Hydrometallurgy 104 (2010) 471–476

Contents lists available at ScienceDirect

Hydrometallurgy

journal homepage: www.elsevier.com/locate/hydromet

Comparative genomics begins to unravel the ecophysiology of bioleaching

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ARTICLE INFO

Article history:
Received 22 January 2010
Received in revised form 26 March 2010
Accepted 31 March 2010
Available online 22 June 2010

Keywords:
CO_2 fixation
Acidithiobacillus
Bioleaching
Biomining
Bioinformatics
Comparative genomics
Metagenomic
Ecophysiology

ABSTRACT

A comparison of the metabolic potential of 20 bioleaching microorganisms and their close relatives from the Eubacteria and Archaea kingdoms permits the prediction of inter- and intra-species physiological interactions (ecophysiology) during spatial and temporal changes that are known to occur within industrial bioleaching heaps. Genome analysis has allowed preliminary models to be built for genes and pathways involved in key processes such as nitrogen and carbon cycling, sulfur and iron uptake and homeostasis, extra-cellular polysaccharide biosynthesis, heavy metal resistance and energy metabolism. This paper will focus on the diverse ways that bioleaching microorganisms obtain carbon from their environment with a particular emphasis on elucidating how these processes might be expected to vary over space and time during the lifetime of a bioleaching operation. It is anticipated that this knowledge will improve our understanding of fundamental biological processes in extremely acidic environments.

1. Introduction

With the sequences of over 1000 microbial genomes completed and more than 2400 in progress (http://microbialgenomics.energy.gov/databases.shtml), comparative genomics has become a powerful tool to improve gene identification and to predict metabolic potential and its regulation. At least sixteen genomes of bioleaching-related bacteria and archaea have been completed or are in progress. These include genomes from type strains such as *Acidithiobacillus ferrooxidans* (Valdes et al., 2008a), *Acidithiobacillus caldus* (Valdes et al., 2009), *Acidithiobacillus thiooxidans*, *Acidiphilium cryptum*, *Metallosphaera sedula* (Auernik et al., 2008), *Acidimicrobium ferrooxidans*, *Leptospirillum ferrooxidans*, *Ferroplasma* sp., *Sulfolobus metallicus* (Bathe and Norris, 2007) and *Acidianus brierleyi* and from several other microorganisms isolated from bioleaching environments (Levican et al., 2008). In addition, gene and microbial community information is being derived from several relevant metagenomics projects (Tyson et al., 2004; Allen et al., 2007). This wealth of data is yielding valuable insight into the metabolic capabilities and interactions that help develop and sustain microbial community structure (microbial consortia) and function (ecophysiology) during bioleaching.

In previous work, we compared the genomes of *A. ferrooxidans* ATCC 23270, *A. thiooxidans* ATCC 19377 and *A. caldus* ATCC 51756 (Valdes et al., 2008b). This allowed the prediction of metabolic and regulatory models for a group of related bacteria known to be involved in the early stages of bioleaching. We have now extended this comparison to include an additional 13 bioleaching microorganisms whose genome sequences are publicly available (Table 1).

Genes and pathways for eleven metabolic processes, including electron transfer pathways and other characteristics have been predicted. These include: carbon assimilation, TCA cycle, sulfur oxidation, sulfur reduction, iron oxidation, iron assimilation, quorum sensing via the acyl homoserine lactone mechanism, hydrogen oxidation, flagella formation, Che signaling (chemotaxis) and nitrogen fixation. In addition, transcriptional and metabolic interplay between pathways has been predicted, allowing the identification of possible coordinated responses to environmental signals such as energy source and oxygen and nutrient limitations. Predictions have also been made for lateral gene transfer events and other aspects of genome evolution.

In this paper, we use an example selected from this information to demonstrate the power of comparative genomics to advance our understanding of the biology of bioleaching. The first is a large-scale comparison of a number of bioleaching microorganisms, focusing on an analysis of the different mechanisms that they exploit to derive carbon for metabolic processes. We speculate as to how these strategies could dynamically influence the composition of microbial consortia as bioleaching proceeds. The second example shows how comparative genomics can help build models of genome evolution and how it reveals that two microorganisms, defined as the same species by classical rDNA

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doi:10.1016/j.hydromet.2010.03.028

Table 1
Bioleaching microorganisms and their respective genomes/metagenomes projects (finished and in progress). L.I.A: limited information available. *: no genome sequence publicly available.

Organism	Domain	Genome status	Opt. temp (º C)	Opt. pH	Carbon assimilation pathway
Acidianus brierleyi DSM 1651	Archaea	In progress	65	1.5	3-hydroxypropionate cycle
Acidimicrobium ferrooxidans DSM 10331	Bacteria	Draft	45	1.9	Facultative heterotrophy (Calvin)
Acidiphilium cryptum JF-5	Bacteria	Finished	35	1.8	Facultative heterotrophy (Calvin)
Acidithiobacillus caldus ATCC 51756	Bacteria	First draft	45	2.0	Calvin Cycle
Acidithiobacillus ferrooxidans ATCC 23270	Bacteria	Finished	30	2.0	Calvin Cycle
Acidithiobacillus ferrooxidans ATCC 53993	Bacteria	Finished	30	2.0	Calvin Cycle
*Acidithiobacillus ferrooxidans DSM 16786**	Bacteria	Draft	30	1.8	Calvin Cycle
*Acidithiobacillus thiooxidans DSM 17318**	Bacteria	Draft	30	1.8	Calvin Cycle
Acidithiobacillus thiooxidans ATCC 19377	Bacteria	Draft	30	2.5	Calvin Cycle
Ferroplasma acidarmanus fer1	Archaea	Draft	42	1.2	Mixotroph
Leptospirillum ferriphilum	Bacteria	Metagenome	40	1.5	Reverse TCA
*Leptospirillum ferriphilum DSM 17947**	Bacteria	Draft	30	1.8	Reverse TCA
Metallosphaera sedula DSM 5348	Archaea	Finished	70	2.0	3-hydroxypropionate cycle
Picrophilus torridus DSM 9790	Archaea	Finished	60	0.7	Heterotroph
Sulfobacillus thermosulfidooxidans DSM 9293*	Bacteria	L.I.A	50	2.3	Facultative heterotroph (Calvin)
Sulfolobus acidocaldarius DSM 639	Archaea	Finished	70	2.0	3-hydroxypropionate cycle
Sulfolobus solfataricus P2	Archaea	Finished	80	3.5	3-hydroxypropionate cycle
Sulfolobus tokodaii strain 7	Archaea	Finished	80	2.5	3-hydroxypropionate cycle
Thermoplasma acidophilum DSM 1728	Archaea	Finished	55	1.0	Heterotroph
Thermoplasma volcanium GSS1	Archaea	Finished	60	2.0	Heterotroph

typing, can actually exhibit different metabolic potential. We show how this information impacts our understanding of microbial diversity in bioleaching heaps.

2. Methods

Genome sequences used in this study: the complete genome sequences of *Acidimicrobium ferrooxidans* DSM 10331, *Acidiphilium cryptum* JF-5, *Acidithiobacillus caldus* ATCC 51756, *Acidithiobacillus ferrooxidans* ATCC 23270, *Acidithiobacillus ferrooxidans* ATCC 53993, *M. sedula* DSM 5348, *Picrophilus torridus* DSM 9790, *Sulfolobus acidocaldarius* DSM 639, *Sulfolobus solfataricus* P2, *Sulfolobus tokodaii* strain 7, *Thermoplasma acidophilum* DSM 1728 and *Thermoplasma volcanium* GSS1 were downloaded from complete genomes website at NCBI (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). The metagenomic sequences corresponding to *Leptospirillum ferriphilum* were obtained from the NCBI website. The genome sequences of *Acidithiobacillus thiooxidans* ATCC 19377, *Ferroplasma acidarmanus* fer1 were obtained from the Center for Bioinformatics and Genome Biology (CGBG, www.cienciavida.cl/CBGB.htm) and Joint Genome Institute (http://genome.jgi-psf.org/ferac/ferac.home.html) respectively.

Gene annotation and pathway analyses: candidate protein coding genes were identified in genome sequences using Glimmer (Salzberg et al., 1998), Critica (Badger and Olsen, 1999) and BlastX (Altschul et al., 1997). The 5′ and 3′regions of each ORF were inspected to define initiation codons using homologies, position of ribosomal binding sites, and transcriptional terminators.

The following bioinformatic programs were used to further characterize candidate genes and their predicted protein products: BlastP and PsiBlast (Altschul et al., 1997), the suite of protein comparison and classification programs available in InterproScan (Mulder and Apweiler, 2007). Model metabolic pathways were reconstructed using PRIAM (Claudel-Renard et al., 2003) and compared to those obtained from BIOCYC (Caspi et al., 2008), KEGG (Kanehisa et al., 2008).

3. Results and discussion

3.1. Genomics of carbon assimilation in bioleaching operations

It has been firmly established that the composition and dynamics of microbial populations (consortia) inhabiting bioleaching heaps vary spatially within the heap and as a function of time as bioleaching proceeds (Demergasso et al., 2005; Coram-Uliana et al., 2006; Rawlings and Johnson, 2007; Remonsellez et al., 2007). The composition of the consortia is thought to be driven by the types of energy source present, the mineralogy of the ore, the availability of oxygen and CO_2 and the temperature of the heap. Current dogma suggests that a bioleaching heap undergoes a progression where mesophilic microorganisms, dominated by bacteria such as *A. ferrooxidans* and *Leptospirillum* spp., initiate the bioleaching process. These microorganisms are succeeded by moderate thermophilic bacteria such as *A. caldus* and *Sulfobacillus* spp. that, in turn, give way to extremely thermophilic archaea such as *S. metallicus* as the temperature of the bioleaching heap rises due to exothermic oxidative reactions.

Little attention has been given to the possibility that the composition and dynamics of bioleaching microbial consortia may, in part, be driven by their access to CO_2 for autotrophs or organic carbon for heterotrophs. Since no organic carbon is present when a bioleaching heap is first started, the microbial initiators of bioleaching are exclusively chemolithoautotrophs since no phototrophs have been detected. However, increasing bacterial biomass resulting from autotrophic activity, could potentially feed heterotrophic and mixotrophic microorganisms either by excretion of organic compounds or via products from cell death.

Another consideration is the bioavailability of CO_2 for autotrophs in the bioleaching heap. Air is typically pumped into the heap and distribute throughout by ducts. The CO_2 in the air should be in equilibrium with the CO_2 in the acidic solution that irrigates the heap. The latter is most likely to be the main distributor of CO_2 to the microorganisms and so the solubility of CO_2 in the liquid phase is an important factor influencing autotrophic growth. As can be seen in Fig. 1, CO_2 is present mainly as a dissolved gas at pHs below 4 and might be expected to be freely available to autotrophs in bioleaching heaps which normally operate around pH 2. On the other hand the solubility of bicarbonate (HCO_3^-) is negligible at pHs below 4 and so would not be available for microbial carbon fixation (Carroll and Mather, 1992). However, the solubility of CO_2 at pH 2 decreases as a function of temperature and its availability at the higher temperatures found in the later stages of bioleaching might influence microbial growth kinetics and hence could impact thermophilic consortia composition.

In an attempt to understand the physiology of microbial carbon assimilation during bioleaching, we reviewed the literature for information on CO_2 fixation pathways in known bioleaching microorganisms or

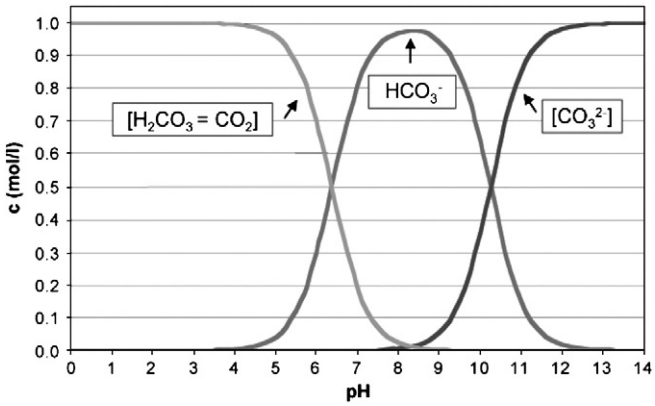

Fig. 1. Solubility and speciation of CO_2 in water as a function of pH at 25 °C.

their close relatives and filled in the gaps in this knowledge by detailed genome analyses using bioinformatics techniques. Although the majority of the sequenced bioleaching microorganisms are obligate autotrophs, several are heterotrophs and one (*F. acidarmanus* fer1) is a facultative mixotroph that obtains its electrons either from an inorganic electron source (Fe (II)) or an organic source but uses only organic compounds as a carbon source (Table 1). Because of the latter, *F. acidarmanus* fer1 will be considered to be equivalent to a heterotroph for the rest of this paper.

The obligate autotrophs under study are predicted to use one of three routes for CO_2 fixation (Fig. 2): (1) the reductive pentose phosphate cycle (Calvin–Bassham–Benson cycle) represents the CO_2 fixation pathway in most aerobic autotrophic bacteria and is characterized by the presence of the CO_2-fixing enzyme ribulose-1,5-bisphosphate carboxylase (RuBisco) whose distribution, activity and regulation have been extensively investigated in several obligate and facultative autotrophs (Wood et al., 2004; Bowien and Kusian, 2002), (2) the reductive citric acid cycle has been found in anaerobic and microaerophilic sulfur oxidizing bacteria from several environments including thermal vents, deep marine subsurface and anaerobic sulfidic environments (Campbell and Cary, 2004). This pathway is characterized by the enzymes ATP citrate lyase, 2-oxoglutarate: acceptor oxidoreductase (2-oxoglutarate synthase), and pyruvate synthase (Hugler et al., 2005) and (3) in the 3-hydroxypropionate cycle, CO_2 is fixed by acetyl-CoA and propionyl-CoA carboxylases in a cycle ultimately forming acetoacetyl-CoA in archaea, which is then split to gain 2 molecules of acetyl-CoA, one of which replenishes the cycle and the other is used for biosynthesis. Past research had demonstrated this pathway only in Chloroflexus (Menendez et al., 1999), a nonsulfur photosynthetic bacterium, but recent work has detected the pathway in several autotrophic archaea (Auernik et al., 2008), so it seems that the pathway is more widespread than as previously thought.

The distribution of these autotrophic and heterotrophic bioleaching microorganisms was plotted as a function of temperature and status (time) of an idealized bioleaching heap (Fig. 3). Several conclusions and hypotheses can be derived from an inspection of Fig. 3:

- Both autotrophs and heterotrophs populate all temperature ranges. It is hypothesized that the major source of organic carbon to feed the heterotrophs comes from autotrophic activity, although external sources such organic solvents in recycled leach liquor may also contribute. The synergism/antagonism between autotrophs and heterotrophs and how these might affect bioleaching are not understood and deserves further investigation.
- The dominant species are bacteria at mesophilic temperatures. In contrast, archaea dominate at high temperatures, while both are present at moderate temperatures.
- At mesophilic temperatures, the major route for CO_2 fixation is via the Calvin cycle, in which all the acidithiobacilli studied exhibit multiple gene clusters involved in CO_2 fixation and all exhibit the carboxysome method for concentrating CO_2. In the case of *A. ferrooxidans*, it has been experimentally demonstrated that different gene clusters respond at the transcriptional level to different CO_2 concentrations suggesting that it, and presumably the acidophiles, can respond to changing environmental concentrations of CO_2 (Esparza et al., 2009). Under laboratory conditions, genes encoding carboxysomes are activated at lower levels of CO_2 suggesting that the acidophiles can inhabit environments low in CO_2, but whether such conditions occur during bioleaching at mesophilic temperatures is not known. It is hypothesized that concentration of CO_2 via carboxysomes becomes an important asset to survival when the acidithiobacilli encounter low concentrations of CO_2.
- At intermediate temperatures, the bacteria fix CO_2 by the Calvin cycle and the reductive TCA cycle. The reductive TCA cycle is typically exploited in aerobic or microaerophilic conditions, suggesting that oxygen may be limiting in parts of the bioleaching heap despite being pumped into the heap.
- At higher temperatures (>60 °C), bioleaching consortia are dominated by Archaea that principally use the modified 3-hydroxyproprionate cycle to fix CO_2 (Fig. 2, Table 1). This discovery was unexpected because it has been shown that this cycle uses bicarbonate as the input source of carbon (Berg et al., 2007), which would be at very low concentrations at pH 2 (Fig. 1). One possibility is that this assumption is incorrect and that bicarbonate is present in the soluble form in biofilm environments, where the pH may be high enough to support soluble bicarbonate. Alternatively, CO_2 is the main inorganic source of carbon for these microorganisms but is converted to bicarbonate

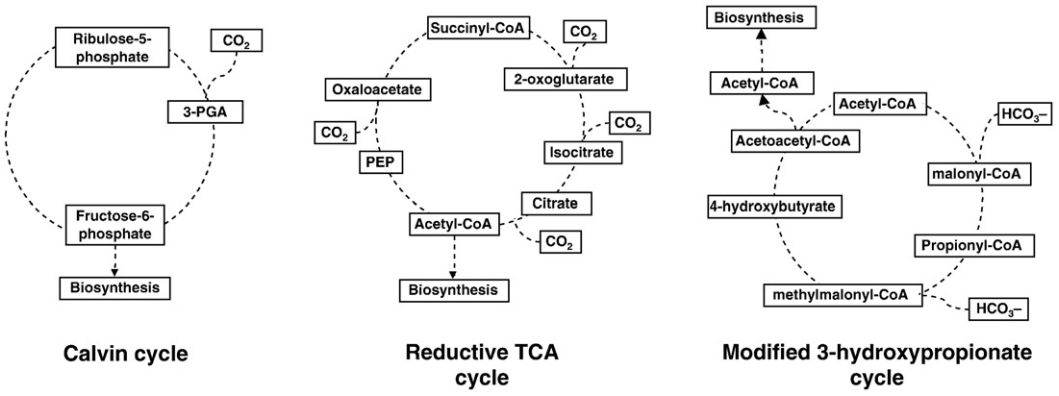

Fig. 2. Three routes for CO_2 fixation predicted in bioleaching bacteria by bioinformatics analyses.

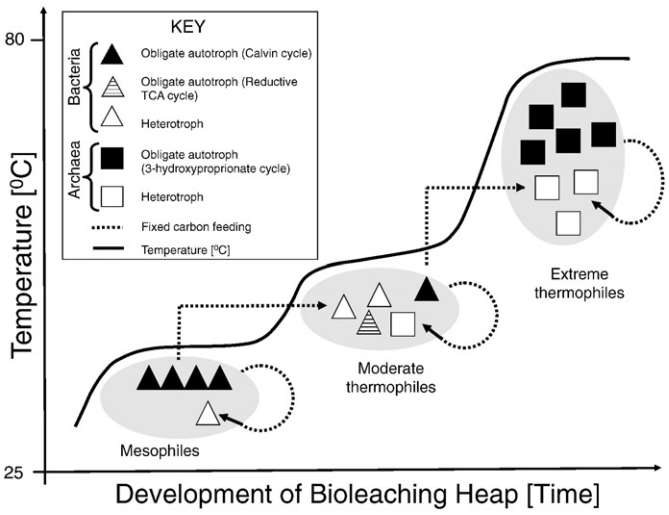

Fig. 3. Distribution of microorganisms as a function of temperature and development of an idealized bioleaching heap, distinguishing bacteria and archaea, autotrophs and heterotrophs and the different pathways of CO_2 fixation used by the autotrophs. A list of the respective microorganisms and their metabolic routes for obtaining carbon can be found in Table 1. The distribution of microorganisms with respect to temperature and time of development of the bioleaching heap was derived from experimental and genomic information available.

inside the cell prior to loading into the 3-hydroxyproprionate cycle. Future research is required to resolve these issues.

The following four bioleaching thermophilic archaea (or closely related species): *A. brierleyi*, *Sulfolobus acidocaldarius DSM 639*, *S. solfataricus P2* and *M. sedula DSM 5348* are predicted to use the modified 3-hydroxypropionate cycle for CO_2 fixation (Table 1), whereas eight thermophilic archaea not thought to be involved in bioleaching are predicted to use predominately the reverse TCA cycle (rTCA): *Aeropyrum pernix K1* (rTCA), *Ignicoccus hospitalis KIN4/I* (dicarboxylate/4-hydroxybutyrate cycle), *Pyrobaculum aerophilum IM2* (rTCA), *Pyrobaculum arsenaticum DSM 13514* (rTCA), *Pyrobaculum calidifontis JCM 11548* (rTCA), *Pyrobaculum islandicum DSM 4184* (rTCA), *and Thermoproteus neutrophilus V24Sta* (rTCA). If this difference in CO_2 fixation pathways is supported by further analyses as more genomic information becomes available, it will be important to determine why bioleaching thermophilic Archaea preferentially use the reverse TCA cycle method. The solution to this question is expected to throw light on the biology of bioleaching at high temperatures.

Whereas this study provides insight into the metabolic potential of carbon production by laboratory strains of autotrophic and heterotrophic bioleaching microorganism, it will be necessary to evaluate the significance of these findings to industrial scale mineral recovery. In order to address this issue, additional information will be required regarding the metabolic potential of microorganisms that are present in a bioleaching heap. Considerable attention is currently being given to the identification of microorganisms present in a heap using a range of molecular techniques including rDNA typing. Whereas the latter provides a rapid overview of the species present, we posit that it can underestimate the true metabolic variation of the microorganisms in a bioleaching heap. Conventional wisdom suggests that microorganisms with >98% identity of 16s rDNA sequence belong to the same species. This level of identity is often equated with similarity of gene content in general. In the next section, we show how two strains of *A. ferrooxidans* that, by the definition of rDNA typing (100% in this case), are considered to be the same species actually exhibit significant differences in gene content when analyzed by comparative genomics. This discovery raises the necessity of identifying a set of gene markers that could provide a better means of estimating the true metabolic potential of the microorganisms in a bioleaching operation that cannot be obtained by using rDNA typing alone.

3.2. Comparative genomics uncovers aspects of genome evolution and suggests that bioleaching microorganisms might be more diverse than previously suspected

The genome sequences of *A. ferrooxidans* ATCC 23270 (Valdes et al., 2008a) and ATCC 53993 were compared. They are 100% identical at the ribosomal DNA level, but have two large genome segments (indels), close to 300 kb in ATCC 23270 and 200 kb in ATCC 53993, not shared between the two genomes (Fig. 4). They also display several minor non-homologous genome regions. These major regions account for about 16% difference in gene content between the two strains of *A. ferrooxidans*.

The first is a genomic island found exclusively in *A. ferrooxidans* ATCC 53993 that contains genes encoding heavy metal resistance components including: mercury detoxification (merA), extrusion (merC) and the transcriptional regulator (merR); a copper translocating P-type ATPase; a three-gene cluster consisting of an outer membrane protein, membrane fusion protein and the efflux pump predicted to be involved in heavy metal extrusion and a five gene cluster potentially encoding arsenic resistance components including an efflux pump, membrane fusion protein, outer membrane efflux protein, heavy metal P-type translocating ATPase and an *arsR* transcriptional regulator.

The second indel is exclusively found in *A. ferrooxidans* ATCC 23270 and contains a cluster of 36 tRNA genes that cover the 20 possible amino acids. Expression and correct loading of some of these tRNAs has been experimentally verified (Levican et al., 2009) opening new challenges for the study of adaptive mechanisms that laterally transferred genetic material can adopt in order to adjust to its residence in a new host.

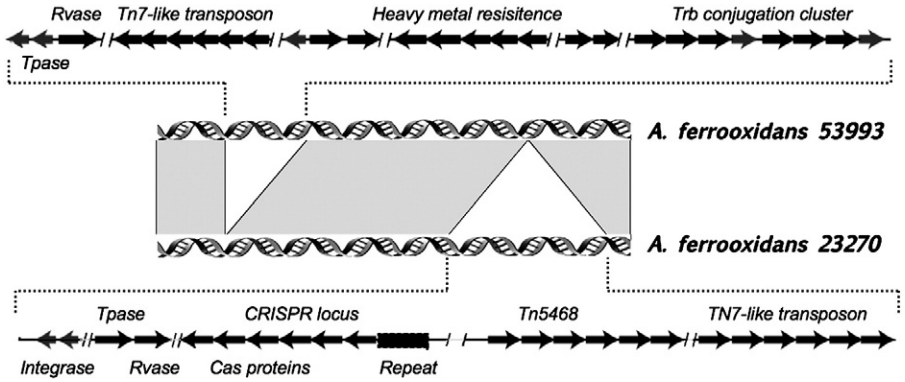

Fig. 4. A comparison of two regions of the genomes of *A. ferrooxidans* ATCC 53993 and ATCC 23270 reveals two indels encoding heavy metal resistance and 35 tRNA genes into the genomes of ATCC 53993 and ATCC 23270, respectively. Grey areas between the genomes represent shared genes and the white areas represent genes present in one genome but not the other. Tpase = transposase; Rvase = resolvase; CRISPR locus (clustered regularly interspaced short palindromic repeats) potentially encoding viral protection functions.

Both indels are flanked by genes known to be involved in lateral gene transfer events, such as a phage integrase, genes involved in conjugation, transposases and several genes involved in DNA mobilization. A CRISPR locus is also present in the indel of *A. ferrooxidans* ATCC 23270 suggesting that this strain has adaptive mechanisms for resistance to viruses. These observations, and the discovery of other potential deletions, insertions and rearrangements of the genomes (data not shown), suggest that the acidithiobacilli are genetically and metabolically flexible, receiving, and perhaps donating, genetic material with other microorganisms in a flux of genetic exchange. This has the potential of providing considerably more physiological diversity than had previously been considered giving rise to the concept of the "pangenome" in which each species has a core genome encoding characteristic functions of the species and additional genetic information encoding additional functions such as metal resistance that provide advantages in specific environments. This observation is in agreement with what has been observed in the Iron Mountain metagenomic projects (Andersson and Banfield, 2008). It also strongly suggests that standard molecular techniques for detecting species within a bioleaching heap, such as rDNA typing (Johnson and Hallberg, 2007) could significantly underestimate the genetic and metabolic diversity of bioleaching microorganisms.

4. Conclusions

The current pace of microbial genome sequencing projects provides a continuously increasing body of biological information that enables to experimentalists and engineers the generation of testable models of microbial metabolism and even the elucidation of ecophysiological interactions in the environment. The generation of these metabolic models from genomic information has been shown as a valuable source of information in the study of biomining microorganisms providing a comprehensive picture of the potential biogeochemical cycles carried out by each microbial representative. A bioinformatic analysis of the available genome sequences of 20 microbial representatives associated with biomining operations has identified different strategies for carbon assimilation and other properties, suggesting in conjunction with the experimental information available, a preliminary model for microbial succession in extreme acidic environments that can be applied to the study of biomining operations. The conceptual translation of genomic information to biological knowledge is a key process for understanding the microbial complexity in extreme environments and also provides new and unexpected opportunities for the development of new strategies for the management of biomining operations. These genome-guided strategies can take in account microbial composition, metabolic potential and the mineralogical nature of the ores for the generation of optimal conditions for the maintenance of an active microbial consortium that can proceed across the operational time of a bioleaching process.

We posit that our models provide an initial framework to help unravel the ecophysiology of bioleaching, but a more accurate picture of "who is doing what to whom, where, when and under what conditions" during bioleaching will require a deeper analysis of the microorganisms present. We suggest that current molecular techniques might significantly underestimate the true genetic and metabolic diversity of microorganisms present in bioleaching heaps. The data required for a more complete understanding of the biology of bioleaching will require novel approaches such as metagenomic analyses.

Acknowledgments

Innova 08CM01-03, Fondecyt 1050063 and 1100887, DI-UNAB 34-06, DI-UNAB 15-06/I and a Microsoft Sponsored Research Award.

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