Genomics, metagenomics and proteomics in biomining microorganisms

Lissette Valenzuela[a], An Chi[b], Simon Beard[a], Alvaro Orell[a], Nicolas Guiliani[a], Jeff Shabanowitz[b], Donald F. Hunt[b], Carlos A. Jerez[a,*]

[a] *Laboratory of Molecular Microbiology and Biotechnology, Department of Biology, Faculty of Sciences, University of Chile, Santiago, Chile*
[b] *Department of Chemistry, University of Virginia, Charlottesville, Virginia, U.S.A.*

Abstract

The use of acidophilic, chemolithotrophic microorganisms capable of oxidizing iron and sulfur in industrial processes to recover metals from minerals containing copper, gold and uranium is a well established biotechnology with distinctive advantages over traditional mining. A consortium of different microorganisms participates in the oxidative reactions resulting in the extraction of dissolved metal values from ores. Considerable effort has been spent in the last years to understand the biochemistry of iron and sulfur compounds oxidation, bacteria–mineral interactions (chemotaxis, quorum sensing, adhesion, biofilm formation) and several adaptive responses allowing the microorganisms to survive in a bioleaching environment. All of these are considered key phenomena for understanding the process of biomining. The use of genomics, metagenomics and high throughput proteomics to study the global regulatory responses that the biomining community uses to adapt to their changing environment is just beginning to emerge in the last years. These powerful approaches are reviewed here since they offer the possibility of exciting new findings that will allow analyzing the community as a microbial system, determining the extent to which each of the individual participants contributes to the process, how they evolve in time to keep the conglomerate healthy and therefore efficient during the entire process of bioleaching.

Keywords: Biomining; Acid mine drainage; Genomics; Metagenomics; Proteomics

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* Corresponding author. Departamento de Biología, Facultad de Ciencias Universidad de Chile, Santiago 1, Casilla 653, Santiago, Chile. Tel.: +56 2 978 7376.

E-mail address: cjerez@uchile.cl (C.A. Jerez).

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1. Introduction: biomining and acid mine drainage generating microorganisms and their mode of action

The use of acidophilic, chemolithotrophic microorganisms capable of oxidizing iron and sulfur in industrial processes to recover metals from minerals containing copper, gold and uranium is a well established biotechnology (Torma, 1983; Brierley, 1982, 1997; Acevedo, 2000; Rawlings, 2002; Olson et al., 2003; Suzuki, 2001). The insoluble metal sulfides are oxidized to soluble metal sulfates by the chemical action of ferric iron, the main role of the microorganisms being the re-oxidation of the generated ferrous iron to obtain additional ferric iron (Rohwerder et al., 2003; Rawlings, 2002; Olson et al., 2003).

Currently, there are operations using mesophilic and thermophilic microorganisms (Torma, 1983, 1988; Brierley, 1982, 1997; Lindström et al., 1992; Acevedo, 2000; Rawlings, 2002, 2005a,b; Olson et al., 2003). Biomining has distinctive advantages over the traditional mining procedures. For example, it does not require the high amounts of energy used during roasting and smelting and does not generate harmful gaseous emissions such as sulfur dioxide (Rawlings, 2002). Nevertheless, acid mine drainage can be generated, which if not properly controlled, pollutes the environment with acid and metals (Rohwerder et al., 2003; Rawlings, 2002, 2005a,b; Olson et al., 2003). Biomining is also of great advantage since discarded low-grade ores from standard mining procedures can be leached in an economically feasible way. There are complete previous reviews regarding methods of bioleaching and their implementation in several countries (Torma, 1983; Brierley, 1982, 1997; Acevedo, 2000; Lindström et al., 1992; Rohwerder et al., 2003; Rawlings, 2002, 2005a,b; Olson et al., 2003). This biotechnology is especially important in Chile (Gentina and Acevedo, 1985; Acevedo et al., 1993), since this country is the principal copper producer in the world, generating about 400,000 ton of copper per year by using biomining.

There is a great variety of microorganisms capable of growth in situations that simulate biomining commercial operations (Rohwerder et al., 2003; Rawlings, 2002; Olson et al., 2003; Hallberg and Johnson, 2001). Many different species of microorganisms are living at acid mine drainage sites (Baker and Banfield, 2003). The most studied leaching bacteria are from the genus *Acidithiobacillus* (Kelly and Wood, 2000). *Acidithiobacillus ferrooxidans*, *A. thiooxidans* are acidophilic mesophiles and together with the moderate thermophile *A. caldus*, they belong to the Gram-negative γ-proteobacteria (Hallberg and Johnson, 2001; Rohwerder et al., 2003). In addition, the members of the genus *Leptospirillum*, other important biomining bacteria belong to a new bacterial division (Hippe, 2000; Coram and Rawlings, 2002). Some Gram-positive bioleaching bacteria belonging to the genera *Acidimicrobium*, *Ferromicrobium* and *Sulfobacillus* have also been described (Clark and Norris, 1996; Johnson and Roberto, 1997; Norris et al., 1996). Biomining extremely thermophilic archaeons capable of oxidizing sulfur and iron (II) have been known for many years, and they are mainly from the genera *Sulfolobus*, *Acidianus*, *Metallosphaera* and *Sulfurisphaera* (Fuchs et al., 1995, 1996; Kurosawa et al., 1998; Norris et al., 2000). Recently, some mesophilic iron (II)-oxidizing archaeons belonging to the *Thermoplasmales* have been isolated and described: *Ferroplasma acidiphilium* (Golyshina et al., 2000) and *F. acidarmanus* (Edwards et al., 2000).

As already mentioned, *A. ferrooxidans* is a chemolithoauthotrophic bacterium that obtains its energy from the oxidation of ferrous iron, elemental sulfur, or partially oxidized sulfur compounds and it has been considered as a model biomining microorganism (Olson et al., 2003; Harrison, 1984; Lundgren, 1980; Suzuki, 2001; Rawlings, 2002).

The reactions involved in ferrous iron oxidation by *A. ferrooxidans* have been studied in detail (Ingledew, 1982; Blake and Shute, 1994; Cox and Boxer, 1986; Rawlings, 2002 and references therein); however, the electron pathway from ferrous iron to oxygen has not been entirely established (Yarzábal et al., 2002b). The terminal electron acceptor is assumed to be a cytochrome oxidase anchored to the cytoplasmic membrane. The transfer of electrons would occur through several periplasmic carriers, including at least the blue copper protein rusticyanin, and cytochrome c552. Recently, a high molecular weight c-type cytochrome, Cyc2, has been suggested

to be the prime candidate for the initial electron acceptor in the respiratory pathway between ferrous iron and oxygen. This pathway would be Cyc2→rus-rusticyanin→Cyc1(c552)→aa3 cytochrome oxidase (Yarzábal et al., 2002b). In addition, there is an apparent redundancy of electron transfer pathways via bc(1) complexes and terminal oxidases in *A. ferrooxidans* (Brausser et al., 2004).

The aerobic oxidation of elemental sulfur by *A. ferrooxidans* and other microorganisms is carried out by a sulfur dioxygenase (Rohwerder and Sand, 2003; Silver and Lundgren, 1968b; Muller et al., 2004; Sugio et al., 1987). Recently, thiosulfate has been postulated as a key compound in the oxidation of the sulfur moiety of pyrite (Schippers and Sand, 1999). Iron (III) ions are exclusively the oxidizing agents for the dissolution. Thiosulfate would be consequently degraded in a cyclic process to sulfate, with elemental sulfur being a side product. This explains why only Fe(II) ion-oxidizing bacteria are capable of oxidizing these metal sulfides (Schippers and Sand, 1999). All reactions comprising this oxidation have been shown to occur chemically (Sand et al., 1995, 2001). However, sulfur compounds oxidizing enzymes such as the tetrathionate hydrolase of *A. ferrooxidans*, *A. thiooxidans*, or *T. acidophilus* may also be involved in the process (De Jong et al., 1997; Kelly et al., 1997; Suzuki, 1999; Friedrich et al., 2001). In addition, enzymes for thiosulfate or sulfite oxidation of *A. ferrooxidans* or *A. thiooxidans* may successfully compete with the chemical reactions with iron (III) ions as an oxidizing agent (Schippers and Sand, 1999). It is important to remark here, that the ultimate oxidizing agent for iron (II) and reduced inorganic sulfur compounds is oxygen, since often the transport of dissolved oxygen is the rate limiting steps in commercial bioleaching operations.

Very complete previous reviews related to molecular biology and genomics of *A. ferrooxidans* (Rawlings and Kusano, 1994; Rawlings, 2001), therefore we will concentrate here in the few global and integrative approaches that have been recently used to study the biomining and acid mine drainage community of microorganisms. The use of genomics, proteomics and metagenomics (Fig. 1), together with metabolomics, will have a key role in the understanding of the molecular mechanisms by which the microorganisms attack and solubilize the ores. This, taken together with the physicochemical, geological and mineralogical aspects of the process, will allow to improve the efficiency of this important biotechnology.

2. Genomics and bioinformatics

Initial studies on the isolation, cloning and sequencing of mostly individual chromosomal genes and plasmids of *A. ferrooxidans* and expression of some of them in *Escherichia coli* to generate functional proteins have been reviewed (Rawlings, 2001). All these efforts have contributed greatly to the identification and isolation of the various components of the iron-oxidation system. Very recently, Rawlings (2005a,b) reported on the evolution of the plasmids pTF-FC2 and pTC-F14 isolated from two related bacteria, *A. ferrooxidans* and *A. caldus*, respectively. These two plasmids encode for several genes related with their replication, addiction and mobilization and belong to the IncQ-like plasmid family and must have originated from the same ancestral plasmid. However, only two of the five mobilization genes have remained highly conserved, whereas the other three genes appear to have evolved such that each plasmid is mobilized most efficiently by a different self-transmissible plasmid (Rawlings, 2005a,b). These plasmids are very important, since they may help in the development of an efficient conjugational system for genetic studies of these microorganisms.

Effective tools for the study of *A. ferrooxidans* genetics and physiology are not in widespread use and, despite considerable effort, an understanding of its unusual physiology remains at a rudimentary level. An efficient and reproducible technique for DNA transfer is still missing. *A. ferrooxidans* was the first biomining microorganism to have its genome almost entirely sequenced (Selkov et al., 2000). In addition a complete genome sequence of *A. ferrooxidans* is available from TIGR (http://www.tigr.org) and although the annotation of all its genes has not yet been published, this information has been so far very useful to many researchers using this microorganism. The genome-wide search for candidate genes for important metabolic pathways and several important physiological functions can now be addressed and predictions for the functions of many new genes can be done. The main focus of research has been the energetic metabolism which is directly responsible of bioleaching. Some researchers used “chromosome walking” to find genes involved in phosphate, sulfur and iron metabolisms and quorum sensing. Using bioinformatic analysis, the genetic contexts of these genes was proposed and these findings were supported by experimental research. New thiosulfate sulfur transferases (TSTs) whose expression are regulated depending on the growth substrate and are probably related to sulfur metabolism and/or oxidation

were found (Ramirez et al., 2002, 2004). Eight nucleotide sequences containing a single rhodanese domain were found in the genome of *A. ferrooxidans* ATCC 23270: *p11*, *p14*, *p14.3*, *p15*, *p16*, *p16.2*, *p21* and *p28* (Acosta et al., 2005). Amino acids sequence comparisons allowed us to identify the potentially catalytic Cys residues and other highly conserved rhodanese family features in all eight proteins. The genomic contexts of some of the rhodanese-like genes and the determination of their expression at the mRNA level by using macroarrays suggested their implication in sulfur oxidation and metabolism, formation of Fe–S clusters or detoxification mechanisms. Several of the putative rhodanese genes were successfully isolated, cloned and overexpressed in *E. coli* and their thiosulfate:cyanide sulfurtransferase (TST) and 3-mercaptopyruvate:cyanide sulfurtransferase (MST) activities were determined. Based on their sulfurtransferase activities and on structural comparisons of catalytic sites and electrostatic potentials between homology-modeled *A. ferrooxidans* rhodaneses and the reported crystal structures of *E. coli* GlpE (TST) and SseA (MST) proteins, two of the rhodanese-like proteins (P15 and P16.2) could clearly be defined as TSTs, and P14 and P16 could possibly correspond to MSTs (Acosta et al., 2005). Nevertheless, several of the eight *A. ferrooxidans* rhodanese-like proteins may have some different functional activities yet to be discovered.

A Pho regulon which is greatly induced during phosphate starvation (Vera et al., 2003) and which is related to metal resistance (Alvarez and Jerez, 2004) was described. Copper ions stimulated polyphosphate degradation and phosphate efflux in *A. ferrooxidans*,

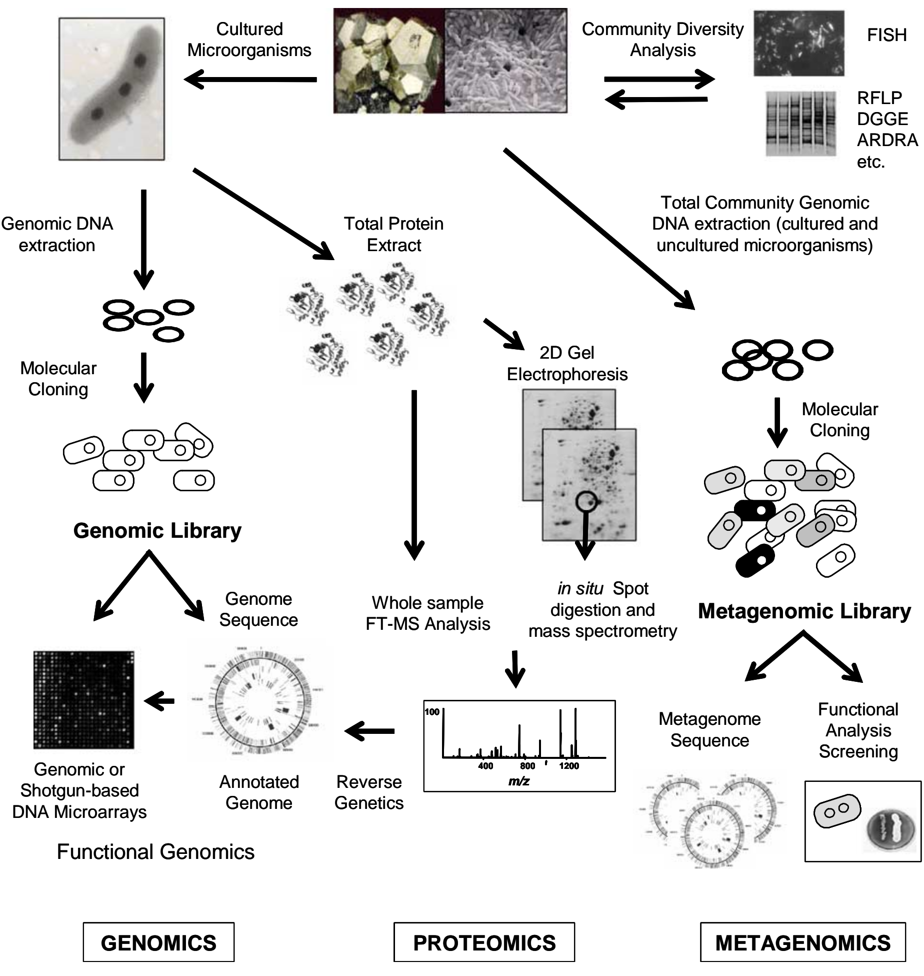


Fig. 1. Overview of the application of genomics, proteomics and metagenomics to biomining microorganisms.

supporting a model for metal detoxification in which heavy metals stimulate polyphosphate hydrolysis and the metal–phosphate complexes formed would be transported out of the cell as part of a possibly functional heavy metal tolerance mechanism in this bacterium (Alvarez and Jerez, 2004 and references therein). The gene expression of *A. ferrooxidans* in response to copper was investigated by using RNA arbitrarily primed polymerase chain reaction (RAP-PCR). By this procedure, more than 100 genes were differentially expressed. Cloning and sequencing of the RAP-PCR products that presented the highest levels of induction or repression in the presence of copper allowed the identification of some putative genes. However, none of these genes clarified the possible effects of this toxic element on the cells (Paulino et al., 2002). Other researchers analyzed the genomic data available to search for the genes involved in iron oxidation and supported their findings experimentally. Eleven genes encoding putative cytochromes *c* were identified and this number of proteins was higher than the eight cytochromes identified by heme staining and immunoblotting in *A. ferrooxidans* ATCC 33020 (Yarzabal et al., 2002a). This clearly indicates the importance of having the genomic sequence available since it is then possible to formulate hypothesis about the regulation of the expression of the pool of cytochromes *c* depending on the environmental growth conditions. The regulation of the expression of the rus operon which encodes two cytochromes *c*, a cytochrome oxidase and rusticyanin was also studied (Yarzabal et al., 2004). They found that this operon was more highly expressed in ferrous iron-than in sulfur -grown cells, in agreement with our findings of rusticyanin and other proteins synthesis during growth of *A. ferrooxidans* in ferrous iron, elemental sulfur and metal sulfides (Ramirez et al., 2004).

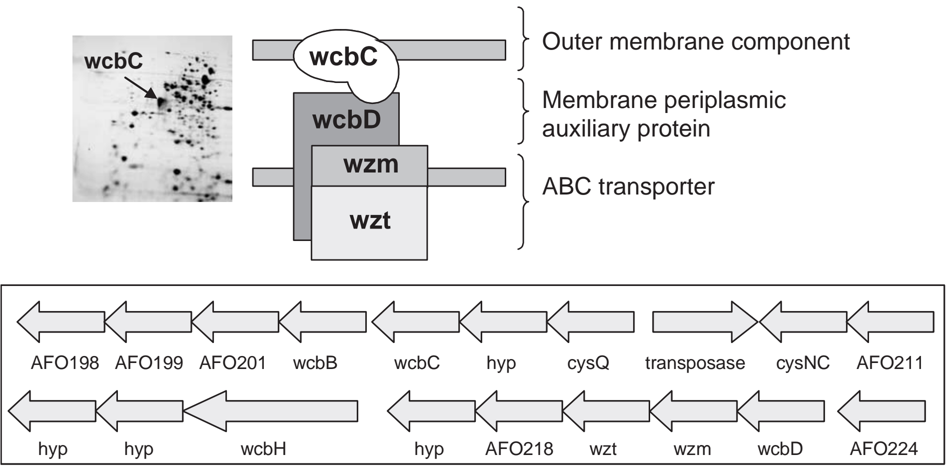
Using an entirely bioinformatic approach other researchers found candidate genes potentially involved in several other functions such as metal resistance, amino acid biosynthesis pathways and others (Barreto et al., 2003). In addition, a cluster of five genes proposed to be involved in the formation of extracellular polysaccharide (EPS) precursors via the Leloir pathway have been identified recently in *A. ferrooxidans* (Barreto et al., 2005).

Genome sequence analysis have revealed that *A. ferrooxidans* contains homologs of almost all the *trb* and *tra* genes required for conjugal transfer of DNA in *Agrobacterium tumefaciens*, and these genes conserved the genetic organization in both microorganisms (Christie and Vogel, 2000; Barreto et al., 2003). Biofilm formation, which is important for mineral leaching (Rohwerder et al., 2003) has been highlighted too by genome sequence analysis. A large set of genes required for pilus formation (*pilA-D*, *pilS* and *pilR*) and exopolymeric substances (EPS) for attachment (Barreto et al., 2003) (see also Fig. 2) and a quorum sensing loci which are associated with biofilm formation in other microorganisms have been identified (Farah et al., 2005).

With the first gapped genome sequence reported it was possible to boinformatically reconstruct the amino acid metabolism pathway almost entirely (Selkov et al., 2000). This information has been exploited to reconstruct in silico aspects of the sulfur metabolism in this bacterium (Valdes et al., 2003). Two candidate mechanisms for sulfate uptake from the environment were detected but both belong to large paralogous families of membrane transporters and their identification remains tentative. Prospective genes, pathways and regulatory mechanisms were identified that are likely to be involved in the assimilation of sulfate into cysteine and in the formation of Fe–S centers. Genes and regulatory networks were also uncovered that may link sulfur assimilation with nitrogen fixation, hydrogen utilization and sulfur reduction. Potential pathways were identified for sulfation of extracellular metabolites that may possibly be involved in cellular attachment to pyrite, sulfur and other solid substrates (Valdes et al., 2003). Metabolic modeling provides an important preliminary step in understanding the unusual physiology of this extremophile especially given the severe difficulties involved in its genetic manipulation and biochemical analysis. However, all these predictions will have to be demonstrated experimentally.

3. Gene function analysis

One of the most used techniques to study differential genome expression or functional analysis of new and characterized genes is the use of DNA microarrays (Duggan et al., 1999). However, a prerequisite to apply microarrays is to know the genomic sequence of the organism to be analyzed. The genome sequences of most biomining microorganisms are not known. A very interesting approach was used to analyze gene function in environmental isolates without knowing the sequence of the microorganism of interest. A random genomic library from an isolate of *L. ferrooxidans* was printed on a microarray (Parro and Moreno-Paz, 2003) (see Fig. 1). The authors analyzed gene expression by using total RNA extracted from *L. ferrooxidans* grown in the presence or absence of ammonium as



ORF	Enzyme Activity	Accession Number
AFO 198	mannosyltransferase, putative [*Pseudomonas putida* KT2440]	AE016780
AFO 199	SAM-dependent methyltransferases [*Thermobifida fusca*]	ZP_00058499
AFO 201	GDP-D-mannose dehydratase [*Aquifex aeolicus*]	AE000722
wcbB	Glycosyl transferases group 1 [*Burkholderia mallei*]	CP000010
wcbC	wcbC, Polysaccharide export protein [*Burkholderia mallei*]	AF285636
cysQ	CysQ, 3'-Phosphoadenosine 5'-phosphosulfate (PAPS) 3'-phosphatase	AE012433
cysNC	ATP sulfurylase/adenylylsulfate kinase [*Xanthomonas campestris*]	CR543861
AFO 211	3'-phosphoadenosine 5'-phosphosulfate sulfotransferase (PAPS reductase)/FAD synthetase [*Acinetobacter sp*]	
wcbH	mannosyltransferase A (mtfA) [*Archaeoglobus fulgidus* DSM 4304]	AE001103
AFO 218	SAM-dependent methyltransferases [*Xylella fastidiosa* Dixon]	AY253301
wzt	ABC (ATP-binding cassette) transporter nucleotide-binding domain	AAK26458
Wzm	putative ABC-2 transporter Wzm [*Burkholderia mallei*]	AAK26457
wcbD	WcbD, Polysaccharide export protein [*Burkholderia mallei*]	AE004649
AFO 224	phosphomannose isomerase/GDP-mannose pyrophosphorylase homolog [*Pseudomonas aeruginosa*]	

Fig. 2. An example of the use of reverse genetics in *A. ferrooxidans*. The WcbC protein was isolated from a 2D PAGE gel and identified by MS/MS as a putative member of an exopolysaccharide exporter system. The genomic context of the *wcbc* gene supports the existence of the putative exporter system.

nitrogen source under aerobic conditions. With this approach, they could select and sequence only those clones bearing the genes that showed an altered expression pattern. Most of the genes of the nitrogen fixation regulon in this microorganism were then determined by sequence comparison, such as the *nifHDKENX* operon, encoding the structural components of Mo–Fe nitrogenase and the *nifSU-hesBhscBA-fdx* operon for Fe–S cluster assembly and other genes involved in nitrogen fixation (Parro and Moreno-Paz, 2003). These results clearly showed that shotgun DNA microarrays are very powerful tools to study gene expression with environmental microorganisms whose genome sequence is still unknown.

As already mentioned, the genome sequence of *A. ferrooxidans* was done by TIGR (http://www.tigr.org) but it is not yet annotated. However, the analysis of the relative variations in mRNA abundance of some of the genes related with sulfur metabolism in this bacterium grown in different oxidizable substrates, was started by performing a preliminary pilot DNA macroarray formed with 70 different genes (Acosta et al., 2005). By previous proteomic analysis, it was shown that the proteins coded in several of these selected genes change their expression according to the growth conditions (sulfur versus ferrous iron), and follow the same patterns of induction/repression that we observed with the macroarrays (Ramirez et al., 2002, 2004; Vera et al., 2003). Gene *p21* codes for a putative thiosulfate sulfur transferase protein (see Standard proteomics section below) and all the putative genes upstream of it have been found forming a cluster (Acosta et al., 2005). They were all highly expressed in cells grown in sulfur compared to the levels seen in ferrous iron (Table 1). This clearly supports our previous proposal based on proteomic analysis that the rhodanese-like gene *p21* forms part of a group of genes related with sulfur oxidation (Ramirez et al., 2002). In addition, the macro-

Table 1
DNA macroarray differential expression of some genes from *A. ferrooxidans* ATCC 23270 grown in elemental sulfur or ferrous iron

ORF	Spots		Relative intensity[a]		Expression ratio	Schematic genetic context
	S°	Fe(II)	S°	Fe(II)	[S/Fe(II)]	
p21			16.35	0.15	109.65	
doxDA-1			2.41	0.19	12.60	
modA-1			10.32	0.05	191.98	*p21* *doxDA* *modA* *hyp4* *tehA*
hyp4			24.19	1.31	18.44	
tehA			16.32	0.79	20.58	
moaA			1.57	0.10	15.01	
p11			5.24	0.14	37.65	*moaA* *p11* *sirA*
sirA			3.32	0.39	8.44	
afeI			13.22	0.68	19.38	*afeI* *orf3* *afeR*
afeR			6.46	4.13	1.57	

Unpublished results.
[a]Relative spot intensities calculated using the net mean intensity for each spot relative to the *exp-1* gene from *Prunus persica*.

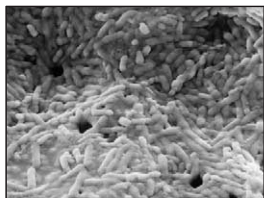
array results obtained for *p21* were validated by our previous RT-PCR studies, both indicating the same behaviour of induction of expression by growth in sulfur compounds both, at the transcriptional and translational levels (Ramirez et al., 2002, 2004).

Also genes *p11* (a rhodanese-like protein), *moaA* (a molybdenum cofactor biosynthesis enzyme) and *sirA* (a predicted redox protein, regulator of disulfide bond formation) (Acosta et al., 2005) were all expressed in higher levels when the cells were grown in sulfur. The normalized quantitation of the expression ratios S°/Fe (elemental sulfur/ferrous iron) in the corresponding oxidizable substrates is also shown (Table 1).

Quorum sensing (QS) type AI-1 is a phenomenon that enables Gram-negative bacterial cells to establish a cell–cell communication mediated through acyl-homoserine lactone molecules and to regulate the expression of specific genes in response to local changes in cell-population density (Bassler, 2002; Whitehead et al., 2001). During bioleaching *A. ferrooxidans* adheres to the solid substrates by means of extracellular polymeric substances (EPS) such as exo/lipopolysaccharides. *A. ferrooxidans* is also able to develop biofilm structures when growing in solid substrates such as elemental sulfur (see Fig. 3A) or metal sulfides, and presents morphological modifications during the cellular adhesion process (Blais et al., 1994; DiSpirito et al., 1982; Karamanev, 1991; Rohwerder et al., 2003). Considering that Acyl-homoserine lactone mediated gene regulation has been shown to influence exopolysaccharide production and biofilm formation in many proteobacteria (Gonzalez and Marketon, 2003; Winans and Bassler, 2002), it was of great interest to study this phenomenon in a biomining bacterium such as *A. ferrooxidans*. A quorum sensing locus was identified and called *afeIR* (Farah et al., 2005). *AfeI* and *afeR* genes were expressed in *A. ferrooxidans* grown in sulfur, whereas *afeI* was overexpressed when the bacterium was grown forming a biofilm over sulfur prills (Table 1). Additionally, it was demonstrated that *AfeI* is an acyl-homoserine lactone synthase (Farah et al., 2005). These results suggest the existence of a functional quorum sensing type AI-1 in *A. ferrooxidans* which could be part of a regulon controlling some physiological functions such as exopolysaccharide synthesis and biofilm formation.

The first DNA macroarrays used with *A. ferrooxidans* have been very useful for the partial study of gene expression in this microorganism (Acosta et al., 2005). However, in the near future, the use of microarrays

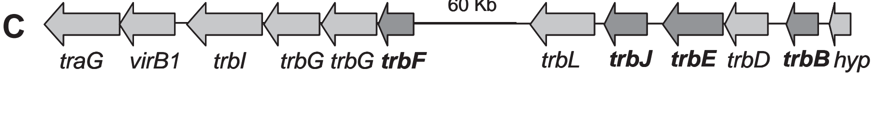
A



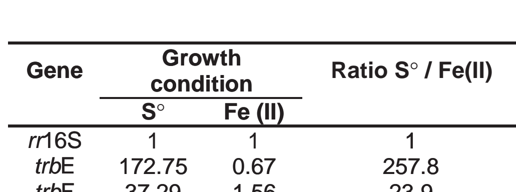
B

FT scan	mass+1	FT counts	Charge	FT m/z	LCQ scan	Protein names
1803	969,52	3629380	2	485,26	3971	TrbE [*Rhizobium rhizogenes*]
1493	1985,19	2379479	3	662,4	3203-993	TrbF [*Agrobacterium tumefaciens*]
1882	1254,78	12291584	2	627,89	4163	TrbJ [*Brucella suis 1330*]

C



D



Gene	Growth condition		Ratio S° / Fe(II)
	S°	Fe (II)	
*rr*16S	1	1	1
*trb*E	172.75	0.67	257.8
*trb*F	37.29	1.56	23.9

E

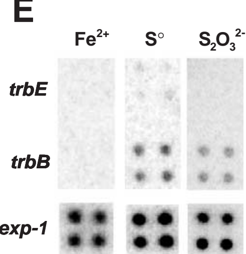


Fig. 3. Differential expression of putative Trb proteins in *A. ferrooxidans*. A. Biofilm of *A. ferrooxidans* grown in a sulfur prill. B. Differential FT-MS partial data for some of the putative proteins present in the extract from *A. ferrooxidans* grown in elemental sulfur and not in ferrous iron. C. Genomic context of the genes *trbE*, *trbF* and *trbJ*. D. Real time PCR quantitation of the levels of *trbE*, and *trbF* genes expression in cells grown in sulfur (S°) or ferrous iron (FeII). E. DNA macroarray showing the expression of genes *trbE*, and *trbB* in cells grown in thiosulfate, sulfur and ferrous iron (unpublished results).

based on the entire genome of *A. ferrooxidans* and other microorganisms will allow having a nearly complete view of gene expression of the members of the microbial community under several biomining conditions, helping to monitor their physiological state and adjustment made during the bioleaching process.

4. Metagenomics

Metagenomics is the culture-independent genomic analysis of microbial communities (Streit and Schmitz, 2004; Schloss and Handelsman, 2003; Handelsman, 2004). In conventional shotgun sequencing of microbial isolates, all shotgun fragments are derived from clones of the same genome. However, if the genomes of an environmental microbial community are to be analyzed (Fig. 1), the ideal situation is to have a low diversity environment. Such a system was found analysing the microbial communities inhabiting a site of extreme acid mine drainage production, in which few organisms types were present (Bond et al., 2000). Still, variation within each species population might complicate assembly of the DNA fragments. Nevertheless, Tyson et al. (2004) used random shotgun sequencing of DNA from this natural acidophilic biofilm. They could reconstruct the near-complete genomes of *Leptospirillum* group II and *Ferroplasma* type II and partial recovery of three other genomes. The extremely acidic conditions of the biofilm (pH about 0.5) and relatively restricted energy source combine to select for a small number of species. Furthermore, the frequency of genomic rearrangements and gene insertions or deletions was very low, making this community ideal for testing these new culture-inde-

pendent genomic approaches in the environment (Tyson et al., 2004).

The analysis of the gene complement for each organism revealed the metabolic pathways for carbon and nitrogen fixation and energy generation. For example, genes for biosynthesis of isoprenoid-based lipids and for a variety of proton efflux systems were identified, providing insights into survival strategies in the extreme acidic environment. However, this information will have to be confirmed by biochemical and physiological approaches.

Clearly, the metagenomic approach for the study of microbial communities is a real advancement to fully understand how complex microbial communities function and how their component members interact within their niches (Streit and Schmitz, 2004; Tyson et al., 2004; Handelsman, 2004). A fully understanding of the biomining community also will require the use of all these current molecular approaches (Fig. 1).

5. Standard proteomics

Proteomics provide direct information of the dynamic protein expression in tissue or whole cells, giving us a global analysis. Together with the significant accomplishments of genomics and bioinformatics, systematic analysis of all expressed cellular components has become a reality in the post genomic era, and attempts to grasp a comprehensive picture of biology have become possible.

One important aspect of proteomics is to characterize proteins differentially expressed by dissimilar cell types or cells imposed to different environmental conditions. Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) in combination with mass spectrometry is currently the most widely used technology for comparative bacterial proteomics analysis (Fig. 1) (Gygi et al., 2000). The high reproducibility of 2D PAGE is particularly valuable for multiple sample comparisons. In addition, it directly correlates the changes observed at the peptide level to individual protein isoforms.

Several studies have used 2D PAGE to study changes in protein expression of *A. ferrooxidans* under different growth conditions. Proteins induced under heat shock (Varela and Jerez, 1992), pH stress (Amaro et al., 1991), phosphate limitation (Seeger and Jerez, 1993; Vera et al., 2003) or presence of copper (Novo et al., 2003) have been reported.

A set of proteins that changed their levels of synthesis during growth of *A. ferrooxidans* ATCC 19859 in metal sulfides, thiosulfate, elemental sulfur and ferrous iron was characterized by using 2D PAGE (Ramirez et al., 2004). N-terminal amino acid sequencing or MS/MS of these proteins allowed their identification and the localization of the corresponding genes in the available genomic sequence of *A. ferrooxidans* ATCC 23270. The genomic context around several of these genes suggests their involvement in the energetic metabolism of *A. ferrooxidans*. Two groups of proteins could be distinguished: proteins highly upregulated by growth in sulfur compounds (and downregulated by growth in ferrous iron): a 44 kDa outer membrane protein, an exported 21 kDa putative thiosulfate sulfur transferase protein, a 33 kDa putative thiosulfate/sulfate binding protein, a 45 kDa putative capsule polysaccharide export protein (WcbC), and a putative 16 kDa protein of unknown function. It is known that most leaching bacteria grow attached to the surface of the solid substrates such as elemental sulfur and metal sulfides. This attachment is predominantly mediated by extracellular polymeric substances (EPS) surrounding the cells and that are adjusted according to the growth substrate (Rohwerder et al., 2003). Thus, planktonic cells grown with soluble substrates such as iron(II) sulfate produce almost no EPS (Gehrke et al., 1998, 2001).

In agreement with these facts, the putative capsule protein that was found induced by cells grown in the solid elemental sulfur (Ramirez et al., 2004) was coded by a putative *wcbC* gene of *A. ferrooxidans* whose context includes the genes *wcbD*, *wzm* and *wzt*, all of them known in other microorganisms to form a polysaccharide ABC exporter (DeShazer et al., 2001). Other neighbor putative genes appear to be related with the synthesis of EPS (Fig. 2). This is a typical “reverse genetics” approach in which after isolating from a 2D PAGE gel an individual protein differentially expressed in a condition of interest, the amino acid sequence of a peptide from the protein is obtained to identify its possible homolog in databases. With this information, its coding gene and genomic context can be searched using the genome DNA sequence. Depending on these results, a suggested function could be hypothesized. It will be of great importance to demonstrate the expression of these putative genes possibly related to EPS synthesis in *A. ferrooxidans* cells grown in different metal sulfides and find out if they are involved in cell attachment and biofilm formation. In this regard, *A. ferrooxidans* is known to form biofilms on solid substrates (see Fig. 3A). It is not known in the acid mine drainage biofilm analyzed by Tyson et al. (2004), which microorganisms are responsible for the production of the polymer embedding the community. However, the

presence of numerous glycosyltransferases and polysaccharide export proteins in *Leptospirillum* group II also suggest a role in biofilm formation.

The second group of proteins analyzed by 2D PAGE (Ramirez et al., 2004) were those downregulated by growth of *A. ferrooxidans* in sulfur (and upregulated by growth in ferrous iron) were rusticyanin, a cytochrome *c*552, a putative phosphate binding protein (PstS), the small and large subunits of RuBisCO, a 30 kDa putative CbbQ protein, amongst others. The results suggest in general a separation of the iron and sulfur utilization pathways. Rusticyanin in addition of being highly expressed in ferrous iron was also newly synthesized as determined by metabolic labeling, although at lower levels during growth in sulfur compounds and iron-free metal sulfides. These results are in agreement with those of Yarzabal et al. (2004). The capacity of *A. ferrooxidans* to oxidize thiosulfate and tetrathionate was found to be inhibited by the presence of ferrous iron (Das et al., 1993). However, we found that during growth of *A. ferrooxidans* in metal sulfides containing iron, such as pyrite and chalcopyrite, proteins upregulated both in ferrous iron and sulfur compounds were synthesized, indicating that the two energy-generating pathways are simultaneously induced depending on the kind and concentration of the available oxidizable substrates (Ramirez et al., 2004). In agreement with these results, it has been previously suggested that *A. ferrooxidans* can simultaneously utilize both ferrous iron and elemental sulfur as energy sources (Espejo and Romero, 1987).

A rhodanese activity has been previously reported in *A. ferrooxidans* (Tabita et al., 1969). This enzyme is a TST, which breaks the S–S bond present in thiosulfate, generating sulfur and sulfite. Other enzymes may also participate in the mechanism proposed by Schippers and Sand (1999), such as the thiosulfate-oxidizing enzyme of *A. ferrooxidans* (Silver and Lundgren, 1968a).

By using 2D PAGE, it was possible to identify an exported rhodanese-like protein (P21) whose levels are increased when *A. ferrooxidans* is grown on metal sulfides and different sulfur compounds but is almost entirely absent during growth on ferrous iron (Ramirez et al., 2002, 2004). Unlike cytoplasmic rhodaneses, P21 was located in the periphery of *A. ferrooxidans* cells and was regulated depending on the oxidizable substrate. If P21 and some of the proteins coded by its adjacent genes are involved in thiosulfate metabolism, one should expect an increased expression of these proteins when the cells are grown in pyrite, thiosulfate or sulfur, as we have observed by proteomics (Ramirez et al., 2002) and by mRNA expression by macroarrays as shown here (Table 1). Protein P21 may not be a periplasmic rhodanese enzyme but rather part of a possible complex in charge of thiosulfate oxidation. This putative complex could be different from the Sox model proposed for sulfur oxidation in many bacteria (Friedrich, 1998) since we did not find any *sox*-like genes in the genome of *A. ferrooxidans* (Ramirez et al., 2004). However, *A. ferrooxidans* harbors duplicated *doxDA* genes that are homologous to the genes encoding thiosulfate : quinone oxidoreductase in *A. ambivalens*, an enzyme oxidizing thiosulfate with tetrathionate as product and ferricyanide or decylubiquinone as electron acceptors (Muller et al., 2004). As pointed out very recently by Friedrich, this gene duplication points to a yet undemonstrated significance in thiosulfate metabolism in *A. ferrooxidans* (Friedrich et al., 2005). *doxDA-1* is part of the putative transcriptional unit containing *p21* (Acosta et al., 2005) and as seen in Table 1, its expression determined by DNA macroarrays is enhanced 12-fold in cells grown in elemental sulfur and 3-fold in cells grown in thiosulfate compared to the expression levels of cells grown in ferrous iron (Acosta et al., 2005). These results support the significance of at least one of the two *doxDA* genes in thiosulfate metabolism in *A. ferrooxidans* and are in favor of the idea that acidophilic bacteria oxidize sulfur by a system different from the Sox enzyme system.

Recently, the term "metaproteomics" was proposed for the large-scale characterization of the entire protein complement of environmental microbiota at a given point in time (Wilmes and Bond, 2004). These authors applied 2D PAGE and downstream analysis to a mixed community of prokaryotic microorganisms, extracting and purifying from it the entire proteome to map it. The highly expressed proteins were identified by MS. This is the first report of this nature, and will be very interesting to apply it to communities such as the biomining one.

6. High throughput proteomics and metaproteomics

In the past decade, an increasing number of sequenced genome provides good options for high throughput functional analysis of proteomes (Celestino et al., 2004). Proteomic studies are well advanced for diverse bacteria, such as model bacteria *E. coli* (Vollmer et al., 2003) and *Bacillus subtilis* (Eymann et al., 2004) as well as many pathogenic bacteria including *Mycobacterium tuberculosis* (Mattow et al., 2004). Nevertheless, there is still a lack of data for identification of proteins from organisms with unannotated or unsequenced genomes, which leads to large-scale

microbacterial proteomics analysis remain challenging. With the development of highly sensitive and accurate computational gene-finding methods such as GeneMark (Borodovsky and Mcininch, 1993) and GLIMMER (Salzberg et al., 1998), new microbial genomes could be explored and scientific knowledge of them could be maximized.

Traditional 2D gel electrophoresis coupled with MS is time consuming as a result of the nature of spot-by-spot analysis and it is biased against low abundance proteins, integral membrane proteins, and proteins with extreme p*I* or MW (Gygi et al., 2000). Alternatively, solution-based approaches offer unbiased measurement of relative protein expression regardless of their abundance, subcellular localization, or physicochemical parameters (Fig. 1). This methodology, however, results in extremely complex samples. For instance, of the 4191 predicted genes in the complete genome of *Escherichia coli*, 2800 of them are believed to be expressed at any one time (Corbin et al., 2003). Additional complexity is introduced upon enzymatic digestion, which generates multiple peptide species for each protein. To obtain comprehensive protein expression information in the samples, a chromatographic separation step prior to mass spectrometry (MS) protein analysis is often necessary. HPLC coupled with online electron spray ionization MS (ESI-MS/MS) has been proved to be a valid approach for analyzing protein expression in complex samples (Washburn et al., 2001). Alternatively, Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR MS) is well suited for the differential analysis of protein expression due to the high mass accuracy and high resolution as well as its inherent wide dynamic range. Peptide charge state can be readily derived with the accurate isotopic peak distribution information provided by FT MS experiment, and co-eluting species with the same nominal m/z ratio can be resolved. The differentially expressed m/z values identified can be assigned to the peptide sequences and, subsequently, differentially expressed proteins can be identified.

Cell lysates were extracted (Gygi et al., 2000) from only 5E10 cells of *A. ferrooxidans* grown up to stationary phase in three different growth media: iron, thiosulfate or sulfur and subjected to trypsin digestion. The resulting peptides were separated on reverse phase column and then analyzed on a ThermoElectron LCQ^{Deca} liquid chromatography ion trap mass spectrometer to confirm the suitability of the samples for further differential analysis. Computational method GLIMMER (Salzberg et al., 1998) was utilized to find the genes in the coding region of *A. ferrooxidans* genome and construct the putative *A. ferrooxidans* protein database. Database search was performed with SEQUEST (Eng et al., 1994), and peptide sequence was then assigned for each MS/MS spectra. Several peptides identified were manually confirmed and used as markers for the subsequent differential FT-ICR analysis and the targeted LCQ analysis. Mass differences that appeared consistently between each pair of analysis were manually investigated. Based on accurate mass-to-charge ratio (m/z) measurements and relative retention times, a correlation of FT-ICR scan number with LCQ retention time was determined and m/z differences identified in the FT-ICR analysis were then correlated to the peptide sequence assignments obtained from preliminary LCQ analysis. Target analysis on LCQ^{Deca} ion trap mass spectrometer at differentially expressed m/z values was performed to obtained peptide sequence information. Differentially expressed proteins were then identified with sequence database search and confirmed manually. Fig. 3A shows a typical biofilm of *A. ferrooxidans* grown on the surface of a sulfur prill. When the total proteins extracted from these cells were analyzed by FT-MS and compared with those seen in ferrous iron or thiosulfate, the highest scores were obtained for peptides derived from proteins that were identified as TrbB and TrbJ amongst others (Fig. 3B). The genes coding for these proteins and their genomic context is seen in Fig. 3C, and was in agreement with that reported previously for this group of genes (Barreto et al., 2003). These proteomic results were validated by using RT-PCR (not shown), real time PCR and DNA macroarrays as seen in Fig. 3D, E. In all cases an increased expression of the *trb* genes analyzed was seen when *A. ferrrooxidans* was grown in a solid substrate such as elemental sulfur. These unpublished results strongly suggest the existence of a functional type IV secretion system in *A. ferrooxidans.* Although it is not yet clear what the function of that system may be in this bacterium, it is likely related to biofilm formation and exchange of macromolecules such as DNA during conjugation. Obviously, an experimental demonstration of conjugal transfer of DNA between *A. ferrooxidans* strains or between this microorganism and other biomining bacteria will have to be demonstrated.

Very recently, high throughput MS has been used in a very interesting metaproteomic approach to study the community proteomics in a natural acid mine drainage microbial biofilm (Ram et al., 2005). These authors were able to detect 2033 proteins from the five most abundant species in the biofilm, including 48% of the predicted proteins from the dominant biofilm organism, *Leptospirillum* group II. Although these results are very

promising, they should be validated, since when ultra-conservative filters were applied for the peptides assigned per gene only one in 4 was a real assignment with a strict criterion. The authors also determined that one abundant novel protein was a cytochrome central to iron oxidation and acid mine drainage formation in the natural biofilm (Ram et al., 2005). This novel approach together with functional metagenomics will offer an integrated study of a microbial community to establish the role each of the participant plays and how they change under different conditions.

7. Concluding remarks

Systems microbiology, which is part of systems biology, is a new way to approach research in biological systems. By this approach it may be possible to explore the new properties of microorganisms that arise from the interplay of genes, proteins, other macromolecules, small molecules, and the environment (Buckley, 2004). This is particularly possible today due to the large numbers of genomic sequences which are becoming increasingly available. However, additional genomic sequences of the different biomining microorganisms will be required to define the molecular adaptations to their environment and the interactions between the members of the community. The idea is to treat the microorganism or community as a whole, integrating fundamental biological knowledge with genomics, metabolomics, and other data to obtain an integrated picture of how a microbial cell or a community operates and how these interactions can be converted into more efficient rates of bioleaching.

The development of an efficient transformation or conjugation system to introduce DNA and a gene knockout system are still lacking for biomining microorganisms. These tools will be essential not only to perform functional genomics and have experimental demonstrations for the suggested gene functions based on bioinformatics analysis of the new post genomic data that is progressively more available, but also to improve bacterial capacity to oxidize iron and sulfur, to form biofilms on the surface of minerals and other properties by the introduction of the corresponding key genes involved. This, together with proper manipulations of the bacterial environment will provide further improvements in the bioleaching rates of biomining operations.

Acknowledgments

This work was supported in part by grant 1030767 from FONDECYT, and ICM-P99-031-F. L.V. was the recipient of a CONICYT Ph.D. scholarship. Spiked RNA *exp1* from *Prunus persica* was a kind gift of M. Gonzalez, from INTA. Sequence data for the *A. ferrooxidans* strain 23270 was obtained from The Institute for Genomic Research website at http://www.tigr.org.

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