

The Use of Bioinformatics and Genome Biology to Advance Our Understanding of Bioleaching Microorganisms

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1. Summary

With about 300 fully sequenced bacterial and archaeal genomes and with additional information of hundreds of thousands of DNA and protein sequences in public databases it is possible to predict genes and their putative protein products in DNA sequences derived from genome sequencing projects. In about 60% of the cases putative functions can be assigned to the predicted proteins. These assignments can range from near certainty to deep speculation, highlighting the need for subsequent experimental validation of the predictions. High-throughput sequencing, microarray screening and protein expression profiling technologies drive discovery efforts in today's genomics and proteomic laboratories. These tools allow researchers to generate massive amounts of data, at a rate orders of magnitude greater than scientists ever anticipated.

In this chapter, we provide a general overview of how bioinformatics and genome biology can provide insight into the genomic organization and function of biomining microorganisms with a special reference to *Acidithiobacillus ferrooxidans* about which most is known. Bioinformatics and genome biology are effective tools for making preliminary inroads into how an otherwise uncharacterized organism functions. It is particularly powerful in cases where it is difficult to implement conventional genetic tools such as in the case of several bioleaching microorganisms. Bioinformatics and genome biology are effective tools for making preliminary inroads into how an otherwise uncharacterized organism functions. It is particularly powerful in cases where it is difficult to implement conventional genetic tools such as in the case of several bioleaching microorganisms and results are beginning to emerge to support this view (e.g. Selkov et al., 2000; Barreto et al., 2003; Appia-Ayme, 2005; Quatrini^a et al., 2005)

2. Introduction

Commercial biomineral recovery (bioleaching, biooxidation) of copper and gold is a cost effect and environmentally friendly alternative for metal recovery from low grade ores. The complete or nearly complete genome sequences of a number of bioleaching bacteria and archaea are publicly available including genomic information derived from an environmental sample. A study of these genomes and predicted metabolic and regulatory pathways is beginning to provide novel and exciting insights into the metabolism of these microorganisms and how they might work synergistically in tank reactors and in heap bioleaching operation to promote the recovery of metals.

The objective of this chapter is to provide a brief overview of recent progress in the areas of bioinformatics and genome biology as they relate to bioleaching microorganisms. After a short introduction to the principle themes of bioinformatics, metagenomics and genome biology, we will outline different cases of how these approaches have been exploited to reveal novel information. By deconvoluting metabolic potential through bioinformatic analysis new kinds of information can be revealed that not only connect vast amounts of data, but can also capture usable knowledge in the form of biologically valid relations that can subsequently be applied to biotechnological applications such as biomining.

Many questions regarding the biology of microorganisms can normally be addressed by a range of genetic and biochemical experiments. Unfortunately, *A. ferrooxidans* has proved recalcitrant to standard genetic manipulation and genetic analysis of other bioleaching microorganisms is only recently being developed. There is only one report of transformation in.

ferrooxidans and this may be strain specific and not of general use [Kusano et al., 1992] and transduction is unknown. Only recently have techniques for conjugation been established and these remain difficult to control and are of low efficiency [Liu et al., 2000, reviewed in Holmes and Bonnefoy, 2006]. Exacerbating the problem is the difficulty of obtaining sufficient cell mass for many biochemical assays. Given these experimental hurdles, metabolic models derived from bioinformatic analyses offer an especially attractive starting point for unraveling the interesting physiology of bioleaching microorganisms.

3. Introduction to bioinformatics, genome biology, metagenomics and comparative genomics

For the purpose of this chapter bioinformatics is defined as the creation and use of computer algorithms to predict genes, proteins and metabolic pathways, principally in sequenced genomes. Genome biology is the application of these programs to understand the biology of the organism under study or how organisms collaborate in the environment. The distinction is fuzzy at most times but bioinformatics can be thought of as essentially a collection of analytical tools whereas genome biology is more the study of the corpus of information revealed by bioinformatic analyses. Several relevant areas of bioinformatics and genome biology are outlined in Figure 1. Bioinformatic algorithms can predict potential genes in a newly sequenced genome and can suggest regulatory sites for these genes, including potential promoters and transcription factor binding sites. Other bioinformatic algorithms can suggest functions for about 60% of the genes in any genome. The power of bioinformatics is that it is capable of making thousands of predictions for any organism and can yield insight into gene function and metabolism in a way that goes far beyond what is possible using only conventional tools of genetics and molecular biology. However, it is what you do with the data that counts and there are bioinformatic applications that can store, compare, and analyze the voluminous quantities of data generated by the use of new technologies.

Another advantage of bioinformatics is that it can reveal answers to questions that are not formulated as hypotheses because of our ignorance of the system under study – we simply do not know enough to ask the right question sometimes – this is the so-called paradigm shift in biology that has taken place in the last ten years. This chapter will illustrate this point. However, bioinformatic predictions are just that – predictions – and should be subjected to experimental validation whenever possible. This is not always possible given the sheer number of predictions that can be derived from a bioinformatics analysis and in this chapter we provide an example of how bioinformatics can save time and money and steer the laboratory scientist towards the validation and experimental investigation of certain key predictions while leaving aside, for the moment, other bioinformatic predictions that are more certain or, perhaps, of lesser interest.

Validation of bioinformatic predictions can be accomplished using standard tools of genetics and molecular biology such as RT-PCR (reverse transcription polymerase chain reaction), complementation of mutants in heterologous hosts, the use of spectroscopy coupled with the use of electron transport inhibitors, etc. However, the revolution in genomics has also spawned the need for high throughput analytical techniques such as the analysis of the transcription of all the genes of an organisms (transcriptome) by microarray analysis or all the proteins (proteome) by 2-D gel techniques or by mass spectrometry (Figure 1) and this chapter will also explore a few examples of the use of these techniques.

Bioinformatics is an effective tool for making preliminary inroads into how an otherwise uncharacterized organism functions. It is particularly powerful in cases where it is difficult to implement conventional genetic tools or where no such tools have yet been devised, such as is the case for most bioleaching microorganisms. Bioinformatics can open the black boxes that most bioleaching microorganisms represent to the biologist.

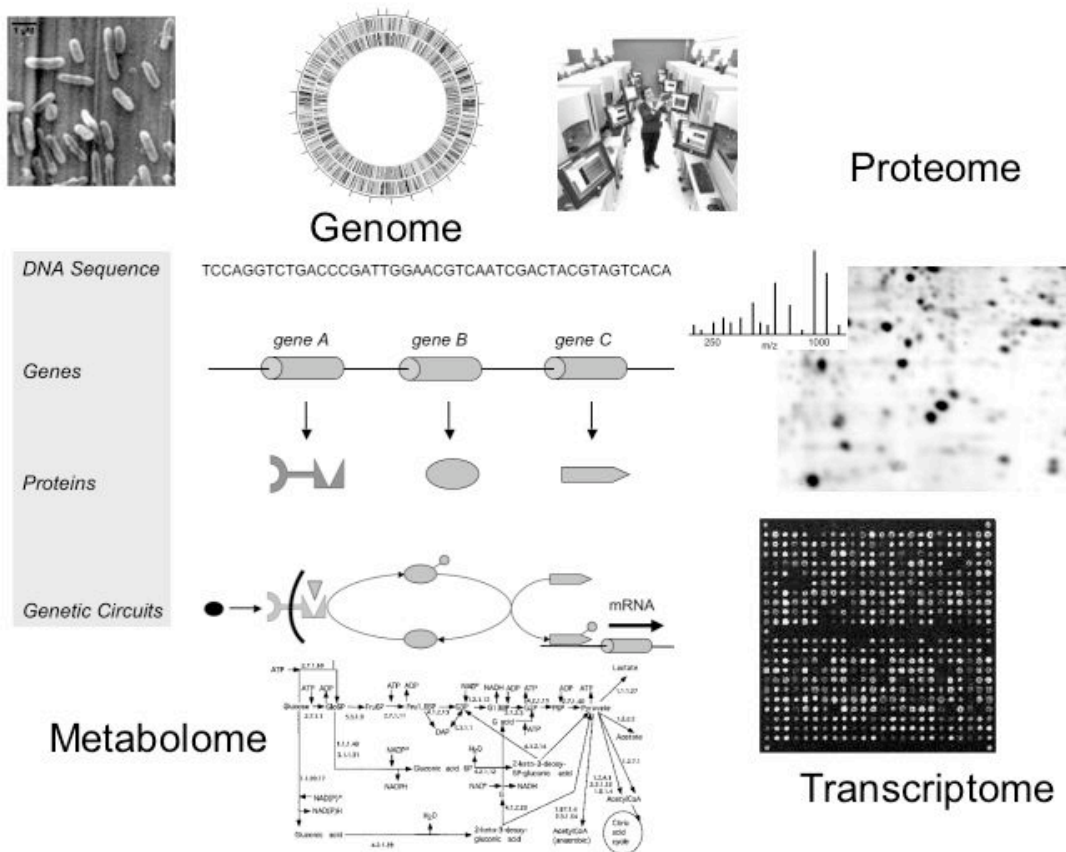


Figure 1. Whole genome sequencing projects (Genome) are enabling the identification of potential genes, proteins and regulatory pathways using a wide range of bioinformatics tools. Further analysis of gene transcription at the mRNA level by microarray analysis (Transcriptome) and proteins by mass spectrophotometry and 2-D gels (Proteome) can suggest metabolic pathways involved in the oxidation of iron and sulfur, microbe-metal interactions, heavy metal resistance and other properties of microorganisms involved in mineral bioleaching.

Other potent uses of bioinformatics are in the emerging fields of metagenomics and comparative genomics. Metagenomics is the culture-independent genomic analysis of microbial communities. The term is derived from the statistical concept of meta-analysis (the process of statistically combining separate analyses) and genomics (the comprehensive analysis of an organism's genetic material) (Schloss et al., 2003). Comparative genomics is, as its name suggests, the comparison of the genetic information and metabolism between different organisms in order to reveal fundamental insights into biological processes. For example, the comparison between pathogenic and non-pathogenic strains of *Escherichia coli* can suggest what genes and functions makes the latter a health hazard. Similarly, a comparison between *A.*

ferrooxidans and *A. thiooxidans* could, in principle, suggest which genes of *A. ferrooxidans* confer on it the capacity to oxidize iron.

An exciting potential of metagenomics is to provide community-wide assessment of metabolic and biogeochemical function. Analysis of specific functions across all members of a community can generate integrated models about how organisms share the workload of maintaining the nutrient and energy budgets of the community. The models can then be tested with genetic and chemical approaches.

Microbiology has experienced a transformation during the last few years that has altered microbiologists' view of microorganisms and how to study them. The realization that most microorganisms cannot be grown readily in pure culture has forced microbiologists to question their belief that the microbial world had been conquered. They have been forced to replace this belief with an acknowledgment of the extent of ignorance about the range of metabolic and organismal diversity. This change fomented a revolution in microbiological thought. At the heart of this revolution was the convincing demonstration that the uncultured microbial world far outsized the cultured world and that this unseen world could be studied (Ward et al., 2005). This change in thinking was prompted by another, equally important realization: microorganisms underpin most of the geochemical cycles that were previously thought to be driven by inorganic processes. The glimmers of insight into the influence that microorganisms exert on the world propelled microbiologists to pursue the uncultured world. In a few years, the study of uncultured microorganisms has expanded beyond asking "Who is there?" to include the difficult question and one that is particularly relevant to the present proposal "What are they doing?"

4. Bioinformatics Can Predict Metabolic Function, Reveal Unexpected Findings and Suggest Industrial Innovations.

In this section, we provide an example of the use of bioinformatics to predict a metabolic function (biofilm formation) in *Acidithiobacillus ferrooxidans* in which an unanticipated result (the presence of a galactose uptake system) was revealed by the analysis and subsequently confirmed by experimental approaches. In addition, the chosen example illustrates the occasional failure of bioinformatics to detect a gene encoding an enzymatic activity for which there is experimental evidence of its existence (GalT). This section also shows how bioinformatic analysis can make predictions that might be of relevance to the industrial use of *A. ferrooxidans* in mineral recovery (the addition of galactose or other sugars might stimulate biofilm formation).

A knowledge of the fundamental physical and biological interactions between a bacterium and a mineral surface are central to understanding the intricacies of interfacial phenomena such as bacterial recognition and attachment to specific mineral surfaces and biofilm formation. To obtain its energy and electron requirements from the oxidation of various forms of reduced sulfur and ferrous iron, and so contribute to mineral dissolution, *A. ferrooxidans* must attach and firmly adhere to mineral surface. This is achieved through the production of an extracellular polysaccharide (EPSs) matrix in which the cells divide and eventually develop into complex biofilms.

Whereas the role of biofilm formation in metal solubilization has been actively evaluated in *A. ferrooxidans* (Gehrke et al., 1998; Sand and Gehrke, 2001), few studies have addressed its biochemical and genetic basis. In this respect, bioinformatic analysis of the genome sequence of the type strain of *A. ferrooxidans* ATCC 23270 has proved particularly useful. The occurrence of several clusters of candidate genes encoding enzymes potentially involved in the metabolism of glucose-1-phosphate via the Leloir pathway and in the subsequent conversion to the extracellular

The Leloir pathway is involved in the conversion of galactose to glucose-1-P. Through this pathway galactose is phosphorylated by galactokinase (GalK) to yield galactose-1-P, which is then converted into glucose-1-P by hexose-1-P uridylyltransferase (GalT), UTP-glucose-1-P uridylyltransferase (GalU) and UDP-glucose epimerase (GalE). The resulting glucose-1-P can then enter the glycolytic pathway or yield the precursor needed for EPS biosynthesis.

Also, a supplementary function catalysed by the enzyme aldose 1-epimerase (GalM) feeds α -galactose into the pathway through the interconversion of the α - and β -galactose. Candidate genes *galK* and *galM* were also found in *A. ferrooxidans* genome and were shown to be organized as part of an operon (*gal* operon) together with *galE*, resembling other well-described *gal* operons (Grossiord et al., 2003). As in other genomes the ortholog of *galU* is transcribed from a discrete and independent promoter. All these genes proved to complement the respective *E. coli* mutants supporting the proposed function of the *gal* operon in this bacteria (Barreto et al., 2005a). In spite of some variation in gene composition and gene order (e.g.: *galPgalMTKE* in *L. lactis*; *galETKMpgm* in *E. coli*; *galKETRM* in *L. casei* and *galETK* in *K. pneumoniae*), the *gal* operon typically includes a *galT* ortholog. Interestingly, an orthologous *galT* gene could not be detected in the genome sequence of *A. ferrooxidans* by bioinformatic analysis, while an enzymatic activity complementing the growth of a *galT* mutant of *E. coli* (S491) was experimentally detected (Barreto et al., 2005a). The associated gene proved to be distinct from other *galT* orthologs.

In most bacteria where the Leloir pathway is functional, galactose enters the cell via GalP permease. The presence of a putative gene (*galP*) potentially encoding a permease in the *A. ferrooxidans* genome with significant similarity to other known galactose importers was, however, unexpected. The functional proficiency and specific role of this permease seemed questionable given that the organism is considered a strict autotroph and that small sugars are known to inhibit its growth. However, gene expression data demonstrated that *galP* is expressed at the level of RNA (both in iron and in sulfur growing cultures) and that transcription is higher in cells grown in iron in the presence of galactose. Also, ¹⁴C-galactose uptake experiments during growth in the presence of sulfur showed that galactose can be taken up by *A. ferrooxidans* and incorporated into EPS (Barreto et al., 2005a,b). Furthermore, the addition of galactose to the growth medium of *A. ferrooxidans*, even though it did not promote growth, stimulated the rate and extent of attachment of cells to pyrite, the amount of EPS formed and the extent of biofilm formation in laboratory conditions (Barreto et al., 2005a,b). This illustrates the paradigm shift in Biology where the powerful new tools of bioinformatics and genome biology allowed the investigators to detect a possible galactose permease where conventional wisdom suggested that none should exist.

The following step in EPS formation is the polymerization of modified sugars on a lipid anchor that is situated in the inner membrane (Broadbent et al., 2003). *A. ferrooxidans* has a candidate operon that potentially encodes for the undecaprenyl pyrophosphate synthetase UppS and the associated anchor formation genes *cdsA*, *dxr* and *pirH*. The modified sugars are attached onto the lipid anchor and polymerized forming chains of sugars of varying length and composition by a variety of glycosyltransferases (GTFs). *A. ferrooxidans* encodes a significant number of different glycosyltransferases, including *epsDEFGH* and *wbaZ*, that could confer a wide range of sugar and linkage specificities. The repeating sugar units of the EPS assembled onto the lipid anchor are predicted to be exported from the cytoplasm to the outer surface of the outer membrane via the Wz protein complex. The activity of these genes has now been validated by protein expression analysis for *A. ferrooxidans* grown on sulfur (Valenzuela et al., 2005).

As in other bacteria, many of the predicted genes involved in EPS biosynthesis of the repeat unit, polymerization and export in *A. ferrooxidans* are clustered in operons. Many of these operons contain as well redundant functions. This aspect of gene organization is likely to enable the bacteria to exploit different ecological niches and to respond to different environmental stimuli through differential regulation. In fact, the nature of the solid substrate is known to influence the chemical composition of the exopolymers and the mode of adhesion of *A. ferrooxidans* (Gehrke et al., 1998). While sulfur-grown cells exhibit purely hydrophobic surface properties and do not attach to pyrite, positively charged exopolymer-complexed iron(III) ions allow the electrochemical interaction of iron grown *A. ferrooxidans* cells with the negatively charged surface of pyrite particles. Modified glucuronic acid is known to be an important component of EPS in iron grown cells (Schippers & Sand, 1999). Candidate *gdhgA* gene, that potentially encodes an enzyme for the conversion of UDP-glucose to UDP-D glucuronate, is embedded in one of the EPS related operons of *A. ferrooxidans*, the specific activation of which may account for the observation pointed above. Microarray and RT/Q-PCR data support the contention that *A. ferrooxidans* may also be able to differentially regulate its EPS-related operons in response to the nature of the substrate (unpublished results).

Several genes of unknown function were present in the Gal- and EPS-related operons of *A. ferrooxidans*. From a bioinformatics perspective knowing the genetic context of a gene with unknown function is important because it can assist in assigning to it a putative function. An example is the case of an ORF encoding a *luxA-like* gene within the *gal* operon of *A. ferrooxidans*. The predicted gene product exhibits similarity with the LuxA family of bacterial luciferase-like monooxygenases (pfam00296) and with F420-dependent oxidoreductases (COG2141), of which glucose-6-P dehydrogenase from *Rhizobium sp.* is a member (Streit et al., 2004). The similarity to a glucose-6-P dehydrogenase, that catalyzes the conversion of glucose-6-P to 6-phosphogluconolactone, could pinpoint the connection between the glucose-1-P pathway (Leloir pathway) mediated by the *gal* genes and the pentose phosphate pathway. The presence of a *pgm* ortholog (phosphoglucomutase) in the *gal* operon of *A. ferrooxidans* whose role is the conversion of glucose-1-P, the central precursor in nucleotide sugar biosynthesis, to glucose-6-P, an intermediate in sugar breakdown and a major point of entry of carbon into the pentose phosphate pathway, further supports this contention. Glucose-6-P could then be converted to 6-phosphogluconolactone by the hypothetical product of the *luxA-like* gene. Phosphoglucomutase has been shown to play a key role in controlling the flux through the Leloir pathway in yeast, probably due to increased conversion of glucose-1-P to glucose-6-P (Bro et al., 2005). Additionally, if LuxA-like exhibited reverse activity, it could catalyze the conversion of 6-phosphogluconolactone to glucose-6-phosphate, and then it could help channel products of CO₂ fixation towards the formation of EPS precursors.

Bioinformatic reconstruction thus suggests that galactose can be fed to glucose-1-P via the Leloir pathway and diverted towards the formation of EPS precursors, although alternative sources of glucose-1-P such as via gluconeogenesis or glycogen utilization might be called upon to synthesize biofilms when needed. What actually happens in natural environments, where the source and percentage of the galactose that is available and ends up in biofilms is uncertain, remains to be evaluated. A possible source of environmental galactose, however, might be the assorted heterotrophic microorganisms long known to be associated with *A. ferrooxidans* in bioleaching operations (Bacelar-Nicolau & Johnson, 1999; Marchand et al., 2002).

These considerations raise the question as to whether the addition of galactose, or compounds that contain galactose, could enhance the rate and/or extent of mineral leaching in an industrial operation. The addition of galactose to laboratory cultures promotes the formation of

EPS and biofilms (Barreto et al., 2005b), but whether this would occur in an industrial setting and whether this resulted in faster rates and/or higher yields of metal recovery are still issues to be explored. Another issue is how the formation of biofilms by *A. ferrooxidans* is regulated. The discovery of a Lux-like quorum sensing system in this microorganisms might help explain how it senses cell density in its environment (Rivas et al., 2005; Farah et al., 2005)

5. Bioinformatics Can Help Focus The Experimental Biologist and Can Predict Genetic Regulatory Connections

This section provides an example of how bioinformatics can be used to help direct the experimental biologist towards challenging and interesting biological questions and, in this way, can save time and money. It also illustrates the power of bioinformatics to predict genetic regulatory connections.

Prior to the availability of the *A. ferrooxidans* genome sequence, only one gene involved in sulfur assimilation, encoding a possible ATP sulfurylase/kinase activity, had been described in this microorganism (Fry and Garcia, 1989). However, after a bioinformatic analysis of its genome sequence, a much more comprehensive picture of the genes and pathways potentially involved in sulfur (sulfate) uptake and assimilation began to emerge (Valdes et al., 2003). This study also suggested potential interconnections of sulfur metabolism with other core processes such as nitrogen fixation, hydrogen utilization, biofilm formation and amino acid metabolism (Fig. 3).

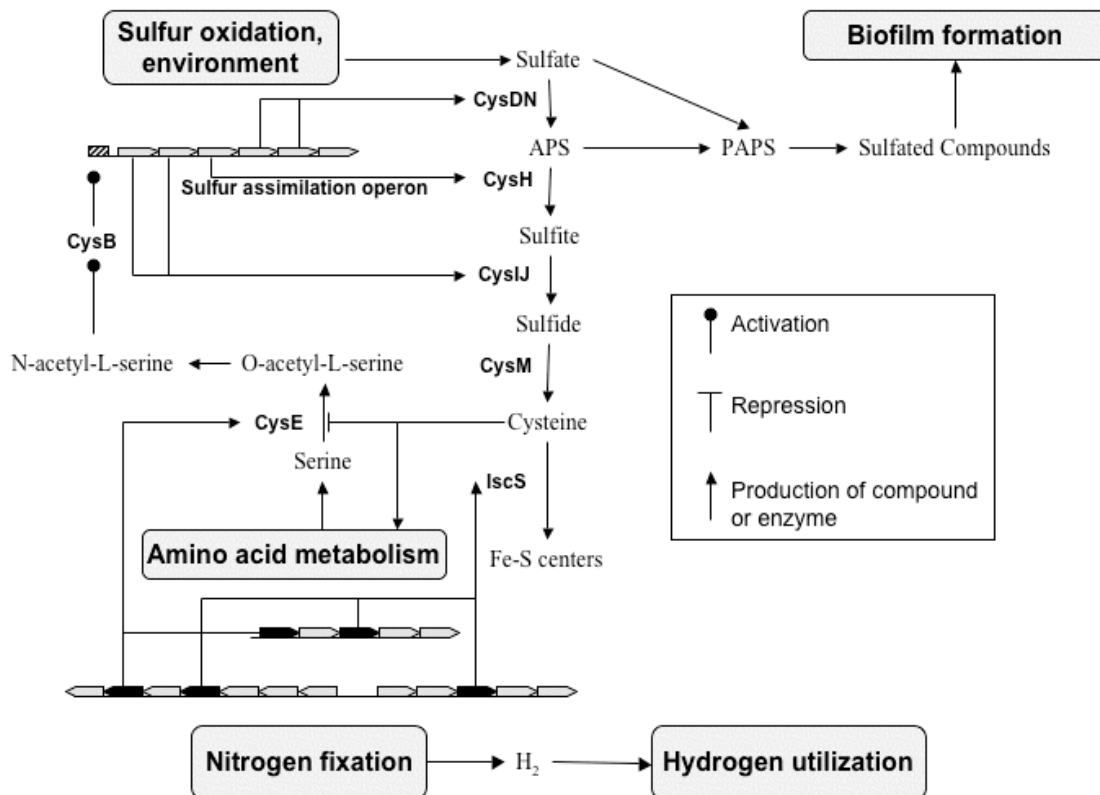


Figure 3. Bioinformatic predictions for sulfate uptake and assimilation in *A. ferrooxidans* with suggested connections to amino acid metabolism, nitrogen fixation and hydrogen utilization (modified from Valdes et al., 2003).

The predicted enzymes that convert sulfate to cysteine in *A. ferrooxidans* exhibit significant similarity with genes in other organisms known to carry out this conversion (Leyh et al., 1998). Further, the potential genes encoding these enzymes are organized in a gene cluster (*cysIJHDN*) that is probably an operon. These bioinformatic predictions are relatively sound and their experimental validation can be placed on a lower priority compared, for example, with the much weaker, but perhaps more interesting, bioinformatic prediction of the link between sulfate and biofilm formation via PAPS or the connection between nitrogen fixation and hydrogen utilization (Fig. 3). These predictions could help explain how *A. ferrooxidans* attaches to sulfur minerals and why its genome contains hydrogenases.

Bioinformatics can also be used to infer gene regulation. Regulation of genes and metabolic pathways are an important processes to understand because they connect cellular processes with external signals from the environment, which can modulate the function of the microorganism promoting or reducing its capacity to bioleach minerals. In the case of sulfur assimilation in *A. ferrooxidans*, an analysis of the predicted genes and pathways involved suggests that sulfate uptake and assimilation can be controlled in this microorganism by processes that are similar to those experimentally validated in other organisms.

For example, it can be predicted that, if sulfur is channeled by IscS to form Fe-S centers in proteins required for nitrogen fixation, then an increased expression of CysE will promote the formation of O-acetyl-L-serine (Fig. 3). This latter compound can spontaneously isomerizes to N-acetyl-serine which is a co-factor for the positive gene regulator CysB (Lochowska et al, 2001). CysB coupled to N-acetyl-serine is predicted to activate the sulfur assimilation operon which will stimulate the uptake of sulfate from the environment, restoring intracellular levels of sulfur depleted by the need to form Fe-S proteins. Conversely, excess cysteine in the cell limits the amount of O-acetyl-L-serine for CysB activation. A reduction in CysB activity reduces the expression of the sulfate uptake operon lowering, in turn, the assimilation of sulfate until intracellular cysteine levels are restored – a beautiful example of a homeostatic process predicted to be at work in *A. ferrooxidans*.

Recent microarray experiments that compare gene transcription profiles of *A. ferrooxidans* grown in either iron or sulfur medium support this model (Quatrini et al., 2005a). Genes proposed to be part of the sulfur assimilation operon *cysIJHDN* are much more expressed in cells grown iron medium compared to sulfur medium, an observation that is consistent with the idea that sulfate is more limiting in the iron medium.

The challenge now is to integrate the regulation of sulfur assimilation for biosynthetic processes such as amino acid formation and the production of Fe-S centers with the use of sulfate as an energy and electron source. The regulation of this latter process is not well understood in any organism, making it a difficult, but not necessarily impossible, task for bioinformatics analysis.

6. Bioinformatics can model full metabolic responses and pinpoint unusual metabolic features that are likely to make functional differences.

This section demonstrates the power of bioinformatics to make predictions not only about genes, proteins and pathways but also how it can suggest global regulatory networks that can integrate major cellular responses, in this case, iron uptake and homeostasis. This example also serves to illustrate how an initial question, how does *A. ferrooxidans* regulate its iron metabolism, can lead to new directions that had not been anticipated. It is shown that *A. ferrooxidans* has an unexpected number of iron uptake systems that led to the suggestion, subsequently validated by experiment, that the microorganism can grow at near neutral pH (unpublished results). The discovery of an extensive repertoire of mechanisms for iron uptake also suggests a possible reason for the sensitivity of *A. ferrooxidans* to high iron loads that limit its ability to bioleach minerals.

A. ferrooxidans grows aerobically at extremely acidic pHs (pH 1-2) in environmental situations where it is confronted with high concentrations of iron. Not only is Fe(II) more stable in these conditions (than at neutral pH in aerobic conditions) but Fe(III) is also much more soluble (>0.1 M), exceeding by seven orders of magnitude typical bacterial iron requirements (10⁻⁸ M). Furthermore, *A. ferrooxidans* uses FeII as an energy and electron source, thus it is presented with a dilemma: how to benefit from the high iron concentrations that the environment offers fulfilling its energy and micronutrient requirements, whilst simultaneously escaping the potentially harmful effects of eventually high intracellular iron loads? How does the microorganism regulate the use of iron as an energy source versus its need as a micronutrient? These aspects intuitively suggested that *A. ferrooxidans* could exhibit novel mechanisms to cope with iron homeostasis, to ensure a tight homeostatic control and that it might have developed iron uptake mechanisms quite distinct from those of neutrophilic organisms who are faced with a limited iron supply.

Contrary to this presumption *A. ferrooxidans* bioinformatic analysis revealed the presence of a number of potential genes and regulatory pathways involved in iron uptake typical of those found in neutrophilic organisms. Given the presence of such genes, the iron homeostatic response in *A. ferrooxidans* was modeled *in silico* based upon known schemes in other organisms. Through this strategy, however, several unusual genetic features were revealed that are thought to reflect special requirements for iron uptake and homeostasis in *A. ferrooxidans* peculiar growth conditions.

Bioinformatic analysis revealed the presence of candidate genes potentially encoding two distinct Fe(II) uptake systems, FeoAB and MntH, that may enable *A. ferrooxidans* to take advantage of the readily bioavailable Fe(II) through direct uptake. As in other microorganisms, (where instead FeoB contributes to iron uptake under anaerobic or mildly acidic microaerophilic conditions), the *feoB* gene in *A. ferrooxidans* is organized in operon with a small open reading frame (ORF) termed *feoA*. However, it has additionally captured in the same locus a predicted gene encoding a porin-like protein (*porA*). This porin exhibits similarity to OprB (COG3659, pfam04966) which has been implicated in the movement of carbohydrates across the outer membrane but that can also transport other ions [Wylie et al., 1993], suggesting that it may contribute to the uptake of Fe(II). The presence of a Fur box-like sequence upstream of this porin suggests a typical Fur mediated iron-dependent repression of the whole gene cluster [Quatrini et al., 2005b,c]. A conserved organization in *Geobacter sulfurreducens* further supports this hypothesis [Rodionov et al., 2004].

The presence of 11 distinct candidate TonB-dependent Fe(III) siderophore outer membrane receptors (OMRs) for high-affinity acquisition of iron in the ferric valence state, came as a surprising finding of the *in silico* metabolic reconstruction. This number rivals or exceeds the complexity found in well-studied neutrophilic bacteria that must scavenge iron from their environment [Quatrini et al., 2005c]. Such a finding argues against the simplistic view that *A. ferrooxidans* has an easy time encountering and taking up readily available soluble iron. It is possible that this complexity reflects the capacity of *A. ferrooxidans* to live at higher pHs than those typically associated with its growth in ferrous sulfate (pH 2) and sulfur (pH 3.5), in which it must scavenge less soluble forms of iron. Demonstrations of the capacity of *A. ferrooxidans* to live at pH 4 in sulfur [Pronk et al., 1991; Vian et al., 1986] and, more recently, at pH 5.5 (Barreto et al., 2005a,b) support this point of view. Furthermore, the diverse range of specificities and pIs encountered for *A. ferrooxidans* OMRs supports the contention that different systems are functioning at different pHs and taking up different iron sources. Consistently, hydroxamate-type siderophores are capable of forming stable iron complexes at low pH, while iron is easily dissociated from catechols-type siderophores under these conditions (Payne, 1994).

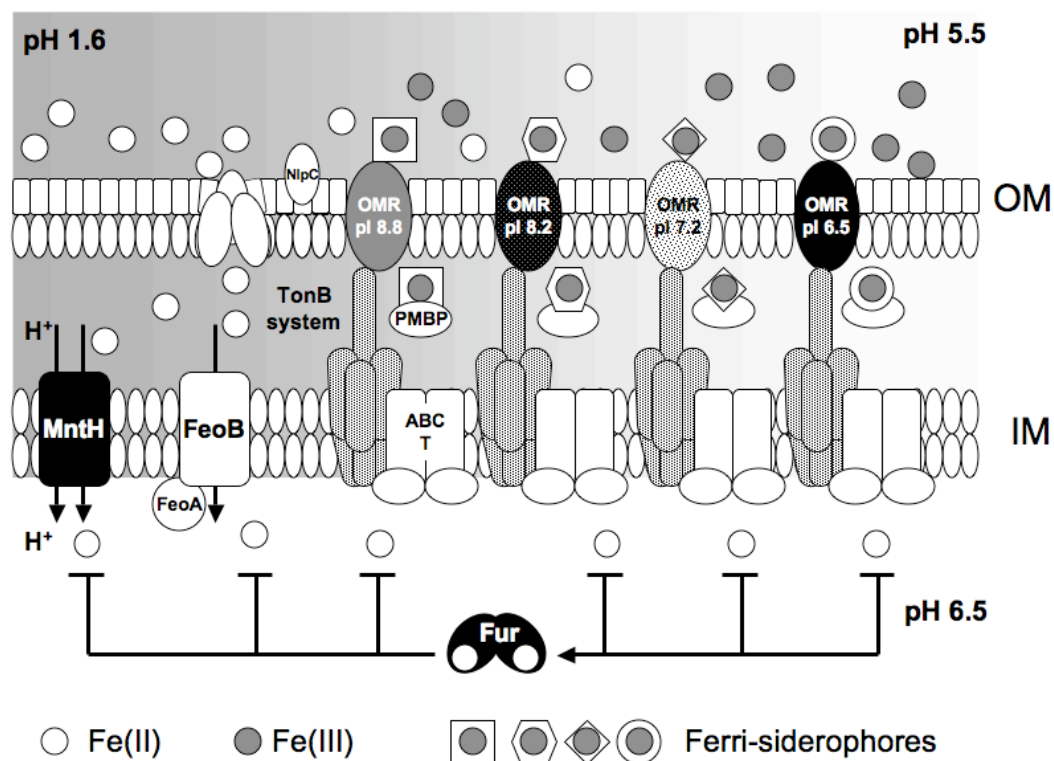


Figure 4. Model of iron uptake and homeostatic mechanisms in *A. ferrooxidans* predicted by bioinformatics analysis. OM, outer membrane; IM, inner membrane; OMR, outer membrane receptor.

Unlike many bacteria that take up iron via siderophores produced and excreted into the environment by themselves, *A. ferrooxidans* does not carry genes that might be involved in conventional siderophore production. Nevertheless, the fact that it bears receptors for dicitrate and heterologous, hydroxamate- and catechol-type siderophores immediately suggests that it can compete efficiently for Fe(III) with siderophore producing organisms within bioleaching

consortia or within biofilm forming bacteria sharing common environmental niches [González-Toril et al., 2003]. The very same feature, might help to explain why *Leptospirillum* strains outgrow *A. ferrooxidans* and dominate the microbial population in the presence of high concentrations of Fe(III) [Rawlings et al., 1999]. The fact that *At. ferrooxidans* possesses more predicted Fe(III) uptake transporters than *Leptospirillum* sp. and thus a different inherent ability to take up Fe(III) could render it more susceptible to higher iron concentrations. In this way the high concentrations of Fe(III) evolve upon Fe(II) biooxidation would confer a selective advantage to *Leptospirillum* strains over *A. ferrooxidans* (Quatrini et al., 2005c).

Taken together this data point out the great complexity and diversity of iron uptake systems potentially expressed by *A. ferrooxidans* that are probably an accurate reflection of its metabolic plasticity and its ability to survive under significantly different environmental conditions.

7. METAGENOMICS AND COMPARATIVE GENOMICS

This section illustrates how metagenomics is being used to determine the community structure of bioleaching operations. It also provides examples of how a metagenomic project can generate novel insights into microbial community interspecies cooperation and how it can suggest ways to culture previously uncultivated microorganisms. How comparative genomics can reveal information regarding the diversity of microbial iron oxidation mechanisms has been recently reviewed in brief (Holmes and Bonnefoy, 2006)

Metagenomics can be used to address the challenge of studying prokaryotes in the environment that are, as yet, unculturable and which represent more than 99% of the organisms in some environments. This approach builds on recent advances in microbial genomics and in the polymerase chain reaction (PCR) amplification and cloning of genes that share sequence similarity (e.g. 16S rRNA, *nif*, *recA*) directly from environmental samples. Such samples can then be analyzed by techniques such as Denaturing Gradient Gel Electrophoresis (DGGE) and Restriction Fragment Length Polymorphism (RFLP) gel electrophoresis providing information as to the species diversity in a sample. Alternatively, the sequences can be labeled with a fluorescent marker and used to locate and, in some cases quantitate, the distribution of species in an environmental sample by FISH (fluorescent in situ hybridization) (Handelsman, 2004).

The need to use non-cultivable techniques to enumerate microorganisms in heap bioleaching operations was first suggested 20 years ago (Yates and Holmes, 1986a,b) and an overview of the process was later published (Holmes, 1991). However, these initial probe approaches were used to enumerate bacteria in cultivation and it was not until recently that the techniques were used to explore the microbial diversity of bioleaching operations and natural communities.

An improved understanding of the microbiology of bioleaching heaps has been identified as key to advancing commercial bioheap operations (Brierley, 2001). However, despite advances in our understanding of the microbiology of stirred tank biooxidation of gold (Rawlings, 2002) and the microbial constitution of some environmental situations (Johnson 1998; Gonzalez-Toril et al., 2003; Tyson et al. 2004), the microbial ecology of copper bioleaching heaps is still poorly comprehended. Early work, involving counts of live, cultivatable microorganisms and indirect measurements such as oxygen uptake, redox potential, pH, etc., provided an initial description of the bulk biological activity in bioleaching heaps (Brierley, 2001). Additional information has come from the laboratory cultivation of pure and mixed cultures of microorganisms associated with industrial-scale heap leaching processes. This has generated information regarding the identification and properties of *Acidithiobacillus* spp., *Sulfurisphaera*-like, *Leptospirillum* spp.,

Ferroplasma spp. and *Sulfobacillus*-like microorganisms (Goebel and Stackebrandt, 1994; Rawlings, 2002; Olson et al., 2003; reviewed in Holmes and Bonnefoy, 2006).

More recently, culture-independent approaches based on PCR amplification and denaturing gradient gel electrophoresis (DGGE) and sequencing of 16S rRNA gene fragments from both Bacteria and Archaea have been used to analyze the microbial community inhabiting a low-grade copper sulfide run-of-mine (ROM) bioleaching test heaps (Demergasso^{a,b} et al., 2005; Coram-Uliana et al., 2005). Three important conclusions can be drawn from these studies. First, although it has proved difficult, due to contamination from rock particles, it is possible to isolate DNA of reasonable quality directly from bioleaching heaps without the intervention of bacterial culturing. This prerequisite is essential for any subsequent metagenomic analysis.

Second, it was shown that heap bioleaching, like tank biooxidation (reviewed in Rawlings, 2005) and heap bioleaching (Demergasso et al., 2005a) proceeds in three stages. These stages result from temperature increases due to exothermic biological oxidation of iron and sulfur: an early stage favoring mesophilic microorganisms (30-40°C) such as *At. ferrooxidans*, *At. thiooxidans*-like bacteria and *Sulfurisphaera*-like archaea.; a second stage when the temperature begins to rise (40-55°C) when *At. caldus*, *Leptospirillum* and *Ferroplasma* groups become dominant and a final stage (55-65°C or higher) where *Sulfobacillus*-like bacteria such as *Alicyclobacillus* (formerly *Sulfobacillus*, Karavaiko et al., 2005) became dominant and archaea such as *Ferroplasma* thrive. This means that the development and interaction of each of these microbial communities, including possible community biofilm formation in the case of heap bioleaching, must be considered in order to fully understand bioleaching processes and suggest ways by which they can be improved.

Third, the microbial diversity in a bioleaching heap, although not as limited as the Richmond Iron Mountain acid mine drainage biofilm (Tyson et al., 2004), is probably not as complex as naturally occurring acidic environments such as the Rio Tinto where thousands of years of natural selection have promoted a quite diverse ecosystem (Lopez-Archilla et al., 2001). From a practical standpoint, this is important because the restricted diversity of microorganisms suggest that a meaningful interpretation of community structure and function of a bioleaching heap can be derived from a metagenomics analysis.

The best example of a metagenomic project that concerns bioleaching microorganisms, or close relatives of them, is the nearly complete sequencing of the metagenome of a biofilm community in acid drainage of the Richmond Iron Mountain mine (Tyson et al., 2004). This biofilm community is very limited in diversity with *Leptospirillum* group II microorganisms accounting for 75%, *Leptospirillum* group III for 10%, Eukaryotic species for 4% and Archaea 1% (including *Ferroplasma* sp.) of the abundance of organisms respectively. One reason for the reduced diversity could be the very restrictive growth conditions imposed by the low pH (<1). Also, the biofilm was located deep in a mine shaft far from the activity of phototrophs who probably supply most of the fixed carbon in environments such as the Rio Tinto. Carbon limitation will select for chemoautotrophs and reduce the biodiversity possible. Nitrogen limitation, as will be described below, also probably restricts biodiversity.

The metagenomic sequence of Iron Mountain challenged a number of significant hypotheses. First, it appears that *Leptospirillum* group III contains genes with similarity to those known to be involved in nitrogen fixation, suggesting that it provides the community with fixed nitrogen. This was a surprise because the previous supposition was that a numerically dominant member of the community, such as *Leptospirillum* group II, would be responsible for nitrogen fixation. However, no genes for nitrogen fixation were found in the *Leptospirillum* group II genome, leading the authors to suggest that the group III organism, now identified as *L. ferrodiazotrophum* (Tyson et

al., 2005) is a keystone species that has a low numerical representation but provides a service that is essential to community function. The identification of *L. ferrodiazotrophum* as the sole nitrogen fixer in the Iron Mountain community also permitted the development of a selective procedure for its isolation and cultivation as a pure strain highlighting how environmental sequence data can provide insights for culturing previously uncultured microorganisms (Tyson et al., 2005).

Furthermore, the prevailing idea that *Ferroplasma* strains, including those found at Iron Mountain, can fix CO₂ has been challenged (Dopson et al., 2005). If it turns out that they are organomixotrophs incapable of fixing CO₂ then some other member of the Iron Mountain community, such as *Leptospirillum*, must be providing the fixed carbon.

Finally, a metaproteome analysis of the Iron Mountain biofilm community, in which proteins were isolated directly from the environment, indicates that a cytochrome predicted to be involved in iron oxidation is a major component of the community proteome (Ram et al., 2005). This protein is encoded by Group II *Leptospirillum* microorganisms that are the dominant community members. Combined metagenomic-metaproteomic approaches are beginning to paint a preliminary picture of autotrophic life at low pH and are likely in the future to yield information regarding the synergistic interactions of microorganisms that will prove useful for improving bioleaching applications

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