microbial communities involved in industrial leaching processes. The application of gene expression studies to industrial samples represents a novel approach, with the potential to improve bioleaching technologies.

Only a few studies have examined the natural communities inhabiting AMD environments such as transcriptomic analysis using DNA microarrays (Parro et al., 2007) and the use of proteomic methods (Ram et al., 2005). These investigations allow the evaluation of gene expression, identification of key biological activities, examination of metabolic functions, and the evaluation of the physiological response of a single bacterial species or consortium when nutrient limitation or other stresses were imposed (Ram et al., 2005; Parro et al., 2007).

The main aim of this study is to detect potential target genes of the microorganisms involved in bioleaching industrial processes and to gain further insights into these processes from a metabolic standpoint. Results are reported of the examination of gene expression as a bioleaching heap aged, and its correlation with other techniques used to evaluate the microbial communities from industrial leaching samples.

2. Materials and methods

2.1. Heap and samples

The Escondida Mine is located 170 km South-East of Antofagasta. The heap was built 2 years ago with run-of-mine (ROM) ore and air was supplied through blowers. The ore was characterized as low-grade sulphide material averaging 0.60% total Cu consisting of chalcocite (40%), covellite (10%) and chalcopyrite (50%). The heap was designed to have one raffinate irrigation solution directed from the solvent-extraction plant that feeds 10 ore strips at a steady-state. Each strip generates its own pregnant leaching solution (PLS) and the start of irrigation of each one was staggered by approximately one month, beginning with strip 1. Samples of PLS from each ore strip and a single raffinate sample were collected in June 2006; and in May, June, and July 2007. Liquid samples were used to study the gene expression in this industrial process as it was not possible to obtain mineral samples. To sample the mineral from a run-of-mine heap implies the drilling of a borehole with the costs and logistical problems involved (for example, the removal of the irrigation line). For the same reason, it was not possible to obtain samples at different heap stages to study the community dynamic during the leaching cycle.

2.2. Oxidation tests in PLS samples

Iron oxidation activity in PLS samples was tested in shake flasks. PLS samples (250 mL) were supplemented with ferrous iron to a final concentration of 4 g/L and incubated between 20 and 25 °C. Prior to ferrous iron supplementation, the ferrous iron contained in the PLS was quantified and shown to be between 0.1 and 0.6 g/L. The oxidation of ferrous iron was analyzed daily by titration using 0.05 N sodium dichromate in a final volume of 15 mL of solution containing 5 mL of sample, 10 mL of acid mixture (15% of H_2SO_4 and 15% of H_3PO_4) and 5 drops of 0.1% diphenylamine (indicator). The time required to completely oxidize the ferrous iron was used as an indicator of ferrous iron oxidation activity. Identical duplicate abiotic control flasks were also supplemented with ferrous iron as described above, but the PLS solutions were filter-sterilized (0.2°μm diameter pore size filters, Whatman®).

2.3. RNA and DNA extraction

To extract RNA, cells were collected by filtering 4–5 L of PLS through a 0.2 μm pore size membrane (Whatman®) and samples were stored at −80 °C. To preserve the RNA during the extraction procedure, 1 mL of RNA protect solution (QIAGEN) was added to each filter. RNA was purified using RNeasy kit (QIAGEN) from the filters. The RNA samples were treated with DNAse RQ-1 (Promega) for 1 h at 37 °C and then stored at −80 °C. The quality of total extracted RNA was confirmed by 1% agarose gel electrophoresis and then quantified using a spectrophotometer model MBA 2000 (PerkinElmer).

DNA was extracted from cells collected by filtering 1 L of PLS through a 0.2 μm pore size membrane (Whatman®) as described previously (Demergasso et al., 2005).

2.4. RAP-PCR

RAP-PCR experiments were performed, with some modifications, following previously described methods (Paulino et al., 2002). The arbitrary primers (Operon Technologies, Alabama, CA, USA) tested in these experiments were: OPJ-12, OPJ-5, OPJ-9, and OPJ-10. The first-strand cDNA was synthesized using the Sensiscript RT kit (QIAGEN), according to the manufacturer's instructions. Reaction mixtures were incubated at 37 °C for 30 min. For synthesis of the second-strand, 2 μL of the first-strand reaction was mixed with 48 μL reaction mixture containing 1× PCR buffer, 2 mM of $MgCl_2$, 0.2 μM of the same arbitrary primer used in the first-strand synthesis, 20 μM of dNTP mix without dCTP, 1 μCi of [α-^{32}P]dCTP and 1.25 U *Taq* DNA polymerase (Invitrogen). Amplification conditions consisted of an initial denaturation at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 40 °C for 2 min, and 72 °C for 1 min. Five μL from each reaction was mixed with

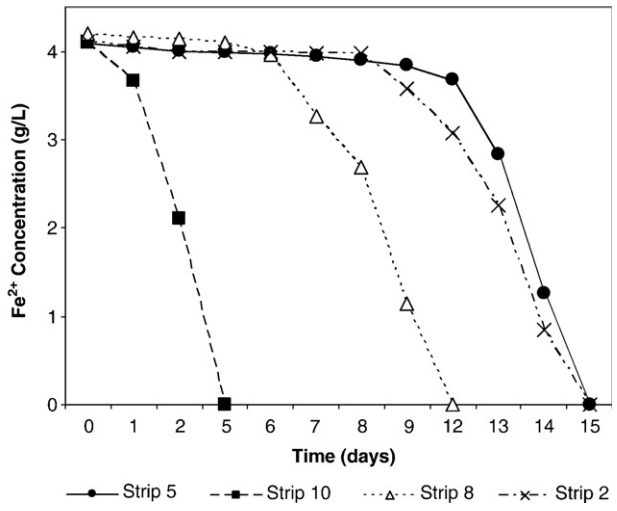

Fig. 1. Ferrous iron oxidation tests of PLS from industrial heap strips analyzed in July of 2007. Strips had been operated for different lengths of time, strip 10 being the youngest analyzed.

5 μL of a solution containing 59.99% deionized formamide, 40% glycerol and 0.01% bromophenol blue, and samples were loaded on a 5% polyacrylamide gel containing 42% urea. Gels were run for approximately 6 h at 1320 V. After electrophoresis, the gels were transferred to 3 mm paper (Whatman®), dried under vacuum, exposed to super resolution type SR film (PerkinElmer) and analyzed in a Cyclone® Plus Storage Phosphor System (PerkinElmer).

2.5. Isolation and reamplification of RAP-PCR products

The differentially expressed RAP-PCR bands were excised from the gel and the DNA was eluted by incubation at room temperature in 20 μL of sterilized nuclease-free water. Two μL of the eluted DNA was added to 48 μL of reaction mixture containing 1× PCR buffer, 2 mM $MgCl_2$, 0.2 μM of the same arbitrary primer used in the cDNA synthesis, 40 μM of dNTP mix and 1 U *Taq* DNA polymerase (Invitrogen) as final concentrations. The amplification was performed as described in Section 2.4.

2.6. Sequence analysis

The identity and similarity of differentially expressed bands were obtained by analyzing the sequences using the BLAST algorithm (Altschul et al., 1997) at the National Center for Biotechnology Information (NCBI, http://ncbi.nlm.nih.gov), both BLASTn and BLASTx functions were used. The genome sequence of *F. acidarmanus* Fer1 (http://genome.jpi-psf.org/draft.microbes/ferac/ferac.home.html) was used to analyze the genomic context of contigs (149, 155, 168) containing the identified genes using the ORF finder program (http://ncbi.nlm.nih.gov/projects/gorf).

2.7. Quantitative real-time PCR

The synthesis of cDNA from RNA samples was performed using the Sensiscript RT kit (QIAGEN), according to the manufacturer's instructions, using 10 pmol of specific primers to *A. ferrooxidans* and *Ferroplasma acidiphilum* 16S rRNA genes. Reaction mixtures were incubated at 37 °C for 30 min. A Rotor-GeneTM 6200 (Corbett Research Pty Ltd.) q-PCR machine and specific Quantimix Easy SYG Kit (Biotools) were used. The reaction mixture contained 10 μL of SYBR® Green PCR Master Mix (Biotools), 1 μL of cDNA, 1 μL of the corresponding oligonucleotide primers, and H_2O added to a total of 20 μL. The amplification program consisted of 1 cycle of 95 °C for 10 min, and then 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 20 s. The standard curves for 16S rRNA genes of *A. ferrooxidans* and *F. acidiphilum* were performed as reported by Remonsellez et al. (2007). Copy numbers of 16S rRNA genes of *A. ferrooxidans* and *F. acidiphilum* were also determined by real-time PCR from DNA extracted from PLS samples as described previously (Remonsellez et al., 2007).

3. Results and discussion

3.1. Microbial iron oxidation activity

It should be emphasized that the operation of the plant was started with strip 1 (oldest) and the heap was enlarged on a monthly basis by the sequential addition of further strips. In this study strip 10 represents the youngest portion of the heap studied and the microbial ferrous iron oxidation activity in PLS solutions was shown to decrease as the heap aged. Ferrous iron consumption tests for PLS samples obtained in July of 2007 are shown as an example (Fig. 1). In addition, the variation in the overall oxidation time is mainly due to the lag phase before oxidation begins. The reason for this is currently being analyzed considering total and active bacterial number and community composition.

Microbial diversity analysis performed by real-time PCR (Remonsellez et al., 2007; manuscript in preparation) indicates that *A. ferrooxidans* strains dominated (between 10^6 and 10^7 cells/mL) in the younger strips (8 and 10) with the shortest delay in the onset of ferrous iron oxidation (Table 1). Meanwhile, in the older strips (2 and 5) the occurrence of *A. ferrooxidans* strains decreased (between 10^4 and 10^3 cells/mL) and microorganisms such as *Leptospirillum* species (data not shown) and the archaeon *F. acidophilum* (Table 1) became predominant showing similar tendency described before for those populations (Rawlings et al. 1999).

The 16S rRNA gene transcription levels of *A. ferrooxidans* and *F. acidiphilum* were determined as indicative of physiological activity (Parro et al., 2007). The results suggested that *A. ferrooxidans* is more metabolically active in strips with a shorter operation time (8 and 10), and that *F. acidophilum* would be more active in the strips with a longer operation time (Table 1). *Ferroplasma* species could be more active in the older strips due to increased organic carbon from dead cells (Rawlings and Johnson, 2007). In conclusion, *A. ferrooxidans* 16S rRNA gene copy numbers and 16S rRNA gene transcript levels are higher in samples with higher iron oxidation activity.

Nevertheless, neither 16S rRNA gene copy number nor 16S rRNA gene expression level alone explains the increasing lag phase in ferrous iron oxidation activity in strips with a longer operation time. One possibility is that *A. ferrooxidans* metabolism changes as the heap ages. Several samples were selected to perform the RAP-PCR analysis to pinpoint different metabolic stages in the microbial communities of strips with different operation times. This technique offers a powerful tool for studying gene expression in the environment, independent of any previous sequence knowledge and without requiring microbial culturing.

Table 1
Copy numbers and transcript levels for 16S rRNA genes from *A. ferrooxidans* and *F. acidiphilum* by real-time PCR in PLS samples of the industrial heap (July 2007)

Strip	*A. ferrooxidans* 16S rRNA gene		*F. acidiphilum* 16S rRNA gene	
	Copy number (copies/mL)	Transcript levels (copies/mL)	Copy number (copies/mL)	Transcript levels (copies/mL)
2	2.6×10^3	2.2×10^4	2.0×10^4	N.D.
5	2.5×10^4	6.7×10^4	2.2×10^5	2.1×10^4
8	1.2×10^6	3.6×10^6	7.7×10^3	4.2×10^3
10	5.4×10^6	5.2×10^7	4.1×10^2	4.2×10^3

N.D. = Analysis undetermined.

3.2. RAP-PCR products cloning and sequencing

Despite the difficulty in obtaining RNA from industrial samples, a large number of bands were identified from the RNA fingerprints, showing that this methodology can be successfully applied to this type of sample. Using the same technique a similar number of bands was previously identified in a pure culture of *A. ferrooxidans* in response to copper stress (Paulino et al., 2002). The length of the retrieved DNA sequences ranged from 100 to 2000 bp. One hundred and twenty RAP-PCR products were reamplified and sequenced. Fig. 2 shows some examples of RNA fingerprinting obtained from the industrial samples. The sequences were compared with sequences from GenBank using the BLAST algorithm (Altschul et al., 1997). The majority of sequences (48.3%) were related to 16S and 23S rRNA genes (Table 2). The sequences obtained from RAP-PCR analyses showed that active microorganisms in this industrial heap were representative of 16S rRNA bacterial sequences of *A. ferrooxidans*, *Acidithiobacillus thiooxidans*, *Leptospirillum ferrooxidans*, and *Leptospirillum ferriphilum*. Archaeal sequences were also detected including *F. acidiphilum*. Sequences of 23S rRNA genes were detected which were distantly related to *Picrophilus torridus* and *Thermoplasma acidophilum* showing similarities of 75% and 92%, respectively (Table 2). This could indicate that some microorganisms inhabiting the system are unknown and metabolically active. These sequences were found in several samples, indicating that they were widely distributed in the system (Table 2). Interestingly, these sequences were not detected by DNA approaches such as 16S rRNA gene clone libraries (Remonsellez et al., 2007). The low similarity of sequences related to 23S rRNA genes could indicate the occurrence of a different group in the system belonging to the archaeal domain or it could be due to the difficulty of identifying 23S rRNA sequences because of poor database sequence coverage, especially for less-well-studied phyla (Hunt et al., 2006).

Thirteen RAP-PCR products showed similarity with 18S rRNA eukaryotic sequences (data not shown). Eleven RAP-PCR products showed similarity with eight different functional genes present in the database (Table 3 and Section 3.3). The remaining 31% of RAP-PCR products showed no similarity with sequences currently in the database. This is consistent with other findings where a high percentage of coding sequences with no similarity to known proteins has been observed in bacterial genomes (Paulino et al., 2002). More recently, a proteomic analysis of 131 periplasmic proteins in *A. ferrooxidans* showed that 10% were proteins with unknown functions and that 26.1% were proteins without homologues in databases (Chi

Table 2
Similarity of non-functional sequences retrieved from RAP-PCR analysis determined by the BLAST homology program

Primer	Related sequence	Gen	Similarity %	Sample	Date
OPJ-12	*A. ferrooxidans* ATCC 19859	16S	96	Strip 3, 6 and 8	May 2007
OPJ-12	*P. torridus* DSM 9790	23S	92	Strip 8	May 2007
OPJ-9	*T. acidophilum*	23S	85	Strip 6	May 2007
OPJ-9	*P. torridus* DSM 9790	23S	84	Strip 3, 6 and 8	May 2007
OPJ-10	*A. ferrooxidans* ATCC 19859	16S	96	Strip 3, 6 and 8	May 2007
OPJ-20	*P. torridus* DSM 9790	23S	88	Strip 6	May 2007
OPJ-12	*P. torridus* DSM 9790	23S	75	Strip 3	June 2007
OPJ-5	*A. ferrooxidans* ATCC 19859	16S	97	Feed	June 2007
OPJ-5	*L. ferrooxidans* Chil-Lf2	16S	99	Feed	June 2007
OPJ-5	*F. acidiphilum*	16S	99	Strip 3	June 2007
OPJ-5	*P. torridus* DSM 9790	23S	89	Feed	June 2007
OPJ-10	*A. ferrooxidans* ATCC 19859	16S	96	Strip 3 and 6	June 2007
OPJ-10	*A. thiooxidans*	16S	98	Strip 6	June 2007
OPJ-10	*L. ferriphilum*	16S	97	Strip 3	June 2007

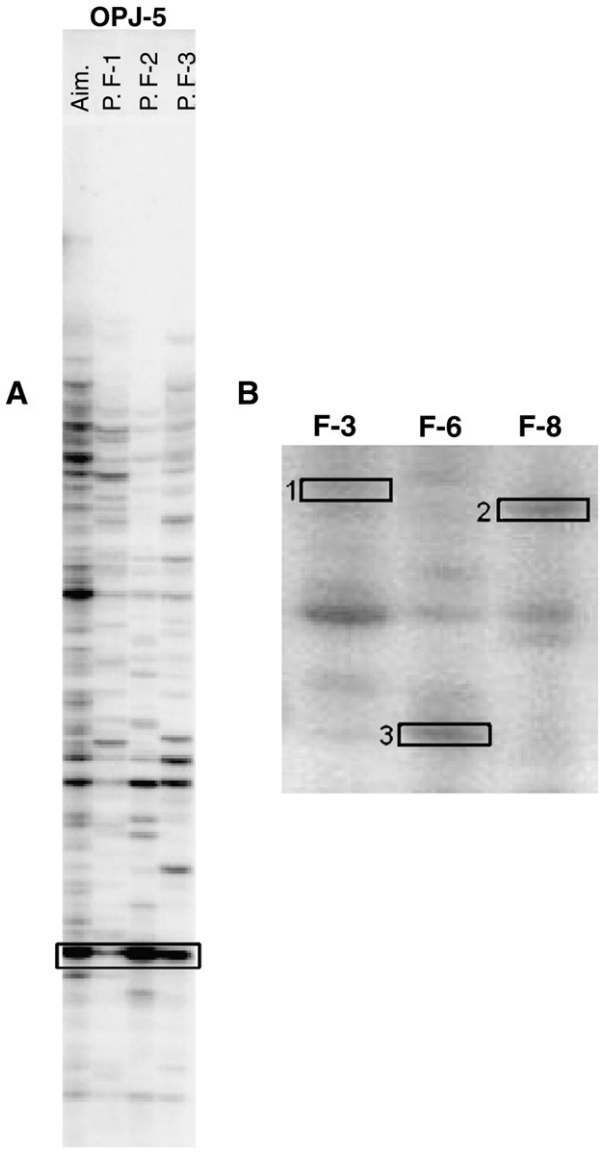

Fig. 2. Examples of polyacrylamide gel electrophoresis of RAP-PCR obtained for industrial heap samples. A) Feed solution (Alim), PLS solutions from strips 1 (P.F-1), 2 (P.F-2) and 3 (P.F-3) analyzed by RAP-PCR (June 2006), the box indicates the band with similar sequence identity to *pdhA-1* gene. B) PLS solutions from strip 3 (F-3), 6 (F-6) and 8 (F-8) analyzed by RAP-PCR (May 2007), boxes 1, 2 and 3 indicate bands with similar sequence identity to *cydA*, RNA polymerase and elongation factor EF-1 genes, respectively.

Table 3
Similarity of functional sequences retrieved from RAP-PCR analysis determined by the BLAST homology program

Primer	Related sequence	Similarity %	Sample, date
OPJ-5	Pyruvate dehydrogenase (*pdhA*), AF362022, *A. ferrooxidans*	86	Strip 1, 2 and 3, June 2006
OPJ-10	Microbial RNA polymerase II, *Thermoplasma* Genome	91	Strip 8, May 2007
OPJ-10	ABC transporter ATP-binding protein, Faci_03000178, *F. acidarmanus*	100	Strip 3, May 2007
OPJ-10	30S ribosomal protein S4, Faci_03001248, *F. acidarmanus*	100	Strip 3, May 2007
OPJ-10	2-methylisocitrate synthase, Faci_03000212, *F. acidarmanus*	100	Strip 3, May 2007
OPJ-10	Mercuric reductase *MerA*, Faci_03001596, *F. acidarmanus*	97	Strip 6, May 2007
OPJ-10	Cytochrome bd quinol oxidase subunit I, Faci_3001025, *F. acidarmanus*	97	Strip 3 May 2007
OPJ-10	Eukaryotic elongation factor EF-1-alpha C	92	Strip 6 and 8, May 2007

et al., 2007). Therefore, the RAP-PCR sequences found in this study, with no known similarity to genes currently available in the database, may play important roles which have not yet been defined.

3.3. Identification of functional sequences

Of the eight different RAP-PCR products that showed similarity with functional genes, two were related to microbial RNA polymerase II and elongation factor EF-1-alpha C (Table 3). In natural communities present in AMD, where *Acidithiobacillus* genus are the dominant species, elongation factor G (EF-G) and Tu (EF-Tu) involved in translation and protein folding were induced under stress conditions (Parro et al., 2007). In the archaeon *F. acidarmanus*, a translation elongation factor EF-1-alpha was up-regulated when the microorganism was grown under chemomixotrophic conditions, and several translation initiation/elongation factors were up-regulated when the microorganism was grown under chemoorganotrophic conditions (Dopson et al., 2005). Also, the translation elongation factor EF-1-alpha of *F. acidiphilum* Y^T was up-regulated when it was grown in the absence of organic carbon (Dopson et al., 2005). Interestingly, we identified one 30S ribosomal protein S4 (Faci_03001248) in strip 3 (Table 3), a strip which showed a relatively low ferrous iron oxidation capacity. In *F. acidarmanus* and *F. acidiphilum* Y^T, several 30S and 50S ribosomal proteins were up-regulated under chemomixotrophic/-chemoorganotrophic conditions (Dopson et al., 2005). Ribosomal proteins were also up-regulated when *F. acidarmanus* was grown in the presence of copper and arsenic (Baker-Austin et al., 2005, 2007). Therefore, the expression of transcription and translation mechanisms could indicate critical conditions during the bioleaching process, such as a scarcity of organic carbon and/or the presence of heavy metals. It is likely, therefore, that certain unfavorable conditions during the bioleaching process could be sensed by *Ferroplasma* species and this could be used as an indicator within the leaching process.

Two sequences with metabolic function, 2-methylisocitrate synthase (*prpD*, Faci_03000212) and pyruvate dehydrogenase (*pdhA*, AF362022), were identified (Table 3). The *prpD* gene forms part of an operon encoding propionate-degrading enzymes that has been described in Gram-negative and Gram-positive bacteria, and some thermophilic archaea (Gerike et al., 1998; Hammelman et al., 1996). Other putative genes encoding enzymes involved in the catabolism of propionate, such as the methylisocitrate lyase (*prpB*, Faci_03000211) and coenzyme methyltransferase (*CobJ*, Faci_03000210), were found in the contig 149 of *F. acidarmanus* Fer1 genome (Fig. 3A). The cluster of genes required for the catabolism of propionate has been investigated previously (Gerike et al., 1998; Hammelman et al., 1996) and one transcriptional unit contains the *prpBCDE* gene cluster encoding propionate-degrading enzymes. Regulation of the expression of the *prpBCDE* operon is complex and requires the synthesis of 2-methylcitrate to signal the presence of propionate in the environment, thereby allowing it to be used as the carbon and energy source (Tsang et al., 1998).

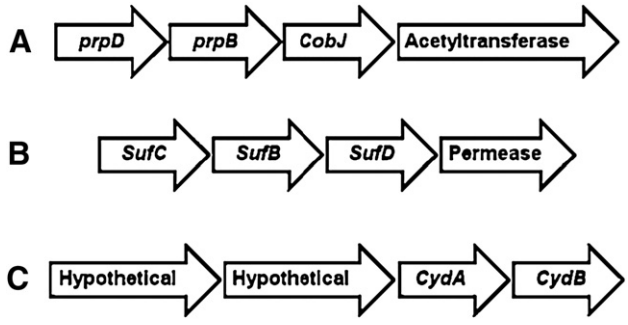

Fig. 3. Proposed genetic organization of putative functional genes of *F. acidarmanus* Fer1 found by RAP-PCR. Arrows indicate proposed transcription of some operons from contigs 149 (A), 155 (B) and 168 (C) of *F. acidarmanus* Fer1 genome (not annotated). The genes *prpD* (Faci_03000212), *SufB* (Faci_03000178) and *cydA* (Faci_3001025) were identified in this study.

The second gene identified encodes for the β-subunit of the pyruvate dehydrogenase (*pdh*) in *A. ferrooxidans*, which catalyzes the conversion of pyruvate to acetylCoA (Table 3). The pyruvate dehydrogenase has been detected and characterized in *A. ferrooxidans* and shares similar features with other *pdh* genes isolated from eukaryotes and Gram-positive microorganisms (Powles and Rawlings, 1997). We determined that *A. ferrooxidans* possesses two copies of this gene in different genomic contexts, corresponding to AFE_0040 and AFE_1272 (according to the TIGR annotation) and they were designated as *pdhA-1* and *pdhA-2*, respectively. Interestingly, the identification of protein mixtures in seven protein spots using proteomic analysis showed an up-regulation of pyruvate dehydrogenase when cells of *A. ferrooxidans* were grown on ferrous iron (Bouchal et al., 2006). In the archaeon *F. acidarmanus* Fer1 a pyruvate 2-oxoglutarate dehydrogenase was up-regulated when cells were grown under chemoorganotrophic conditions (Dopson et al., 2005) and in the presence of high concentration of arsenic (Baker-Austin et al., 2007). In addition, most of the enzymes of the tricarboxilic acid cycle and a putative NAD-dependent isocitrate dehydrogenase were expressed in an AMD environment with extreme iron, sulphate and dissolved oxygen concentrations (Parro et al., 2007). It is probable that the increased expression of genes encoding metabolic functions may reflect an increased bioenergetic demand induced by stress conditions such as the presence of heavy metals or low ferrous iron concentration.

One putative mercuric reductase (*MerA*, Faci_03001596) was also identified (Table 3). The properties of mercuric reductase, a flavoenzyme that reduces Hg^{2+} to less toxic Hg^0 using NADPH as an electron donor, have been widely studied in a range of Gram-negative and Gram-positive bacteria, but the mechanisms responsible for mercury resistance in the archaeal domain remain unknown (Silver and Pheng, 1996). Mercuric reductase activity has also been found in *A. ferrooxidans*, and mercury volatilization by some mercury-resistant strains was reported to be activated in the presence of ferrous iron (Iwahori et al., 2000). It is likely that the mercuric reductase could have been involved in oxidative stress response, as several reductase enzymes that were induced when the acidophilic archaeon *F. acidarmanus* was grown in the presence of heavy metals (Baker-Austin et al., 2005, 2007). Potential roles in protection against oxidative stress have also been suggested for reductases in Gram-positive and Gram-negative bacteria and haloarchaeons (Sitthisak et al., 2005; Rodríguez-Montelongo et al., 2006; Kaur et al., 2006).

One ABC transporter ATP-binding protein (Faci_03000178) was identified (Table 3), and it was determined to be an open reading frame encoding for a Fe–S assembly protein, *SufB*. The biosynthesis of iron–sulphur clusters (Fe–S) depends on a multiprotein system, activated under stressful conditions (Loiseau et al., 2003). The SUF system is formed by six proteins: SufC, an atypical cytoplasmatic ABC-ATPase, which forms a complex with *SufB* and SufD; SufA, which acts as a scaffold protein for assembly of iron–sulphur clusters and delivery to target proteins; SufS, a cysteine desulphurase which mobilizes the sulphur atom from cysteine and provides it to the cluster; and SufE which currently has no associated function (Loiseau et al., 2003; Outten et al., 2003). The components of the system are organized in the operon *sufABCDSE* in the Gram-negative bacterium *Escherichia coli*, and the operon is induced by oxidative stress and iron deprivation (Outten et al., 2003). The main proteins of the SUF complex were found in the contig 155 of the *F. acidarmanus* Fer1 genome, and the putative operon is composed of *sufC* (Faci_03000179), *sufB* (Faci_03000178) and *sufD* (Faci_03000177) genes (Fig. 3B). Some authors have proposed that this sulphur transfer mechanism may be important for limiting sulphide release during oxidative stress

conditions (Outten et al., 2003). It is proposed therefore that this could be a candidate gene to be monitored in bioleaching processes, indicating stress conditions during the leaching cycle.

Finally, a single cytochrome bd quinol oxidase subunit I (*cydA*, Faci_3001025) was identified (Table 3). In *Ferroplasma* species, electron transport proteins were reported to be up-regulated when grown under chemomixotrophic/chemoorganotrophic conditions (Dopson et al., 2005). Interestingly, the putative operon *cydAB*, composed of cytochrome bd quinol oxidase subunit I (*cydA*, Faci_3001025) and cytochrome bd quinol oxidase subunit II (*cydB*, Faci_3001026) was identified in the contig 168 of the *F. acidarmanus* Fer1 genome (Fig. 3C). Several studies carried out on *A. ferrooxidans* indicate that energy source and stress conditions regulate the expression of cytochromes (Paulino et al., 2002; Quatrini et al., 2006; Bouchal et al., 2006). In *A. ferrooxidans* the *cydAB* operon encoding bd ubiquinol oxidase is composed of *cydA* (subunit I) and *cydB* (subunit II) genes and an increased expression was seen when cells were grown on S^0 than when grown on ferrous iron. Other cytochromes such as the operon *cyoBACD* encoding bo_3 ubiquinol oxidase were induced in the same conditions (Quatrini et al., 2006). However, two proteins involved in electron transport, rusticyanin and cytochrome *c-552*, have been reported to be up-regulated in iron oxidizing cells of *A. ferrooxidans* (Bouchal et al., 2006). A gene with high similarity to cytochrome *c* was also identified when cells of *A. ferrooxidans* were exposed to copper and it has been suggested that cytochrome genes may play an important role in responding to environmental stimuli (Paulino et al., 2002). It is suggested, therefore that genes encoding for electron transport proteins could represent good candidates to monitor oxidation activity and stress conditions in bioleaching processes.

3.4. Differential expression of functional genes in industrial leaching system

To gain insights into the changes in the metabolism of the microbial communities during the leaching cycle, the meta-transcriptome fingerprints of PLS samples with different iron oxidation activity were studied.

Even though differential gene expression of RAP-PCR products was observed in polyacrylamide gels, further analysis will be required to quantify them. However, these initial results appear promising and suggest some hypothesis.

Functional genes found in strips with shorter operation times corresponded to functional categories of housekeeping genes such as RNA polymerase and elongation factor EF-1. The RNA polymerase gene was identified in strip 8 (Fig. 2A) and the elongation factor was identified in strip 6 (Fig. 2A) and 8 (data not shown). High relative expression levels of 16S rRNA, translation elongation factor, RNA polymerase and ribosomal proteins genes could be indicative of high physiological activity (Parro et al., 2007). On the contrary, functional genes found in strips with longer operation times corresponded to several functional categories: protein synthesis, stress response, carbohydrate metabolism and energy production and conversion (Table 3). It is suggested that genes encoding 30S ribosomal proteins, mercuric reductase and proteins involved in the biosynthesis of iron–sulphur clusters (Fe–S) are expressed as a consequence of the overall stress environment in the aging strips (Table 3). The expression of these proteins has been associated to stress conditions such as oxidative stress and the presence of high concentrations of heavy metals in *E. coli* and the archaeon *F. acidarmanus* (Outten et al., 2003; Baker-Austin et al., 2005, 2007). Despite the unfavorable conditions in older strips, it was demonstrated that other microorganisms such as the archaeon *F. acidiphilum* showed higher metabolic activity in these strips (Table 1) compared with the younger ones. Therefore, the induction of genes involved in energy production and conversion is necessary to maintain the high energy demand in the bioleaching process. The expression of electron transport proteins has been demonstrated to depend on the energy source available in *Ferroplasma* species (Dopson et al., 2005). A putative *CydA* related sequence of *F. acidophilum* was identified from strip 3 but not from the younger strips (Fig. 2B). It could suggest that the putative cytochrome of *F. acidophilum* play an important role in the response to environmental conditions such as changes in energy source and oxidative stress that are present in the older strips.

A gene related to key metabolic pathways was identified in strips 1, 2 and 3 with 92, 70 and 32 operation days, respectively (Fig. 2A and Table 3). A proteomic study demonstrated the up-regulation of pyruvate dehydrogenase when cells of *A. ferrooxidans* were grown on ferrous iron (Bouchal et al., 2006).

Specific primers targeting *pdhA-1* gene were designed to quantify its level in cDNA samples obtained from PLS during the leaching cycle, and preliminary results by real-time PCR have shown different gene expression of the *pdhA*-1 gene of *A. ferrooxidans* in the aged and new strips (data not shown). 16S rRNA gene transcript levels of *A. ferrooxidans* were used for normalizing as described previously (Yarzabal et al., 2004; Quatrini et al., 2006). The increased expression of genes encoding metabolic functions may reflect an increased bioenergetic demand induced by the low energy source.

Currently other functional genes detected by RAP-PCR in PLS samples from this industrial heap are being analyzed by real-time PCR to confirm the differential expression observed in the polyacrylamide gels and their potential as markers in the process. Constitutive genes in the bioleaching microbial community are also being investigated to normalize the analysis of differential gene expression.

4. Conclusions

This report represents the first study to analyze the global gene expression in an industrial bioleaching process and to elucidate the roles and key metabolic functions of the active microbial community. In addition, the current study highlights the usefulness of the RNA arbitrary primer PCR techniques for preliminary characterization of the differential gene expression in industrial bioleaching samples, from which it is very difficult to obtain optimal concentrations and good RNA quality for subsequent molecular analysis. The data presented suggests that the functional spectrum of expressed genes is consistent with genomic and proteomic reports obtained from pure cultures and environmental AMD samples with similar microorganisms to those involved in bioleaching processes. The functional genes detected by RAP-PCR in PLS samples from the bioleaching heap at the Escondida Mine seems to be related to metabolic changes during the leaching cycle and are now being analyzed as potential indicators for industrial monitoring.

Acknowledgements

This work was supported in part by the FONDEF project D04I1169 from CONICYT. P. Galleguillos is recipient of a CICITEM Ph.D. scholarship and F. Remonsellez is supported by the Technology and Science Bicentennial Programme from CONICYT. We thank Dr. Owen Rowe (Bangor University, UK) and Dr. Cristina Dorador (Centro de Biotecnología, Universidad Católica del Norte, Chile) for critically reading the manuscript.

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