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Genomics update

Bioleaching genomics

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Mineral ores are full of metals, some very precious – but how to extract them? The Hamersley mines in the Pilbara in Western Australia contain such rich iron ore that it can almost be welded as it comes out of the ground. Traditionally, metals are extracted by 'smelting' or pyrometallurgy, the thermal treatment of minerals and metallurgical ores and concentrates to bring about physical and chemical transformations, which then enables recovery of valuable metals. But this process is energy-consuming and generates many undesirable side-products such as toxic gases. Alternatively, bioleaching (or biomining) by microorganisms is used to extract metals from ores by dissolving them into extremely acidic aqueous solution. Bioleaching is a natural process involving acidophilic bacteria and archaea, which have the ability to either oxidize metal sulfides or to oxidize reduced inorganic sulfur compounds (RISCs) to sulfuric acid, or both (Fig. 1, left panel). Acid mine drainage liquors were found to contain bacteria responsible for producing iron-rich acidic waters from coal and metal mines. Bioleaching is used today in commercial operations to process ores of copper, nickel, cobalt, zinc and uranium, whereas biooxidation is used in gold processing and coal desulfurization.

The biomining industry has a long-standing interest in the use of extreme acidophiles for metals recovery from ores (for recent reviews see Rawlings, 2002; 2005; Valenzuela *et al*., 2006; Rawlings and Johnson, 2007). These organisms, with as prime example the mesophilic chemolithotrophic bacterium *Acidothiobacillus ferrooxidans*, can liberate precious (e.g. gold) and base (e.g. copper) metals trapped in metal sulfides (e.g. iron pyrite and chalcopyrite) through dissimilatory oxidative processes. Biological regeneration of Fe^{3+} from Fe^{2+} is the key to chemical attack of metal sulfides. Efficacy in biomining environments also requires tolerance of high levels of toxic heavy metals as well as the ability to assimilate inorganic carbon, as organic sources can be scarce in this environment. A full complement of these desirable traits is not typically present in a single native microorganism, but may be in a consortium. Here we give a brief update of the current status of (meta)genome sequencing and genomics of bioleaching microorganisms and communities.

Fig. 1. (Left) Natural leaching of the mineral chalcopyrite ($CuFeS_2$), which then is precipitated as a mixture of chrysocolla (light blue copper silicate) and malachite (green copper-carbonate-sulfate). The rust-brown colour is a precipitation of iron (oxide/hydroxide), another product of the chalcopyrite leaching reaction. Courtesy of Torbjörn Kjellsson (Kjellsson, 2002). (Right) Large dump-leaching facility at a copper mine operation near Salt Lake City, Utah (http://www.personal.psu.edu/faculty/j/e/jel5/biofilms/leaching.html).

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Table 1. Acidophilic prokaryotes identified in stirred-tank mineral bioleaching and biooxidation operations.

Metal leached	Co	Zn/Pb	Au	Cu/Zn/Fe	Cu/Fe
Mineral ore/concentrate	cobaltiferous pyrite	zinc/lead pyrite	(arseno) pyrite	poly-metallic	chalco-pyrite
Leaching temperature	35°C	35–40°C	40°C	45°C	78°C
Species isolated					
Acidianus sp					*
Acidiphilium cryptum		*			*
Acidithiobacillus caldus				*	*
Acidithiobacillus ferrooxidans		*	*		*
Acidithiobacillus thiooxidans	*	*	*		*
Ferroplasma cupricumulans					*
Ferroplasma acidophilum				*	
Leptospirillum ferrooxidans	*	*	*		
Leptospirillum ferriphilum				*	*
Metallosphaera sp					*
Sulfobacillus sp	*		*	*	
Sulfobacillus thermosulfidooxidans	*				

Adapted from Rawlings and Johnson (2007).

Chemistry of bioleaching

Metal leaching is mainly a chemical process in which ferric iron and protons are responsible for carrying out the leaching reactions. These reactions take place in the extracellular polysaccharide laid down by cells growing in biofilms rather than cells in a planktonic lifestyle. Biofilm formation greatly accelerates the reactions. There are two types of mechanisms, a thiosulfate mechanism proposed for the oxidation of acid-insoluble metals sulfides, e.g. pyrite (FeS_2) and a polysulfide mechanism for acid-soluble metal sulfides, e.g. sphalerite (ZnS) and chalcopyrite ($CuFeS_2$). In the thiosulfate mechanism, ferric iron attack solubilizes the acid-insoluble metal sulfide, producing thiosulfate as an intermediate which is then oxidized to sulfate.

$$FeS_2 + 6\,Fe^{3+} + 3\,H_2O \rightarrow S_2O_3^{2-} + 7\,Fe^{2+} + 6\,H^+$$

$$S_2O_3^{2-} + 8\,Fe^{3+} + 5\,H_2O \rightarrow 2\,SO_4^{2-} + 8\,Fe^{2+} + 10\,H^+$$

The role of the microbes is therefore to produce sulfuric acid for proton attack and to keep the iron in the oxidized ferric state for oxidative attack on the metal (Rawlings, 2005). For overviews of acidophilic microorganisms and their chemistry of carbon, iron and sulfur metabolism see Suzuki (2001) and Johnson and Hallberg (2009).

Biomining processes

The engineering options for biomining encompass relatively unsophisticated, inexpensive irrigated dumps (Fig. 1, right panel), to controlled bioleaching from heaps, or to very expensive and highly controlled stirred reactors (Rawlings *et al.*, 2003). The heap reactors now being constructed are stacked, aerated and irrigated. An example of such heaps is in La Escondida, Chile where there are three stacks all around 2–4 km^2. These are typically used for low-grades ores. These heaps are usually not seeded with any consortia of microbes but are allowed to develop naturally; dormant organisms are present in a quiescent state just waiting for the right conditions. In an industrial setting, bioleaching is started by adding sulfuric acid and aerating the heap. As the leaching progresses, the temperature of the heap increases and so the composition of the microbial communities is constantly changing. The first organisms to act are the mesophilic acidophiles (optimal temperature for growth below 40°C), these are mostly Gram-negative bacteria, next in succession are the moderate thermoacidiophiles (40–60°C) which are mostly Gram-positives and finally the extreme thermo-acidiophiles (> 60°C), which are mostly *Archaea*. The latter have been added to heaps from consortia isolated from high-temperature acidic environments. The metabolism of sulfur and carbon in these acidiophiles has been reviewed by Johnson and Hallberg (2009). The majority of acidiophiles fix carbon dioxide by the Calvin–Benson cycle. The key enzyme here is RUBISCO, and this activity has been detected in these bacteria and confirmed by genome sequencing projects.

Microbial washout from the heap is not a major concern as these microbes tend to grow in biofilms or penetrate into the ore and remain attached. However, the liquor run-off from a heap can be used as the starting culture for a new heap. The liquor is processed by solvent extraction and electro-winning to deposit the metals. Heap leaching takes months rather than years as for dump leaching. Compared with stirred tank reactors, heap reactors form undesired gradients of pH, temperature and reagent levels. Still, the reaction conditions

Table 2. Genome sequencing projects of microbes involved in oxidation/reduction of iron and/or reduced inorganic sulfur compounds; the majority are acidophiles and are capable of carbon dioxide fixation.

Kingdom	Organism	Strain	Phenotype	Energy source	Temperature range	Accession No.	Publication/contact
Complete sequence							
Bacteria	*Acidiphilium cryptum*	JF-5	Iron reducer	Heterotroph	Mesophile	NC_009484	microbes@cuba.jgi-psf.org
Bacteria	*Acidithiobacillus ferrooxidans*	ATCC 53993	Iron oxidizer	Obligate chemoautolithotroph	Mesophile	NC_011206	borolea@ornl.gov
Bacteria	*Acidithiobacillus ferrooxidans*	ATCC 23270	Iron oxidizer	Obligate chemoautolithotroph	Mesophile	NC_011761	Valdes *et al.* (2008a)
Bacteria	*Leptothrix cholodnii*	SP-6	Iron oxidizer	Heterotroph	Mesophile	NC_010524	demerson@bigelow.org
Archaea	*Metallosphaera sedula*	ATCC 51363	Iron oxidizer	Chemolithotroph	Thermophile	NC_009440	Auernik *et al.* (2008a)
Archaea	*Sulfolobus acidocaldarius*	DSM 639	Sulfur oxidizer	Lithotroph	Thermophile	NC_007181	Chen *et al.* (2005)
Archaea	*Sulfolobus solfataricus*	P2	Sulfur metabolizing	Lithotroph	Hyperthermophile	NC_002754	She *et al.* (2001)
Archaea	*Sulfolobus tokodaii*	7	Sulfur metabolizing	Lithotroph	Hyperthermophile	NC_003106	Kawarabayasi *et al.* (2001)
Bacteria	*Sulfurihydrogenibium azorense*	Az-Fu1	Sulfur oxidizer	Heterotroph	Thermophile	CP001229	Reysenbach *et al.* (2009)
Bacteria	*Sulfurihydrogenibium sp.*	YO3AOP1	Sulfur oxidizer	Chemolithoautotroph, Heterotroph	Thermophile	NC_010730	Reysenbach *et al.* (2009)
Bacteria	*Sulfurimonas denitrificans*	ATCC 33889	Sulfur oxidizer, nitrate reducer		Mesophile	NC_007575	Sievert *et al.* (2008)
Bacteria	*Thiobacillus denitrificans*	ATCC 25259	Iron oxidizer, sulfur oxidizer	Chemolithotroph, Lithotroph	Mesophile	NC_007404	Beller *et al.* (2006)
Ongoing/draft sequence							
Archaea	*Acidianus brierleyi*		Iron oxidizer, sulfur metabolizing	Lithotroph	Thermophile		garrett@mermaid.molbio.ku.dk
Archaea	*Acidianus sp*	JP7	Iron oxidizer	Lithotroph	Thermophile		lJAnderson@lbl.gov
Bacteria	*Acidimicrobium ferrooxidans*	ICP	Iron oxidizer	Autotroph	Thermophile		microbes@cuba.jgi-psf.org
Bacteria	*Acidithiobacillus caldus*	ATCC51756	Sulfur oxidizer	Chemolithotroph	Thermophile		Valdes *et al.* (2008b)
Bacteria	*Acidithiobacillus thiooxidans*	ATCC19377	Sulfur oxidizer	Chemolithotroph	Mesophile		Valdes *et al.* (2008b)
Archaea	*Ferroplasma acidarmanus*	Fer1/Fer1env	Iron oxidizer		Mesophile	AABC05000000	Allen *et al.* (2007)
Archaea	*Ferroplasma sp.*	Type II	Iron oxidizer	Heterotroph	Mesophile	AADL00000000	Tyson *et al.* (2004)
Bacteria	*Halothiobacillus neapolitanus*	c2	Sulfur oxidizer	Chemolithoautotroph	Mesophile		microbes@cuba.jgi-psf.org
Bacteria	*Leptospirillum ferrooxidans*	DSM 2705	Iron oxidizer,	Autotroph	Mesophile		microbes@cuba.jgi-psf.org
Bacteria	*Leptospirillum sp.*	Group II UBA	Iron oxidizer		Mesophile	AADL00000000	Tyson *et al.* (2004)
Bacteria	*Sulfobacillus acidophilus*	NAL	Iron oxidizer, sulfide oxidizer	Autotroph, Mixotroph	Thermophile		microbes@cuba.jgi-psf.org
Bacteria	*Sulfobacillus thermosulfidooxidans*	AT-1	Iron oxidizer, sulfide oxidizer	Autotroph, Mixotroph	Thermophile		microbes@cuba.jgi-psf.org
Archaea	*Sulfolobus metallicus*			Chemolithoautotroph, Lithotroph	Thermophile		garrett@mermaid.molbio.ku.dk
Bacteria	*Thiomonas intermedia*	K12	Sulfur oxidizer	Mixotroph	Mesophile		microbes@cuba.jgi-psf.org

Adapted from the GOLD Database (http://www.genomesonline.org; February 2009) and CBGB (http://www.cienciavida.cl/CBGB.htm).

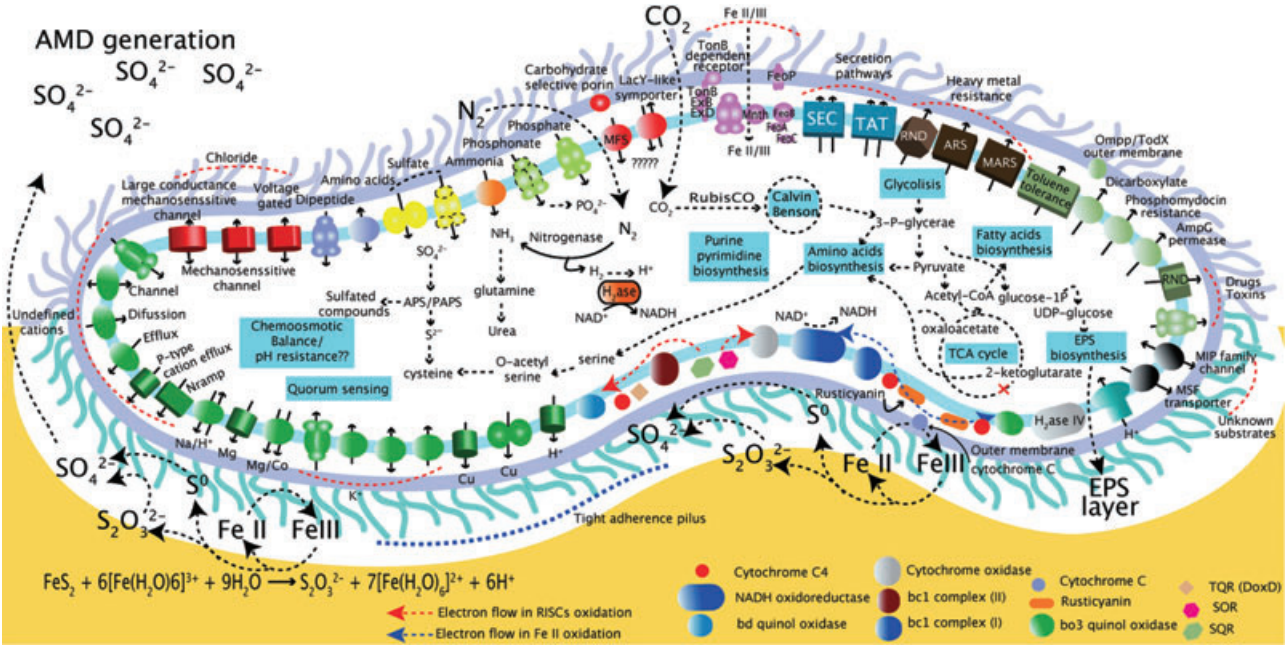

Fig. 2. Whole-cell model for *A. ferrooxidans* ATCC 23270. Genome-based model of the cellular metabolism, including predicted transport systems, chemolithoautotrophic components, carbon/nitrogen/sulfur metabolism, and biogeochemical cycling. Reproduced from Valdes and colleagues (2008a).

are less heterogeneous in a heap than in a dump or *in situ* leaching operations.

Stirred reactors consist of a series of aerated continuous-flow tanks. These are used for the recovery of high-value metals such as gold. These reactors are expensive to construct but do allow a much more controlled and efficient system for metal recovery. Temperature, pH and aeration are all precisely controlled in order to maintain the desired microbial population. The stirred tanks have many similarities with sewage treatment plants (Siezen and Galardini, 2008). Highly efficient consortia of microbes can be selected and maintained, there is continuous flow, rapid degradation of substrate and less cell washout. Sterility is not essential, as all that is wanted is microbes that can degrade the ore. The consortia of microbes in tanks do change with time, so those that were used to seed the tank will change composition and optimize as the process proceeds. Table 1 lists examples of acidophilic prokaryotes identified in stirred-tank mineral bioleaching and bio-oxidation operations.

Genomics of bioleaching microbes

Table 2 summarizes genome sequencing projects of acidophilic microbes involved in oxidation/reduction of iron and/or RISCs, many of which were isolated from bioleaching operations. *Acidithiobacillus ferrooxidans* is a major member of microbial consortia used in the bioleaching industry (Table 1). It is abundant in environments associated with pyrite ore bodies, coal deposits and their acidified drainages. *Acidithiobacillus ferrooxidans* is a chemilitho-autotrophic γ-proteobacterium that acquires energy from the oxidation of iron- and sulfur-containing minerals. It is capable of carbon and nitrogen fixation, and thrives at pH of 1–2. The long-awaited, complete annotated genome sequence of the mesoacidophilic *A. ferrooxidans* ATCC 23270 (3.0 Mb, 58.8% GC) has only recently been published (Valdes *et al.*, 2008a). As expected, the organism was found to have a complete repertoire of genes required for a free-living, chemolithoautotrophic lifestyle, including CO_2 fixation, nucleotide and cofactor biosynthesis. Three copies of the gene cluster for RUBISCO were identified, suggesting the ability to adapt to different levels of CO_2. Electron transport from iron oxidation is through cytochromes and rusticyanin to cytochrome oxidase and NADH dehydrogenase. The organism can also grow anaerobically and the genome suggests that this is by using sulfur as the final electron acceptor. Figure 2 shows a schematic whole-cell model of functions encoded in the *A. ferrooxidans* genome (Valdes *et al.*, 2008a), and a first simple metabolic model using flux balance analysis has been reconstructed (Hold *et al.*, 2009). Based on a high-throughput proteomics study of periplasmic proteins, a detailed model was made of location and putative function of many of the periplasmic proteins, including those involved in iron and sulfur oxidation (Chi *et al.*, 2007).

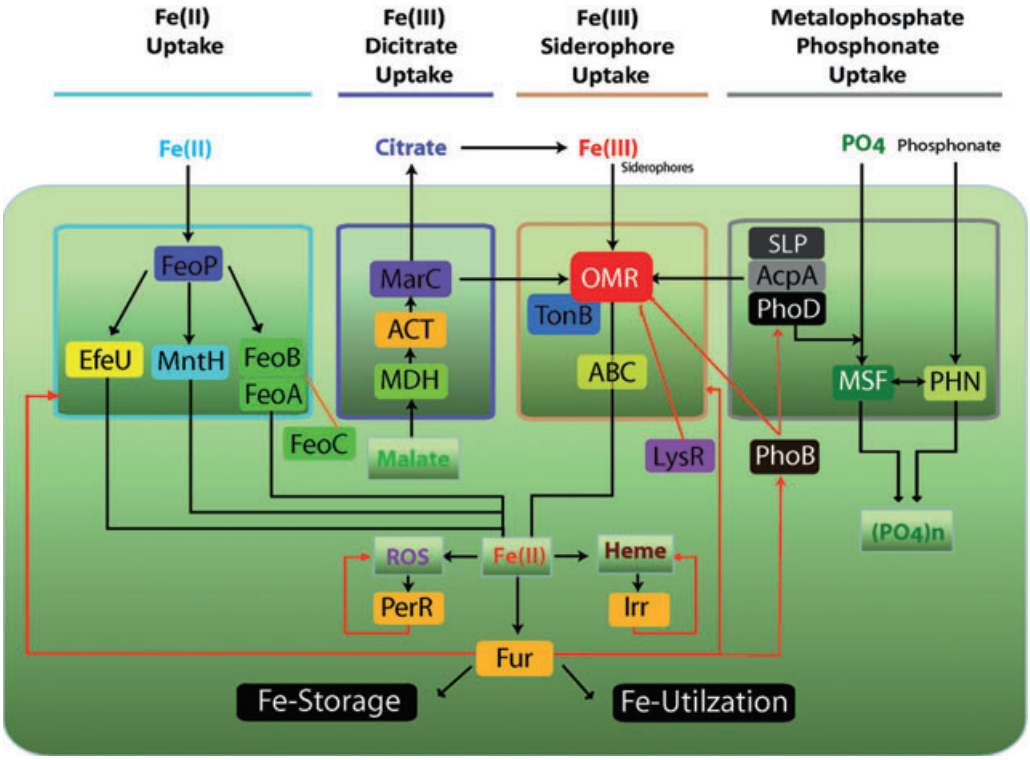

Fig. 3. Diversity of alternative iron acquisition modules and putative regulatory connections in acidophiles. Light blue: ferrous iron uptake module, Violet: Ferric-dicitrate uptake module, Orange: Ferric-siderophore uptake module, Grey: Metalophosphate/phosphonate uptake module, Orange arrows: Regulatory connections. Reproduced from (Osorio *et al.*, 2008a).

In the complete genome sequence of the extremely thermoacidophilic archaeon *Metallosphaera sedula* (2.2 Mb, 46% GC), genes were identified for iron and sulfur oxidation, autotrophic carbon fixation, metal tolerance and adhesion (Auernik *et al.*, 2008a). Comparative genomics with *A. ferriooxidans* showed that *M. sedula* has different respiratory electron transport chain components, as it does not appear to contain cytochromes. Many of the predicted electron chain components, and several new ones, were identified by global transcriptional analysis of *M. sedula* growing in the presence of ferrous iron and RISCs (Auernik and Kelly, 2008; Auernik *et al.*, 2008a).

A classical metagenome sequencing study of a low-complexity, acid-mine drainage microbial biofilm, growing within a pyrite ore body, allowed the reconstruction of near-complete genomes of the iron oxidizers *Leptospirillum* group II and *Ferroplasma* type II (Tyson *et al.*, 2004). A genome dynamics study in a natural population of the acidophilic archaeon *Ferroplasma acidarmanus*, sampled with a 5-year interval from the same acidic mine site, suggested that gene sequence variability was due to frequent recombination, resulting in a mosaic genome pool (Allen *et al.*, 2007). An oligo-nucleotide microarray has been developed that monitors prokaryotic diversity in extremely acidic environments, including members of the *Nitrospira* phylum, *Acidithiobacillus* genus, acidobacteria, sulfur-reducing bacteria, *Actinobacteria* and *Archaea* of the *Ferroplasma* and *Thermoplasma* genera (Garrido *et al.*, 2008).

Comparative genomics

Most recently, comparative genomics studies of bioleaching microbes have identified shared or unique adaptation mechanisms. Comparison of genomes of three *Acidithiobacilli* (*A. ferrooxidans, A. thiooxidans* and *A. caldus)* has led to metabolic and regulatory models for each species of electron transfer pathways, CO_2 fixation, TCA cycle, sulfur oxidation/reduction, iron oxidation, iron assimilation, quorum sensing, hydrogen oxidation, flagella formation, chemotaxis and nitrogen fixation (Valdes *et al.*, 2008b). Predicted interplay between microbes pinpoints possible coordinated responses characteristic of autotrophic microorganisms to environmental signals, such as energy source, oxygen and nutrient limitations, and provides some understanding of how these microorganisms survive and proliferate in extreme environments, including industrial bioleaching operations.

For instance, how do aerobic acidophiles, especially Fe(II)-oxidizers, contend with the paradoxical hazards of iron overload and iron deficiency, each with deleterious

consequences for growth? Some organisms encounter molar concentrations of iron compared with 'normal' conditions 10^{-16} M. Comparative genomics of iron management genes and Fur regulation was carried out for the same three *Acidithiobacilli* and for three extreme acidophilic iron oxidizers of the *Leptospirillum* genus (Osorio *et al*., 2008a,b). Significant differences in abundance and diversity or Fe-management mechanisms are predicted in *Acidithiobacilli* and Leptospirilla, and may represent niche partitioning and ecological successions in a bioleaching environment (Osorio *et al*., 2008a) (Fig. 3). Surprisingly, *Acidithiobacilli* have a very large number of Fe(III)-siderphore uptake systems, but they do not make siderophores – so in conditions of higher pH 4–5, which may occur in industrial bioleaching heaps, they may scavenge the siderophores of other organisms.

Future trends, challenges and spin-offs

What is needed in the future for the biomining industry? As bioleaching heaps get hotter they get more efficient, but when the temperature gets too high then the moderate thermophiles cannot function and the need for seeding with extreme thermophiles becomes necessary. The search is on for microbes which will have a broader temperature range of activity. Will it really be possible to seed a heap of some 15 km^2 the size of the proposed heap at La Escondida in the near future? New isolates from hot, acid environments or a GMO organism may help solve these problems. Extreme thermo-acidophile genomes (Auernik *et al*., 2008b) can be examined for pathways responsible for conferring desirable biomining traits. Taken together with the emerging molecular genetic tools for extreme thermoacidophiles, metabolic engineering of biomining organisms with enhanced properties may soon be a reality. Bioleaching heaps use a tremendous amount of water, and mining operations are not usually in places with much water, so in future much more effort will be spent on ways of remediating the water so that it can be reused in further extractions.

The amount of raw material and accessibility of ores for metal extraction on earth is a finite one. Electronic waste is often full of precious metals and it should also be considered as a potential 'raw material' for biomining. Metallurgical processes using chemical leaching of metals are now commonly used, but bioleaching along with biosorption are now being considered to concentrate the leached metals (Cui and Zhang, 2008).

Is there life on Mars? Or has there ever been life on Mars or anywhere else in the universe for that matter? This may seem a strange question to pose at the end of an article on bioleaching/biomining. Astrobiology is making use of the discoveries of bioleaching. Signs Of LIfe Detector (SOLID2) used a protein chip antibody array to detect microbes, complex molecules and small molecules from mineral deposits (Parro *et al*., 2008). This work showed that SOLID2 would be capable of detecting similar compounds in samples from space.

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