# The use of genomics, proteomics and other OMICS technologies for the global understanding of biomining microorganisms

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A B S T R A C T

The use of genomics, proteomics, transcriptomics, metabolomics and other OMICs approaches together with bioinformatics tools allows a global picture of how a microbial cell operates in its community to be obtained. The so-called Systems Microbiology approach treats the microorganism or community as a whole, integrating fundamental biological knowledge with OMICS research.

The oxidative reactions resulting in the extraction of dissolved metal values from ores is the outcome of a consortium of different microorganisms. Therefore, this bioleaching community is particularly amenable for the application of Systems Microbiology. As more genomic sequences of different biomining microorganisms become available, it will be possible to define the molecular adaptations of bacteria to their environment, the interactions between the members of the community and to predict favorable or negative changes to efficiently control metal solubilization. Some key phenomena to understand the process of biomining are biochemistry of iron and sulfur compound oxidation, bacteria–mineral interactions (chemotaxis, cell–cell communication, adhesion, biofilm formation) and several adaptive responses allowing the microorganisms to survive in a bioleaching environment. These variables should be considered in an integrative way from now on. Together with recently developed molecular methods to monitor the behavior and evolution of microbial participants during bioleaching operations, Systems Microbiology will offer a comprehensive view of the bioleaching community.

OMICS approaches are providing exciting new findings that will allow not only predictions on how to keep the microbial consortium healthy but to make it more efficient during the entire process of bioleaching. Some of these recent discoveries will be briefly reviewed here.

## 1. Introduction

Many acidophilic microorganisms have been isolated and characterized as part of the ore bioleaching consortium (Rawlings, 2005; Watling, 2006 and references therein). Recently, the description in theoretical and practical terms of how to develop and optimize a mineral-oxidizing microbial consortium (naturally isolated or constructed) depending on the type of reactor (open or closed) and mineral being used has been described (Rawlings and Johnson, 2007). However, several other microorganisms are present in mining environments and have not been isolated because of the lack of adequate culture methods or selective enrichment methods used to enrich and isolate these microorganisms (Watling, 2006). Due to different temperatures, ferric iron concentrations, increasing toxic metals (Dopson et al., 2003) or salt concentrations used or generated during bioleaching, different bacteria are selected and can be detected as the dominant species (Romero et al., 2003) which are not always the most important for the process itself. The reduction of high bacterial growth rates due to high ionic strengths or very high concentrations of metals may be rate-limiting steps in industrial operations. What approaches could be used to alleviate these problems? The search for new microorganisms by using classical genetic procedures allows the selection of bacteria with improved sulfur and iron oxidizing activities in normal conditions or in high salt or very high metal concentrations. As one recent example, a new isolated *Acidithiobacillus ferrooxidans* has been claimed to have an increased mineral oxidizing activity (Sugio et al., 2006). However, no real improvement in copper recoveries by using this microorganism in industrial bioleaching operations has yet been reported.

How can molecular biology help to improve this complex community of microorganisms to make them more efficient in biomining? The original idea of generating a super biomining bacterium by genetically modifying a microorganism was actively undertaken by several researchers around 20 years ago. Although it has been possible to change the metabolic or other properties in several heterotrophic microorganisms by the introduction of appropriate genes, it has been very difficult or impossible to do it in bioleaching bacteria. A methodology was reported to transform *A. ferrooxidans* (Liu et al., 2000), but this procedure was not very efficient and in practical terms,

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due to its low and difficult reproducibility, it has not contributed to biomining improvements so far. The great importance of being able to introduce DNA in biomining acidophiles is that such a system could be used to generate knock-out mutations in the genes of interest to do functional genomics and have an experimental proof of the supposed or predicted function of the corresponding gene product. In the case of a moderate thermophilic microorganism such as *Acidithiobacillus caldus* MTH-04, the construction of a conjugative transfer system between this extreme acidophile and *Escherichia coli* has recently been reported (Liu et al., 2007b). This system will not only greatly facilitate the genetic and functional study of an important biomining bacterium but may eventually allow improving a strain by using a genetic engineering approach. Although *Sulfolobus acidocaldarius* is an acidophilic archaeon not important for biomining, the recently reported method to introduce foreign DNA into its cells (Albers et al., 2006) may open an avenue to explore transformation systems in biomining hyperthermophiles. Obviously, before attempting these genetic approaches, much more knowledge about the physiology and genomics of these microorganisms will be required to decide which functions, metabolic or oxidative pathways could be modified. This knowledge is greatly increased by the use of the different OMICS technologies.

*A. ferrooxidans* is the most studied microorganism isolated from biomining operations, being considered a model bacterium although in several cases it may not be the most important microorganism in bioleaching. It is a chemolithoauthotrophic Gram negative γ-proteobacterium that obtains its energy from the oxidation of ferrous iron, elemental sulfur, or partially oxidized sulfur compounds (Rawlings, 2005; Rohwerder et al., 2003; Valenzuela et al., 2006 and references therein).

The reactions involved in ferrous iron oxidation have been studied in detail and have recently been reviewed (Rawlings, 2005; Rohwerder et al., 2003). 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 a cytochrome *c*552. A high molecular weight *c*-type cytochrome, Cyc2, which is located in the outer membrane of *A. ferrooxidans* has been suggested to be the prime candidate for the initial electron acceptor in the respiratory pathway between ferrous iron and oxygen (Yarzábal et al., 2002). The mechanisms for the oxidation of the sulfide moiety in metal sulfides and other sulfur reduced compounds are also very important (Rawlings, 2005; Watling, 2006; Valenzuela et al., 2007a,b). For example, during chalcopyrite oxidation by ferric iron, insoluble elemental sulfur is generated on its surface, causing a passivation that hinders the complete dissolution of this copper sulfide during biomining. Therefore, sulfur oxidizing microorganisms will play a key role in the acceleration of chalcopyrite bioleaching. The aerobic oxidation of elemental sulfur by *A. ferrooxidans* and other microorganisms is carried out by a sulfur dioxygenase (Rohwerder et al., 2003). On the other hand, thiosulfate has been postulated as a key intermediate compound in the oxidation of the sulfur moiety of pyrite (thiosulfate mechanism) (Rohwerder et al., 2003). Sulfur compound oxidizing enzymes such as thiosulfate-oxidizing enzyme in *A. ferrooxidans* (Kelly et al., 1997) may be involved in the process. In *A. ferrooxidans* several thiosulfate–sulfur transferase proteins have been described some of which are highly expressed when the bacterium is grown in pyrite and sulfur, but not in ferrous iron (Ramírez et al., 2004), suggesting their implication in sulfur oxidation and metabolism (Valenzuela et al., 2007a; Acosta et al., 2005; Jerez, 2007a). Most heterotrophic microorganisms use a complex of periplasmic proteins (called Sox from sulfur oxidizing) (Friedrich et al., 2001). However, *A. ferrooxidans* genome sequence does not show the presence of genes equivalent to this thiosulfate oxidizing system (Valenzuela et al., 2006, 2007a; Ramírez et al., 2004), and therefore should oxidize this compound by other means. During the oxidation of a metal sulfide such as pyrite, the two *doxDA* genes (whose products are most likely involved in thiosulfate oxidation) of *A. ferrooxidans* are upregulated, in agreement with the proposed thiosulfate mechanism for pyrite oxidation (Valenzuela et al., 2007a).

Other enzymes involved in metabolizing reduced inorganic sulfur compounds are sulfite oxidoreductase, a sulfide:quinone oxidoreductase and tetrathionate hydrolase (previously reviewed in Rawlings, 2005; Kelly et al., 1997). In *A. ferrooxidans*, tetrathionate hydrolase has been recently purified and characterized and its gene identified (Kanao et al., 2007). This protein was previously reported as an outer membrane protein highly synthesized in sulfur-grown cells but not in ferrous iron-grown cells (Buonfiglio et al., 1999). This enzyme has also been purified from *A. thiooxidans* (Tano et al., 1996). Very recently, a novel gene cluster including the gene encoding for tetrathionate hydrolase (*tetH*), which is involved in metabolism of reduced inorganic sulfur compounds has been characterized in *A. caldus* (Rzhepishevska et al., 2007). In this case, TetH was expressed with tetrathionate, pyrite and thiosulfate as substrates.

What is known about sulfur and iron oxidation mechanisms in thermophilic microorganisms? At temperatures greater than 60 °C *Sulfolobus metallicus* and *Metallosphaera* spp. are most likely the most important biomining thermophilic species (Watling, 2006; Hallberg and Johnson, 2001). However, very little if anything is known about the mechanisms that these archaea use to oxidize iron or sulfur. The sulfur oxidation enzymatic activity structure from *Acidianus ambivalens* has been characterized in detail (Urich et al., 2004). Although this microorganism is known to oxidize sulfur, its role in bioleaching is not clearly defined. On the other hand, by using a cDNA substrative hybridization approach, the genetic basis for iron oxidation in the thermophilic archaeon *S. metallicus* has been described for the first time (Bathe and Norris, 2007). Several iron-responsive genes were identified and their expression was characterized under different growth conditions. These authors also reported preliminary proteomic evidence indicating that some of these genes may form a respiratory oxidase complex in the membrane of *S. metallicus*.

How can the OMICS approach help to improve the efficiency of biomining? By using these technologies it may be possible to discover new non-cultivable microorganisms and explore the new properties of microorganisms that arise from the interplay of genes, proteins, other macromolecules, small molecules, and the environment (Buckley, www.asmusa.org). 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.

In this brief review I will concentrate on 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 OMICS (Fig. 1) will have a key role in the understanding of the molecular mechanisms by which the microorganisms attack and solubilize ores.

## 2. The biomining community

Several groups have contributed to the knowledge of the biomining and acid mine drainage community analysis and cannot be reviewed here due to space limitations. For a recent review of the subject, see Schippers (2007). Of extreme importance is not only knowledge of which microorganisms are present in a bioleaching operation, but to be able to monitor their behaviour during the process, to determine the predominant species and the way they evolve in time with the changing environment as the metals are solubilized. In recent years, several molecular methods have been developed for other microorganisms and these have been succesfully applied to many biomining operations (Schippers, 2007 and references therein). The most common techniques employed to explore bacterial diversity use 16S rRNA and rDNA profiles and are culture-

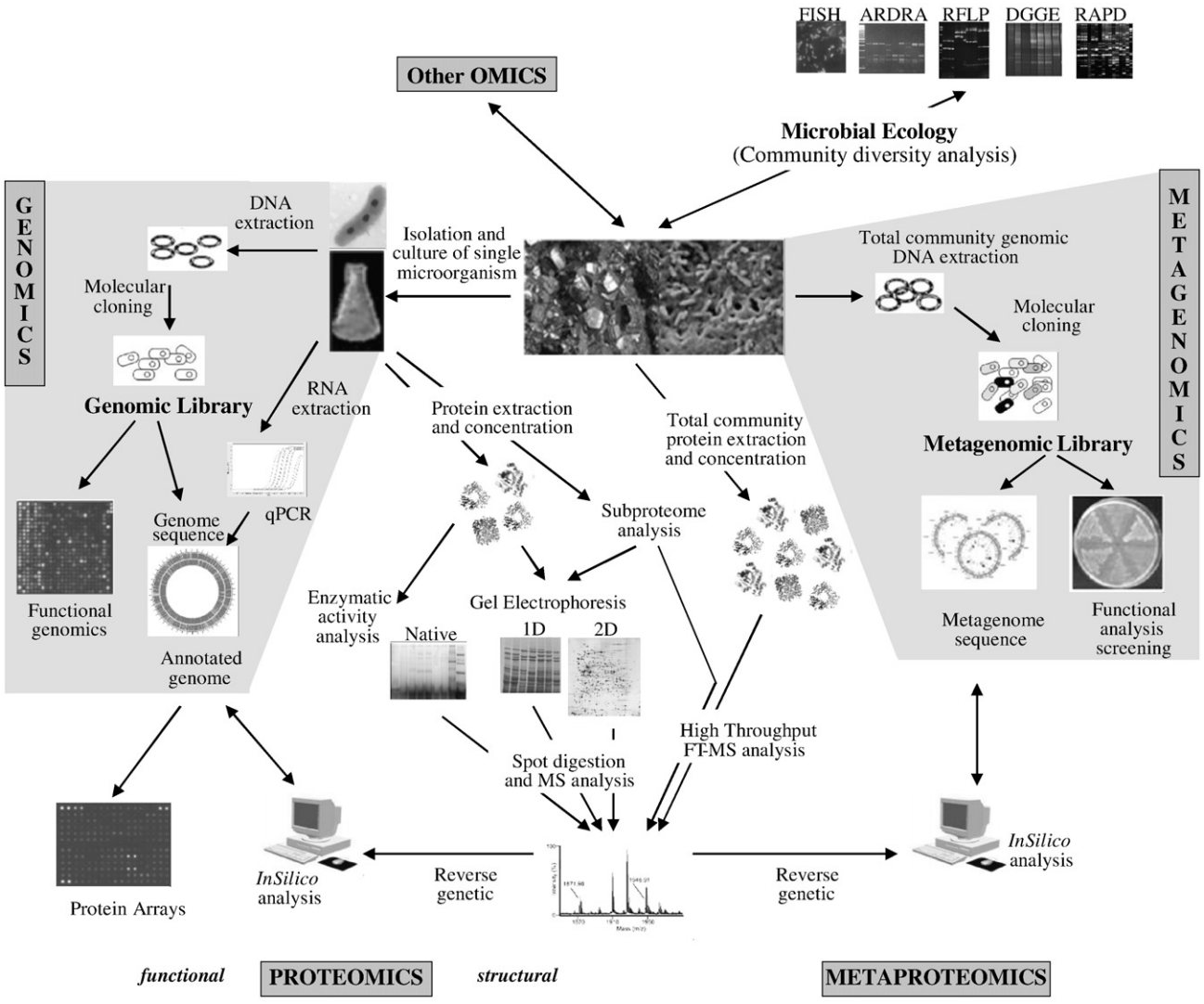


**Fig. 1.** A schematic overview of the main application of OMICS and different molecular biology tools to study the biomining community. Adapted from Valenzuela et al., (2006).

independent methods. Among these methods are included fluorescence *in situ* hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), real time PCR and others (Fig. 1). Very recently, a very promising proteomic approach has been reported for heterotrophic bacteria allowing the identification of the microorganisms present in different samples by determining the dominant proteins synthesized by each bacterium (Liu et al., 2007a). This method can discriminate not only at the species level but also discriminates strains of the same species. It is expected therefore, that this simple, rapid and easy to perform method could also be applied to biomining microorganisms.

So far the genomic sequences of acidophilic microorganisms available are those of *A. ferrooxidans* (TIGR), some reconstructed *Leptospirillum* spp. and *Ferroplasma acidarmanus* (Tyson et al., 2004). *Sulfolobus acidocaldarius* (Chen et al., 2005) and *S. tokodaii* (Kawarabayasi et al., 2001) are also known, although they have not been reported as having a role in bioleaching. Very recently, the genomic DNA sequence of the hyperthermophilic *Metallosphaera sedula* of important use in bioleaching has been reported (Auernik et al., 2008). Actually, there is a great interest in obtaining more genomic sequences of biomining bacteria. A Chilean institution (Fundación Ciencia para la Vida) has already sequenced the genomes of *A. thiooxidans*, and *A. caldus* and the company Biosigma S.A. has isolated some new bacteria from biomining operations and have entirely sequenced their genomes. It is therefore not difficult to predict in the very near future the generation of new DNA microarrays to monitor not only all the microorganisms present in samples from industrial operations but also specific genes such as those indicating the nutritional or stress status of the microorganisms or genes involved in iron or sulfur compound oxidation whose products will be predominant during different stages of active bioleaching.

## 3. Genomics, functional genomics and metagenomics

*A. ferrooxidans* was the first biomining microorganism whose genome was sequenced by TIGR (http://www.tigr.org). This information has been very useful to do genome-wide searches for candidate genes for important metabolic pathways and several important physiological functions. Furthermore, predictions for the functions of many new genes can be done (Fig. 1). The main focus of research has been the energetic metabolism which is directly responsible for bioleaching. Genes involved in phosphate, sulfur and iron metabolism, quorum sensing, those potentially involved in several other functions such as metal resistance, amino acid biosynthesis pathways and those involved in the formation of extracellular polysaccharide (EPS) precursors have been studied. Most of these findings have recently been reviewed (Valenzuela et al., 2006; Jerez, 2007b and references

therein) and therefore mainly the new findings that have appeared after these publications will be summarized here.

Having the genomic sequence of a given biomining microorganism is very important, since it is then possible to formulate hypothesis about the regulation of the expression of most of these genes under different environmental conditions. Metabolic reconstruction and 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 bioinformatic predictions will have to be demonstrated experimentally by using functional genomics, proteomics and other approaches. Some examples seen only when the genome sequence is available is the existence of paralog genes that show sequence similarities but may have different functions in the same microorganism. Several sulfur metabolism related genes coding for thiosulfate–sulfur transferase (TST)-like proteins in *A. ferrooxidans* were found and the activities of some of them were experimentally demonstrated (Acosta et al., 2005). Another example is the presence of two *pet* operons in *A. ferrooxidans* (Bruscella et al., 2007). *A. ferrooxidans* contains a *petIIABC* gene cluster encoding for cytochrome *c*1, cytochrome *b* and a Rieske protein that constitute a bc1 electron-transfer complex. By means of RT-PCR and Northern blotting it was shown that the *petIIABC* cluster was co-transcribed with cycA, encoding a cytochrome *c* belonging to the c4 family, *sdrA*, encoding a putative short-chain dehydrogenase, and *hip*, encoding a high potential iron–sulfur protein, suggesting that the six genes constitute an operon, termed the *petII* operon. The second *pet* operon, *petI* contains a gene cluster that is similarly organized except that it lacks *hip*. Real-time PCR and Northern blot experiments demonstrated that *petI* was transcribed mainly in cells grown in medium containing iron, whereas *petII* was transcribed in cells grown in media containing sulfur or iron. Interestingly, *A. ferrooxidans* is the only organism, to date, to exhibit two functional bc1 complexes (Bruscella et al., 2007). The same authors used genome sequencing and annotation to reconstruct genome-scale metabolic networks in several acidophilic γ-proteobacteria such as *A. ferrooxidans*, *A. caldus* and *A. thiooxidans* (Holmes et al., unpublished). This information has been coupled with published information derived from several other microorganisms including *Leptospirillum* spp., belonging to the deep-branching *Nitrospira* phylum, and the archaea *Ferroplasma* spp. in order to generate novel insights into the biological underpinnings of iron and sulfur oxidation, iron homeostasis, biofilm formation and nutrient recycling in extremely acidic conditions. The recently available genome sequence of *Metallosphaera sedula* DSM 5348, (Auernik et al., 2008) provided insights into biologically catalyzed metal sulfide oxidation. By using comparative genomics, pathways and proteins (in)directly involved with bioleaching were identified. As expected, the genome of this archaeon encoded genes related to autotrophic carbon fixation, metal tolerance and adhesion. The terminal oxidase cluster organization suggested the presence of hybrid quinol-cytochrome oxidase complexes. When compared with *A. ferrooxidans*, the genome of the archaeon *M. sedula*, encoded for at least one putative rusticyanin, involved in iron oxidation and a putative tetrathionate hydrolase (TTH) involved in sulfur oxidation (Auernik et al., 2008). *M. sedula* also contains a gene cluster with homology to *fox* genes in *S. tokodaii* and *S. metallicus* (Auernik et al., 2008), which were demonstrated to be up-regulated on Fe(II) vs S° (Bathe and Norris, 2007). Despite the similarities found in some of the genes involved in the energetic metabolism of other biomining microorganisms, several aspects of the iron and sulfur metabolism of *M. sedula* still remain to be identified and characterized.

The use of microarrays based on the entire genome of *A. ferrooxidans* and other microorganisms will enable a nearly complete view of gene expression of the members of the microbial community under several biomining conditions, helping to monitor their physiological state (lack of nutrients, stressing condition and others) and allow adjustments to be made during the bioleaching process. A first preliminary pilot DNA macroarray formed with 70 different genes was used to study the relative variations in mRNA abundance of some genes related with sulfur metabolism in *A. ferrooxidans* grown in different oxidizable substrates (Valenzuela et al., 2006; Acosta et al., 2005). Using this transcriptomic approach, the genes of C-P lyase, the main bacterial enzyme in charge of degrading phosphonates were recently found to be highly expressed in *A. ferrooxidans* grown in these organic compounds used as the sole phosphorus source (Vera et al., 2008). The existence of a functional C-P lyase system is clearly advantageous for survival under Pi limitation, a condition that may greatly affect bioleaching of ores.

A genome-wide microarray transcript profiling analysis (approximately 3000 genes of the *A. ferrooxidans* ATCC 23270 strain) was later performed (Quatrini et al., 2006; Appia-Ayme et al., 2006). The authors reported the genes preferentially transcribed in ferrous iron growth conditions or in sulfur conditions. These results supported and extended models of iron and sulfur oxidation and supported the possible presence of alternate electron pathways and that the oxidation of these two kinds of oxidizable substrates may be coordinately regulated (Quatrini et al., 2006). By using the same approach, the expression of the genes involved in carbon metabolism of *A. ferrooxidans* was studied in response to different oxidizable substrates (Appia-Ayme et al., 2006). Whole genome transcriptional response analysis was done by using an oligonucleotide microarray for each identifiable protein-encoded ORF in the *M. sedula* genome. This study showed that 88 ORFS were up-regulated 2-fold or more in this archaeon upon addition of ferrous sulfate to the medium. These involved componenents of terminal oxidase clusters predicted to be involved in iron oxidation, as well as genes predicted to participate in sulfur metabolism (Auernik et al., 2008).

As already mentioned, some mining companies are currently interested in doing transcriptomic analysis of their newly isolated microorganisms with improved capacities to leach copper since they already have their genomic sequences.

Metagenomics is the culture-independent genomic analysis of microbial communities (Streit and Schmitz, 2005). In conventional shotgun sequencing of microbial isolates, all shotgun fragments are derived from clones of the same genome. To analyse the genomes of an environmental microbial community (Fig. 1), the ideal situation is to have a low diversity environment. Such systems were found when analyzing the microbial communities inhabiting a site of extreme acid mine drainage production, in which few organisms types were present. Still, variation within each species might complicate assembly of the DNA fragments. Nevertheless, random shotgun sequencing of DNA from this natural acidophilic biofilm was used (Tyson et al., 2004). These authors could reconstruct the near-complete genomes of *Leptospirillum* group II and *Ferroplasma* type II and partially recovered 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, a situation ideal for testing these new culture-independent approaches to the study of environmental communities (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 (Tyson et al., 2004; Streit and Schmitz, 2005; Handelsman, 2004). A full understanding of the biomining community also will require the use of all these current molecular approaches (Fig. 1). Recently new insights for high-resolution ecological studies of microbial activity, *in situ*, have been

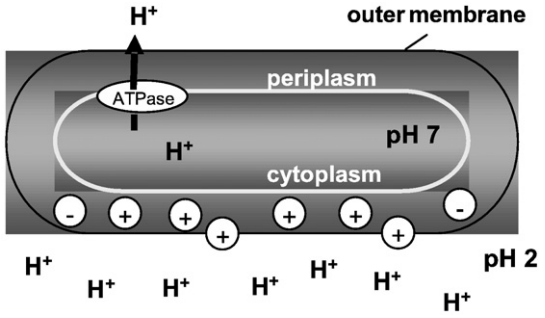


**Fig. 2.** Possible adaptation of acidophilic bacteria to the very acidic external medium. The periplasmic proteins of acidophilic microorganisms such as *A. ferrooxidans* appear to form a positive barrier to retard entrance of protons from outside.

opened by the detection and quantification of gene sequence variability within the studied populations (Allen et al., 2007).

## 4. Proteomics and metaproteomics

Several standard differential proteomic studies have been performed with *A. ferrooxidans* grown under different conditions, especially under sulfur and ferrous iron oxidation conditions, with a good general agreement between these results and those obtained by genomic studies (Valenzuela et al., 2006; Bouchal et al., 2006; Chi et al., 2007). The goal of functional proteomics is to correlate identification and analysis of proteins to the function of genes or proteins. With the discovery of a variety of modular protein domains that have specific binding partners, it has become clear that most proteins occur in protein complexes and that understanding a function of a protein within the cell requires the identification of its interacting partners (Mann et al., 2001). In the case of a biomining bacterium such as *A. ferrooxidans*, the identification of protein complexes involved in oxidative reactions is of high priority. Questions to be addressed are: Are other complexes formed between rusticyanin and proteins in the periplasm? Are there periplasmic complexes involved in sulfur compound oxidation? What other complexes with oxidative reactions are present in this and other microorganisms? Functional proteomics will answer these and many other questions that will help to understand better the metal sulfide oxidation mechanisms (Valenzuela et al., 2007a; Bathe and Norris, 2007). This, together with proper manipulations of the microbial environment (Rawlings, 2005) is expected to produce further improvements in the bioleaching rates of biomining operations.

How can high throughput proteomics studies help in deciphering the nature of unknown genes present in biomining microorganisms? The most relevant reactions for ferrous iron and sulfur oxidation take place at the periplasmic space of *A. ferrooxidans* (Rawlings, 2005; Yarzábal et al., 2002). However, little is known about the periplasm of this acidophile and its components. To further study the proteins that may be involved in the oxidation of sulfur and metal sulfides, we analyzed and characterized by high throughput expression proteomics the proteins present in the periplasmic fraction of this bacterium. A bioinformatic prediction for the localization of all the proteins coded in the *A. ferrooxidans* genome was done (Chi et al., 2007). A theoretical number of around 310 proteins showed predicted export signals typical of those found in the periplasm. Experimentally, by using an ion trap-Fourier transform mass spectrometer, 131 proteins were found in the periplasmic fraction of the acidophilic *A. ferrooxidans* grown in thiosulfate and 220 proteins under all tested growth conditions (Valenzuela et al., 2007a; Chi et al., 2007). The majority (70%) of these periplasmic proteins showed isoelectric points higher than 7. Most of these basic proteins are acid-tolerant and/or acid stable, as illustrated with the well studied basic periplasmic protein rusticyanin. A similar amount of highly basic periplasmic proteins was seen in other acid resistant microorganisms. This was not the case in neutrophiles such as *E. coli* or in alkalophiles, which on the contrary, showed a higher percentage of acidic periplasmic proteins. These results (Chi et al., 2007) strongly suggest a special adaptation of acidophiles such as *A. ferrooxidans* to their acidic environment. The general idea would be that this periplasmic positively charged "barrier" would retard the flux of protons into the periplasmic space (Fig. 2), thus avoiding an excessive acidification of the periplasm.

The periplasmic proteins from *A. ferrooxidans* include many transport and binding proteins, cell envelope proteins, energy metabolism proteins and proteins involved in fate and folding. Fig. 3 shows some of the periplasmic proteins previously described as being associated with iron oxidation and that we confirmed as periplasmic proteins by mass spectrometry analysis (cytochromes *c*4, cytochrome

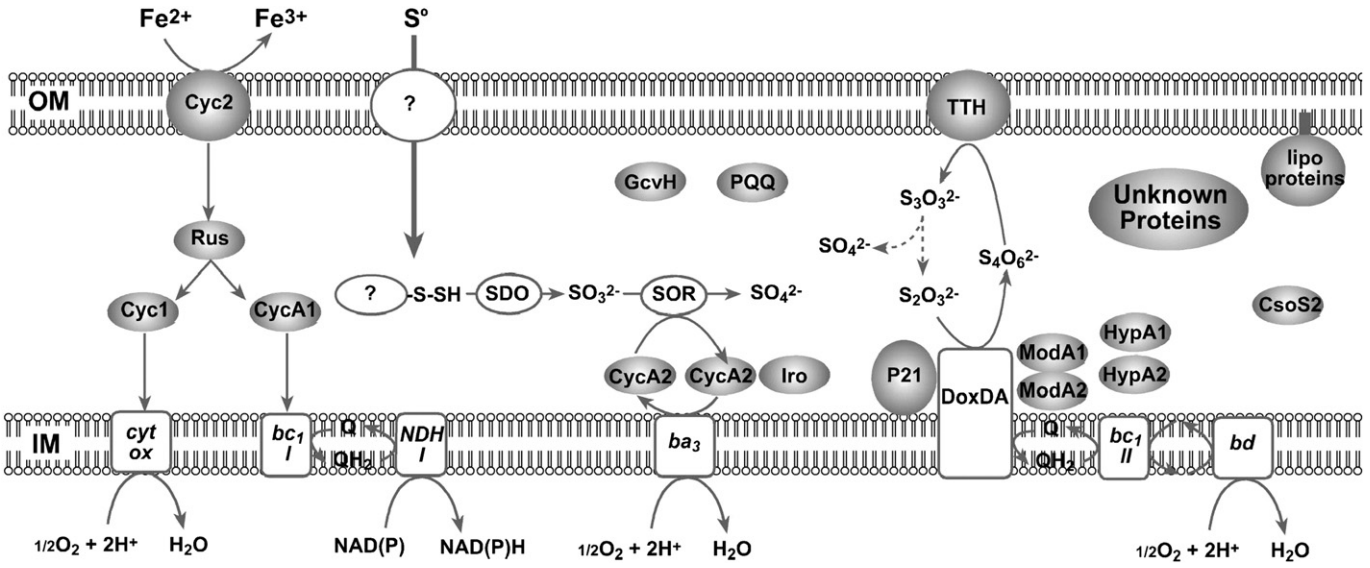


**Fig. 3.** A scheme showing a portion of the surface proteins from *A. ferrooxidans*, most of which are periplasmic proteins involved in energetic metabolism. The proteins shaded in gray were identified experimentally by proteomic analysis of the periplasmic fraction. The unknown periplasmic proteins indicated are most likely unique of *A. ferrooxidans* and may have several different unknown functions. Adapted from Rawlings, (2005) and Chi et al., (2007).

*c*, cytochrome *c*552 and rusticyanin). The putative high redox potential iron–sulfur protein (HIPIP) or iron oxidase (Iro in Fig. 3) has been proposed to be the first electron acceptor in several alternative models of the electron transfer chain between Fe(II) and oxygen (Yamanaka and Fukumori, 1995). Bruscella et al. (2005), cloned the gene encoding for *A. ferrooxidans* HIPIP and overproduced it in the periplasm of *E. coli*. Translocation of this protein into the periplasm of *E. coli* was dependent on the Tat export system. Recent results demonstrated that this protein is present in the periplasm of *A. ferrooxidans*, being the only periplasmic protein with a Tat signal instead of the sec or secP signals present in the rest of the periplasmic proteins (Chi et al., 2007). Fig. 3 also shows some of the proteins involved in sulfur metabolism already mentioned, such as tetrathionate hydrolase (TTH), sulfur dioxygenase (SDO), sulfite oxidoreductase (SOR) and several of the cytochromes involved in both iron and sulfur oxidations.

Several periplasmic proteins were identified as having unknown functions and around 26.1% were proteins with no homologues in databases, many of which may be characteristic of this microorganism (Chi et al., 2007). These findings not only contribute to understanding the physiology of *A. ferrooxidans* but also will be important for the genomic annotation of the new periplasmic proteins from this and other extremophilic bacteria present in the biomining environment. They will also give some clues to their function depending on their differential expression under different oxidizable substrates. For example, the presence of a CopC-like putative copper resistance protein in the periplasm of *A. ferrooxidans* was experimentally confirmed (Chi et al., 2007) and our preliminary results show that the putative *copC* gene is upregulated when the bacterium is subjected to the presence of copper (Navarro and Jerez, unpublished results).

Very recently, high throughput mass spectrometry 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. 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). Lo et al. (2007) used community genomic data sets to identify, with strain specificity, expressed proteins from the dominant member of a genomically uncharacterized natural acidophilic biofilm. Proteomics revealed inter-population genetic exchange, which may be crucial for the adaptation to specific ecological niches within the very acidic metal-rich environment studied. The extremely high sensitivity of novel mass spectrometers can distinguish the behaviour of closely related coexisting microorganisms. This is important since microorganisms grouped together as a single species may have quite different roles in their natural systems (Lo et al., 2007). These novel approaches together with functional metagenomics will offer an integrated study of a microbial community to establish the role each of the participants plays and how they change under different environmental conditions.

## 5. Physiologically important functions in bacteria–mineral interactions

The OMICS procedures summarized in Fig. 1 should be used in close conjunction with the known physiological functions of the microorganisms being studied. It should be possible to better control the activity of the bacteria and archaea by giving them the appropriate nutritional and physicochemical conditions, and by interfering with some of these microbiological functions in order to enhance their action (for metal extraction) in the case of biomining or to inhibit their capacities to control acid mine drainage. Some of these key physiological behaviours are chemotaxis, quorum sensing (QS) and biofilm formation (Fig. 4).

The chemotactic ability of biomining microorganisms is very important for the specific bacterial adherence at the places where the substrates to be oxidized are present. Bacteria such as *L. ferrooxidans* clearly possess chemotactic systems and are attracted by a concentration gradient of ferrous iron (Acuña et al., 1986; Jerez, 2001) such as the one generated on the surface of pyrite (Fig. 4). Although an *A. ferrooxidans* strain has been reported to have a chemotactic response to thiosulfate (Chakraborty and Roy, 1992), it is known that other strains of this microorganism are entirely non-motile. This is the case of *A. ferrooxidans* ATCC 23270. Also, a described phenomenon is the

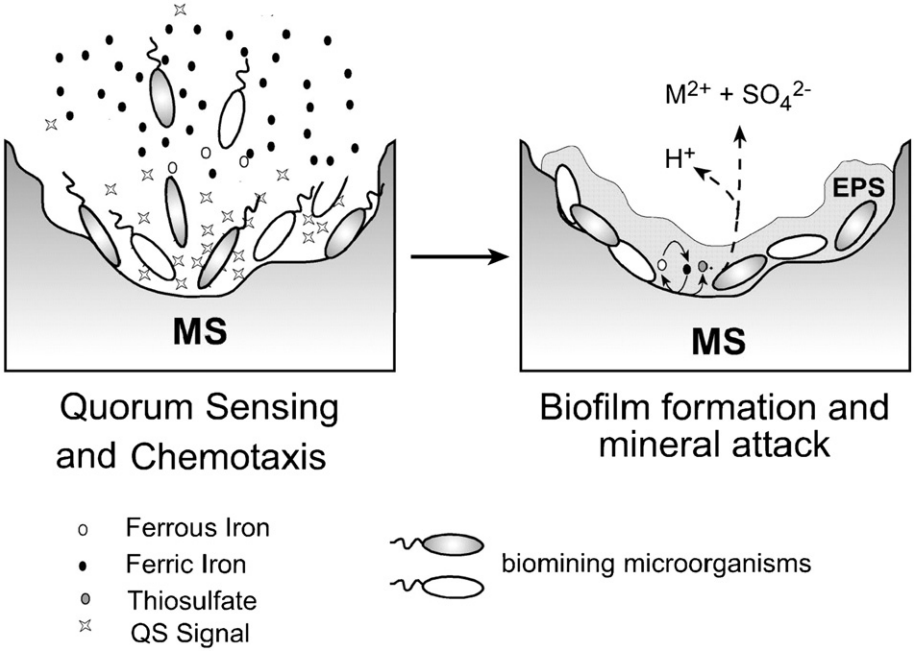


**Fig. 4.** Some physiological responses of bioleaching microorganisms considered important for bacteria–mineral interaction. Interference of these functions may improve or inhibit biological mineral attack. MS, metal sulfide. Adapted from Jerez, (2007b).

lost of flagella and motility in many laboratory strains. A preliminary comparative genomic analysis between the annotated genome of *A. ferrooxidans* ATCC 23270 and draft genome sequences of *A. thiooxidans* ATCC 19377 and *A. caldus* ATCC 51756 indicated that *A. ferrooxidans* lacks genes for flagella formation and also the Che two component signaling transduction system, in agreement with the behaviour seen for this microorganism in the laboratory. The other two thiobacilli contain genes for both properties (Valdés et al., 2007). Nevertheless, as already stressed out, OMICS predictions have to be experimentally demonstrated.

Several microorganisms are known to monitor their own population density through processes collectively described as QS (Miller and Bassler, 2001). In most cases specific genes within the bacteria are switched on at a defined population density (defined bacterial quorum) and the result obtained is the activation of functions required by the population. In almost all cases, the capacity to detect a bacterial quorum depends on the release of a signal molecule from the microorganisms that accumulates in proportion to the cell number (Fig. 4). Thus, QS represents multicellular bacterial action, where cells communicate with each other to coordinate their behaviour. Very recently, it has been demonstrated that *A. ferrooxidans* contains quorum sensor signal molecules (AI) and a QS locus. At least one of these genes was overexpressed when *A. ferrooxidans* was grown on solid elemental sulfur (Valenzuela et al., 2006; Farah et al., 2005), suggesting the possibility that biofilm formation could be controlled in this biomining bacterium by QS.

To date, the role of QS in bioleaching communities is still unknown. However, the characterization of a functional QS type AI-1 system in *A. ferrooxidans* and the recent report of a second AI synthase in *A. ferrooxidans* indicate the importance of these signaling systems in this acidophile (Farah et al., 2005; Rivas et al., 2007). Furthermore, in many heterotrophic microorganisms there is strong evidence indicating that biofilm formation is affected by QS (Gonzalez and Keshavan, 2006).

In the past few years, the importance of the 3′, 5′-cyclic diguanylic acid (c-di-GMP) as second messenger in the regulation of bacterial biofilm formation through the control of the production of extracellular polymeric substances (EPS) has been reported (Hickman et al., 2005; Jenal and Malone, 2006). Preliminary results from our group strongly suggest that *A. ferrooxidans* possesses a c-di-GMP pathway that should be involved in biofilm formation, as it happens in many bacteria (Ruiz et al., 2007). The knowledge of these two important signaling systems will open very interesting opportunities to explore new ways to improve the bioleaching process. Modulation of the attachment of the microorganisms to ores through interference with their QS and communication responses can be envisaged as a new way to control metal extraction by these microorganisms (Valenzuela et al., 2007b).

Biofilms are organized layers of bacteria associated with a solid surface by means of the matrix of EPS. The environment within this community favors intercellular interactions between bacteria. Thus, in the presence of oxygen or aerobiosis, *A. ferrooxidans* can respire molecular oxygen and oxidize Fe(II). Some of the microorganisms in a biofilm are also able to perform Fe(III) respiration under anaerobiosis (Pronk et al., 1992), regenerating Fe(II) that could be used by those microbial cells living in the aerobic zone within the biofilm. Other types of bacteria of the microbial biofilm consortium will generate compounds useful to other members of the community and they can themselves benefit from the metabolic byproducts of other microbes present in the biofilm (Fig. 4).

In fact, *A. ferrooxidans* contains a functional operon which is involved in EPS synthesis and is known to form a biofilm (Barreto et al., 2005). Therefore, as is the case in many gram-negative bacteria (Gonzalez and Keshavan, 2006), the QS type AI-1 of *A. ferrooxidans* could regulate these physiological functions –or some of them– which are key for cellular adhesion and colonization of the mineral.

Until now, in *A. ferrooxidans* or other biomining microorganisms, it is not possible to alter their QS type AI-1 systems by introducing genes through genetic engineering, since as already mentioned, the genetic systems to do it are cumbersome and not easy to use. Therefore, the only practical way actually available for QS interferences in *A. ferrooxidans* is through the chemical approach by using acyl homoserine lactone analogs (Valenzuela et al., 2007b). The use of agonists and antagonists to improve non-specific adhesion and biofilm formation, including exopolysaccharide production will be of great value for bacterial leaching of ores (Fig. 4).

Finally, the development of efficient transformation or conjugation systems to introduce DNA and gene knockout systems are almost entirely 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 postgenomic data, but also to eventually improve some physiological bacterial capabilities. At the same time, the new OMICS methods are greatly helping to monitor more precisely the biomining consortia and it is expected that providing the right physiological conditions to the community together with proper chemical or physical manipulations of the bacterial environment will further improve bioleaching rates in biomining operations.

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