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The bioleaching potential of a bacterial consortium

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HIGHLIGHTS

- A consortium of five bacteria involved in copper bioleaching was sequenced.
- A model of metabolic pathways representing the bioleaching was constructed.
- The consortium showed a high capacity to resist heavy metals.
- This is the first operational industrial bioleaching consortium described to date.

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ABSTRACT

This work presents the molecular foundation of a consortium of five efficient bacteria strains isolated from copper mines currently used in state of the art industrial-scale biotechnology. The strains *Acidithiobacillus thiooxidans* Licanantay, *Acidiphilium multivorum* Yenapatur, *Leptospirillum ferriphilum* Pañiwe, *Acidithiobacillus ferrooxidans* Wenelen and *Sulfobacillus thermosulfidooxidans* Cutipay were selected for genome sequencing based on metal tolerance, oxidation activity and bioleaching of copper efficiency. An integrated model of metabolic pathways representing the bioleaching capability of this consortium was generated. Results revealed that greater efficiency in copper recovery may be explained by the higher functional potential of *L. ferriphilum* Pañiwe and *At. thiooxidans* Licanantay to oxidize iron and reduced inorganic sulfur compounds. The consortium had a greater capacity to resist copper, arsenic and chloride ion compared to previously described biomining strains. Specialization and particular components in these bacteria provided the consortium a greater ability to bioleach copper sulfide ores.

1. Introduction

Research related to microorganisms which inhabit extreme environments has focused on the study of archaic life forms as signs of the origin of life or for applied interests, such as industrial and biotechnological operations (Cavicchioli et al., 2011). Mining environments are characterized by the presence of microbial organisms able to survive despite high concentrations of metals, elevated temperatures, and high levels of acidity (Dopson et al., 2014). Many of these extremophile species are capable of oxidizing iron and reducing inorganic sulfur compounds used as energy sources for different metabolic processes. These properties have being used for economic reasons, where metal recovery from minerals ores cannot be processed by conventional methods and require other procedures for their exploitation.

One of the primary natural processes studied in extremophile organisms is copper bioleaching. This process is defined as the use of microorganisms to solubilize copper from low-grade ores (Rawlings and Johnson, 2007). It is widely known that copper bioleaching involves a complex community of microorganisms, where numerous ferrous iron and sulfur oxidizing bacteria produce the chemical precursors required to generate the scenario for leaching reactions to occur (Mosier et al., 2013).

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In order to better understand the molecular mechanisms involved in this mining process, several genomes of different bacteria isolated from natural (acid drainage) and industrial bioleaching sites (mines) have been sequenced (Mi et al., 2011; Travisany et al., 2014, 2012; Valdes et al., 2008). The γ-proteobacteria *Acidithiobacillus ferrooxidans* is one of the most characterized model of acidophilic bacteria, with studies focused on descriptions of particular metabolic and genomic features (Hodar et al., 2012; Latorre et al., 2016; Orell et al., 2010). These works have contributed to the identification of important mechanisms involved in bioleaching and adaptation to extreme environmental conditions, principally related to metal resistance, iron and sulfur oxidation. However, the function of the bacterial consortium and the specific participation of each microorganism during copper bioleaching are less understood.

In this context, the aim of this work was to identify the molecular basis of a consortium of five efficient copper bioleaching bacteria, all of them frequently found in a mining environment. Using complete sequencing and functional annotation, common and particular genomic features of this extremophile bacteria consortium were identified. An integrative analysis of genomic data allowed the generation of a metabolic consortium model able to indicate the potential of each bacterium and the metabolites required during the bioleach process. The results add experimental data to demonstrate the high capacity of the consortium bacteria to bioleach copper in relation with other previously sequenced mining strains. Finally, the bacterial consortium was composed of the major components of a stable, effective and continuously grown bioleaching consortium operating in a contemporary, large-scale, and fully-operational biotechnology system.

2. Material and methods

2.1. Genome sequencing and assembly

Genomic DNA extracted from *Sulfobacillus thermosulfidooxidans* strain Cutipay, *Acidithiobacillus thiooxidans* strain Licanantay (DSM 17318), *Acidiphilum multivorum* strain Yenapatur, *Leptospirillum ferriphilum* strain Pañiwue and *Acidithiobacillus ferrooxidans* strain Wenelen (DSM 16786) were used for whole genome shotgun sequencing. All sequencing reads were obtained using both Ion Torrent and 454 GS Junior platforms. Sequencing corresponding to *Sb. thermosulfidooxidans* Cutipay and *At. thiooxidans* Licanantay have been previously reported (Travisany et al., 2014, 2012). A trimming process was executed, removing low complexity reads and adaptors. Only reads with per base phred quality value greater than 20 were maintained. These high quality reads were used for the assembly process. Assembly of each of the five bacterial genomes was performed using wgs-assembler (Celera Assembler) version 7.0. A summary of the results of genome assembly and annotation of the five biomining bacteria under study can be found in Supplementary Table S1. All genome data are available at http://biominingdb.cmm.uchile.cl/genomes/.

2.2. Phylogenomic analysis

All bacteria were isolated from a mining environment and their corresponding strains from the same species. To construct the tree, was used the publicly available genomes of 19 biomining bacteria (Supplementary Table S2) retrieved from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) and the DOE Joint Genome Institute (JGI; http://www.jgi.doe.gov) databases. Conserved core genes were obtained as follows: first, orthologs genes between all biomining bacteria were determined using ORTHOMCL v1.4 with an e-value cutoff of $1e^{-5}$. Only orthologs shared among all the considered bacteria were kept. Then, multiple alignments for each ortholog group were carried out using MUSCLE v3.8.31. Ortholog groups that included genes with gaps more than 30% in the group alignment were discarded. Also, for simplicity, orthologs groups with more than one gene per genome were not considered in order to have a unique sequence for each bacterium. Finally, using GBlocks 0.91b, poorly aligned regions from each ortholog group were removed and all the alignments were concatenated in a single large multiple alignment. This concatenated sequence alignment was used to construct a Neighbor Joining tree with 1000 bootstrap replications using PHYLYP. The tree was plotted using the ape R package.

2.3. Genome annotation, manual curation and metabolic pathway identification

A pipeline for prediction and annotation of coding sequences (CDS) was used for the genomes of the five biomining bacteria following protocols described previously (Travisany et al., 2014, 2012). Briefly, an *ab initio* CDS and tRNAs prediction was performed using REGANOR which implements a combination of GLIMMER and CRITICA tools for gene finding and tRNAscan-SE for tRNA gene identification. All predicted CDS were functionally annotated using Hidden Markov Models tools for prediction of transmembrane helices TMHMM and the presence of signal peptide cleavage sites with SignalP. The BLASTP against the NCBI Non Redundant, SWISSPROT, TCDB, OMNIOME, the Kyoto Encyclopedia of Genes and Genomes (KEGG), the Cluster of Orthologous Groups database (COG) and InterproScan using PROSITE, Pfam, ProDom and SMART. The consensus annotation was performed by METANOR and InterPRO hits were used to assign Gene Ontology (GO) terms to genes. Manual curation of automated annotations was performed using the GenDB platform. An enrichment analysis of COG categories was carried out for the gene sets from each of the five biomining bacteria using Fisher's exact test with Benjamini–Hochberg multiple testing correction. Categories with corrected p-value <0.05 were considered enriched.

Based on the gene names of this protein list, a set of corresponding amino acid sequences was retrieved from the SWISSPROT. These sequences were the input for a BLASTP against the eggNOG database to extend the list to other organisms using an e-value of $1e^{-10}$ and sequence identity of 70%. The COG numbers of the high quality proteins were then obtained and clusters of these sequences were retrieved in order to build a database of resistant proteins. This database was used to search for genes related to protein resistance in the Chilean biomining bacteria draft genomes using BLASTP, considering a cutoff e-value of 0.01 and identity of 35%. The matching sequences from the Chilean biomining bacteria obtained were further expanded to include all sequences from their respective ortholog groups. Finally, each of these potential resistant protein sequences was manually curated through multiple sequence alignments to a reference resistant protein sequence and specific motive search when available (Banci et al., 2006; Nies, 2003). For the identification of metabolic pathways, annotated genomes from each bacteria were used to create pathway/genome databases (PGDB) using the Pathway Tools software v 13.0. From those databases, pathways of interest were selected (Supplementary Table S3) and curated following the same protocol used in the characterization of heavy metal resistance proteins.

Comparative genomics strategy was used first to determine ortholog groups that shared a unique set of genes between each of the five native bacteria genomes and a previously released genome from the same species. The following were compared: *At. ferrooxidans* strain Wenelen versus strain ATCC 23270; *At. thiooxidans* strain Licanantay versus strain ATCC19377; *A. multivorum* strain Yenapatur versus strain DSM 11245; *L. ferriphilum* strain Pañiwe

vs strain ML-04; and *Sb. thermosulfidooxidans* strain Cutipay vs strain DSM 9293. The same strategy was applied to identify putative genes involved in chloride ion resistance. The complete set of genes from each of the Chilean biomining bacteria and a counterpart from the same genus or species with significantly lower chloride minimum inhibitory concentration (MIC) were compared. In each case, unique genes from each of the more resistant strains listed in Supplementary Table S4 were selected and analyzed. Genome structural analysis was performed by Mauve and Nucmer programs.

2.4. Experimental assays

Experimental bioleaching assays corresponding to copper recovery were obtained directly from patents US 2006/0094094 A1 and US 2007/0042482 A1 and Bobadilla-Fazzini et al. (2014). The MIC was used to determine the effect of chloride (NaCl and KCl), copper ($CuSO_4$), and arsenic (As_2O_3) on growth of different bacterial strains as described previously (Bobadilla-Fazzini et al., 2014). Briefly, 9 K medium at pH 1.6 was inoculated with $100e^7$ - cells/mL for each strain separately which was supplemented with different concentrations of salts. The medium was incubated at 20 °C and 30 °C for 5 days for *At. ferrooxidans* strains (Wenelen, ATCC 23270 and ATCC 53993), *A. multivorum* strains (Yenapatur and DSM 11245), *At. thiooxidans* Licanantay strain, *Acidiphilium cryptum* DSM2389 strain and *L. ferriphilum* strains (Yagan, BRL075 and DSM1467). For *Sb. thermosulfidooxidans* Cutipay strain and *Sulfobacillus. acidophilus* DSM 10332 strain the medium was incubated at 50 °C. The MIC was defined as the lowest concentration of the respective salt at which no growth was observed. MIC determination assays were performed in triplicate and in three separate experiments.

3. Results and discussion

3.1. Consortium genome features

To determine the microbial diversity present in copper mining bioleaching systems, the biodiversity of culturable bacteria and achaea present in Chilean ore deposits was investigated. More than 10,000 16S sequenced regions from DGGE experiments were collected from native microbiota from different Chilean mining sites (Fig. 1). The results confirm the low complexity (or low species richness) of these extreme environments (Tyson et al., 2004), showing that the most abundant organisms were the gamma-proteobacterium species, along with nitrospirae and clostridia. Similar composition and bacterial diversity has been described in acid mine drainage (AMD)(Baker and Banfield, 2003) and suggests a high potential to oxidize sulfur. Inside this group, five different native Chilean bacteria were isolated according to their higher capacity to extract solubilized copper in comparison to other public bioleaching strains (Fig. 2): *Acidithiobacillus thiooxidans*

A.

Genus	Number	Percent
Acidithiobacillus	3900	40.30%
Ferroplasma	1657	17.10%
Unknown	995	10.30%
Leptospirillum	994	10.30%
Unknown bacteria	659	6.80%
Sulfobacillus	423	4.40%
Alicyclobacillus	313	3.20%
Acidiphilium	107	1.10%
Stenotrophomonas	94	1.00%
Thermoplasma	76	0.80%
Anoxybacillus	58	0.60%
Sulfolobus	44	0.50%
Others under 0.5%	347	3.60%
Total	**9667**	**100.00%**

B.

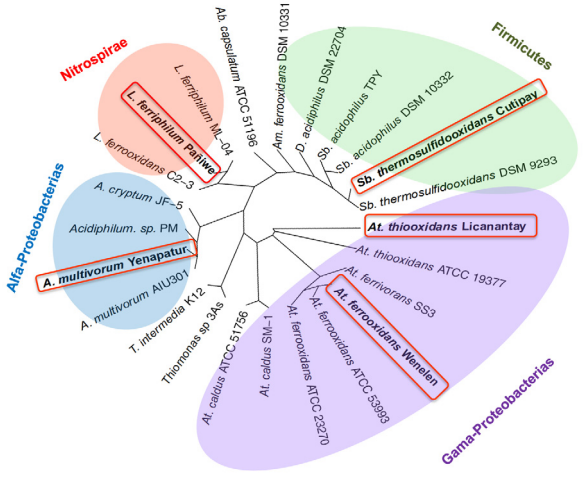

Fig. 1. Biodiversity of cultivabe bacteria in Chilean copper mines. (A) Number of DGGE sequences assigned to each genus. (B) Phylogenetic concatenated tree generated with the core of conserved genes present in the Chilean consortium species and reported bioleaching bacterial strains (Supplementary Table S2).

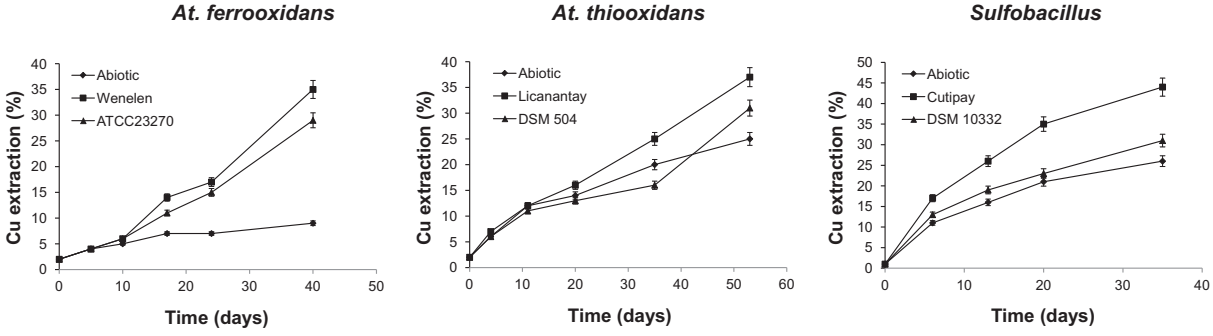

Fig. 2. Copper extraction efficiency between the native Chilean and ATCC strains. Experimental details patents Pub. No.: US 2006/0094094 A1 (Wenelen), Pub. No.: US 2007/0042482 A1 (Licanantay) and Bobadilla-Fazzini et al., 2014 (Cutipay)(Bobadilla-Fazzini et al., 2014).

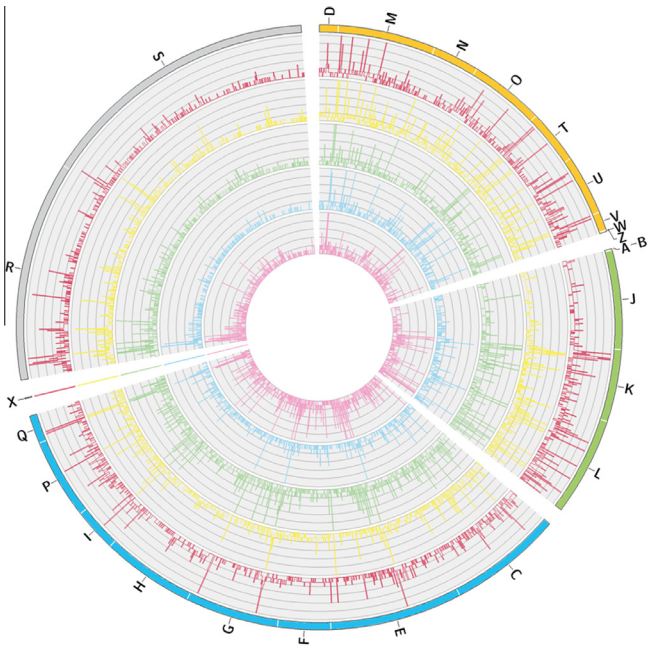

Fig. 3. Circular representation of COG categories of consortium bacteria. The diagram shows the distribution of COG categories in each genome. COG families are grouped as follows: Cellular Processes and Signaling (D, M, N, O, T, U, V, orange); Information Storage and Processing (J, K, L, light blue); Basal Metabolism (C, E, F, G, H, I, P, Q, green); Poorly Characterized (R, S, light grey) and No COG annotation (X, dark grey). Each concentric circles represents a consortium bacteria. From the outer to the inner circle: *At. thiooxidans* Licanantay (yellow): *A. multivorum* Yenapatur (green), *L. ferriphilum* Pañiwe (light blue) *At. ferrooxidans* Wenelen (red), *Sb. thermosulfidooxidans* Cutipay (pink). Bars within each circle represent the number of genes with different COG numbers in each category (details see Supplementary Table S5). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Licanantay (DSM17318), *Acidiphilium* sp. Yenapatur (DSM27270) (here classified as *Acidiphilium multivorum*), *Leptospirillum ferriphilum* Pañiwe, *Acidithiobacillus ferrooxidans* Wenelen (DSM 16786) and *Sulfobacillus thermosulfidooxidans* Cutipay (DSM 27601).

These strains represent the four dominant bacterial taxonomic groups involved in copper mines (Yin et al., 2008), covering more than the 50% of the total bacterial genera present in the ecosystems of Chilean mines. Finally, these five biomining bacteria were selected to grow in a novel bioreactor designed to produce leaching biomass (Patent Registration No. CL 48319), representing the major components of a stable, effective and continuously grown bioleaching consortium operating in a large-scale and fully operational biotechnology system at CODELCO, Radomiro Tomic Division (Antofagasta, Chile).

In order to analyze the molecular features related to the bioleaching of copper, the complete genome sequences of these five biomining bacteria were determined and analyzed. The resulting assemblies indicated that main features of sequenced chromosomes share the same general statistics (contig number, number of annotated genes and assembly size) as average public biomining bacteria genome sequences, discarding the presence of bias during the assembly of the chromosomes (Fig. 3 and Supplementary Table S1).

Whole genome alignments showed notorious structural variations (insertions, deletions, inversions) between the five native bacteria and their corresponding ATCC reference strains (Supplementary Fig. S1). Among them, an elevated set of unique genes that encode unknown proteins and putative transposases were present in the consortium. *At. thiooxidans* Licanantay showed a total assembly 29% higher than that of public *At. thiooxidans* ATCC 19377, a genome structure variation which may impact the bioleaching process (Darling et al., 2008). The five bacteria share a conserved core of 478 genes, 47 of them unique to this

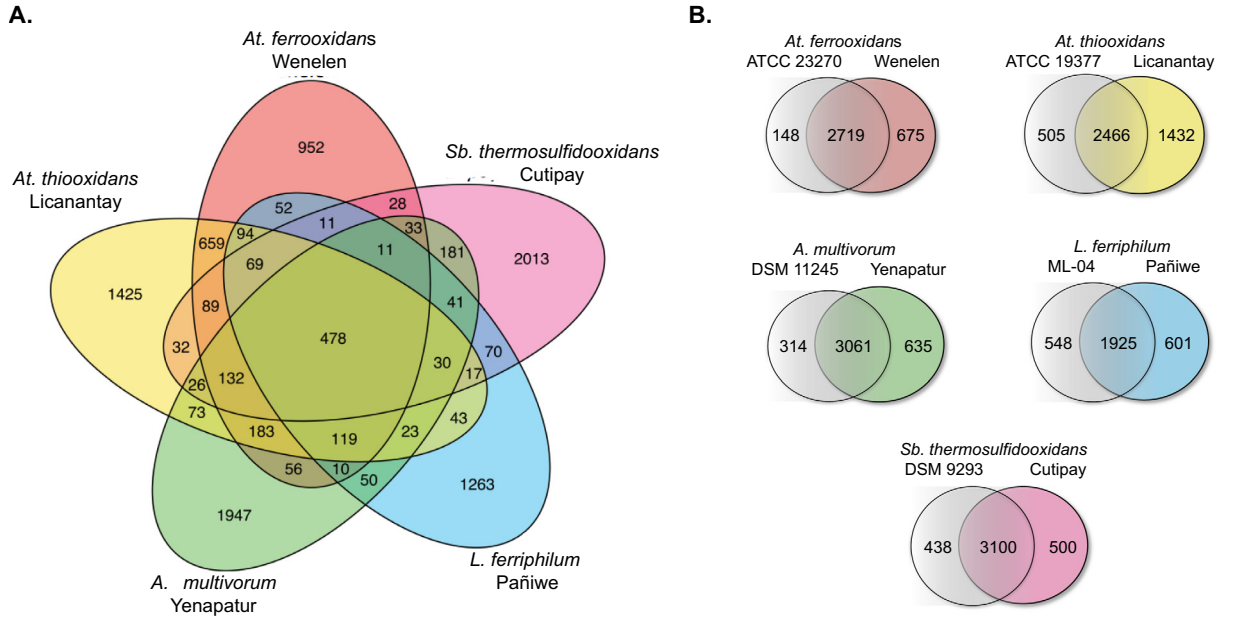

Fig. 4. Gene conservation. (A) Venn diagram showing the unique and shared genes between the five native Chilean bacteria. (B) Total gene distribution between the native Chilean consortium and ATCC strains.

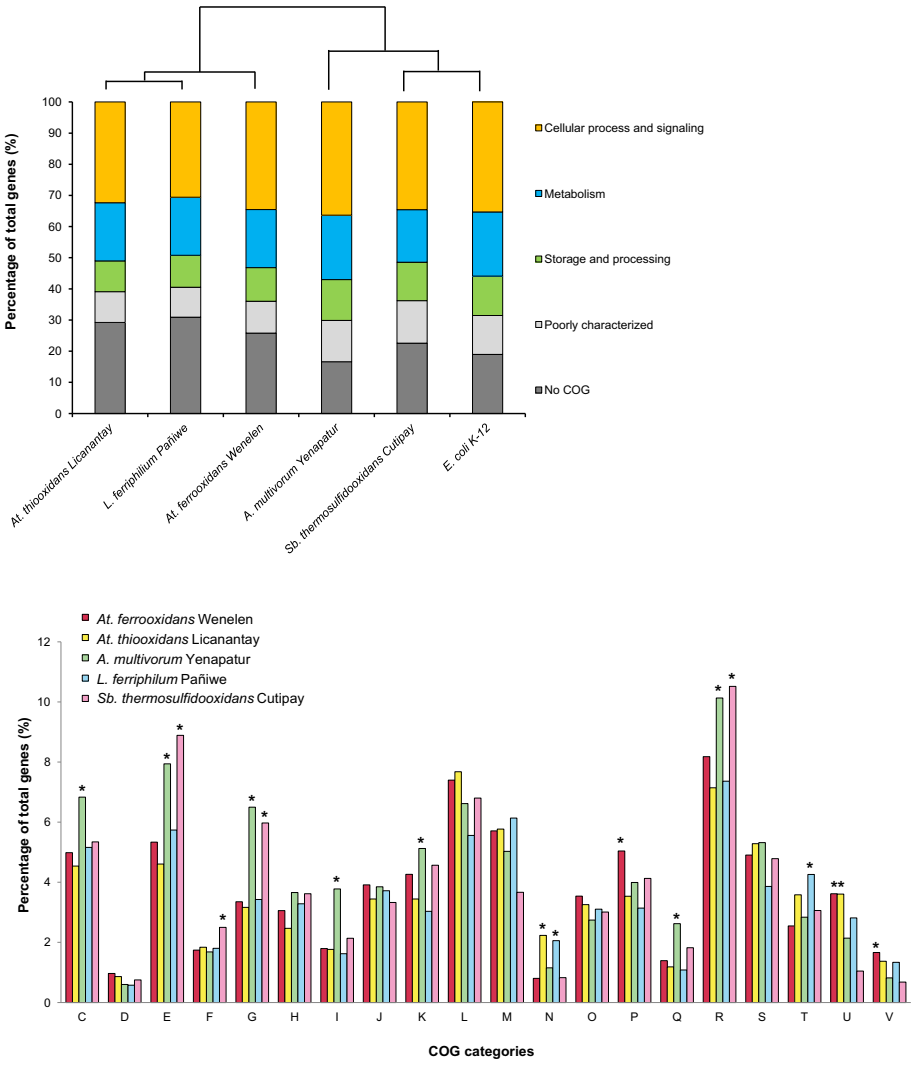

Fig. 5. Individual COG category coverage. (A) percentage of total genes accounted by species for each meta-COG class. Bacterial clustering was performed with total genes of each COG category. (B) percentage of total genes accounted by species and individual COG category ([C] Energy production and conversion, [D] Cell cycle control, cell division, chromosome partitioning, [E] Amino acid transport and metabolism, [F] Nucleotide transport and metabolism, [G] Carbohydrate transport and metabolism, [H] Coenzyme transport and metabolism, [I] Lipid transport and metabolism, [J] Translation, ribosomal structure and biogenesis, [K] Transcription, [L] Replication, recombination and repair, [M] Cell wall/membrane/envelope biogenesis, [N] Cell motility, [O] Posttranslational modification, protein turnover, chaperones, [P] Inorganic ion transport and metabolism, [Q] Secondary metabolites biosynthesis, transport and catabolism, [R] General function prediction only, [S] Function unknown, [T] Signal transduction mechanisms, [U] Intracellular trafficking, secretion, and vesicular transport, [V] Defense mechanisms). Percent of genes not assigned in COG category (NoCOG): *At. ferrooxidans* Wenelen 26%, *At. thiooxidans* Licanantay 29%, *A. multivorum* Yenapatur 17%, *L. ferriphilium* Pañiwe 30%, *Sb. thermosulfidooxidans* Cutipay 23% and *E. coli* K-12 19%. Asterisk denotes significant enrichment ($P < 0.05$) compared to the total proportion in the bacterial genome.

consortium (i.e. not detected in the ATCC reference strains) (Fig. 4). In this group, were highlight proteins involved in energy production and protein biosynthesis (two processes that confer efficiency to extract copper), unique elements probably acquired by horizontal gene transfer events inside the community (Fig. S2 and Supplementary Table S3)(Hemme et al., 2016).

A COG class clustering inside the consortium allowed for classification of the five bacteria into two main clades according to the bacterial abilities to use different energy sources (Fig. 5 and Supplementary Table S5). One clade is composed of the chemolithoautotrophs *At. ferrooxidans* Wenelen, *At. thiooxidans* Licanantay and *L. ferriphilum* Pañiwe, and the other of the chemomixotroph *Sb. thermosulfidooxidans* Cutipay and the heterotroph *A. multivorum* Yenapatur. The main differences in functional categories between these groups relates to the proteins involved in inorganic ion transport and biogenesis. Differences suggest a strong dependence between the bioleaching process and the bacterial ability of using inorganic compounds as sole energy sources. Additionally, they also illustrate the bacterial complementarity that occurred during the bioleaching process, supporting previous experimental strategies used to increase the efficiency to recover copper from ores (Feng et al., 2013; Wang et al., 2012, 2014).

3.2. Integrative bioleaching pathway model

Through the analysis of genome annotations and metabolic pathway reconstructions, species-specific components of key metabolic routes that influence the bioleaching process were revealed. An integrated model of metabolic pathways representing the bioleaching capability of the bacterial consortium was assembled (Supplementary Fig. 3 and Supplementary Table S3).

3.2.1. Iron oxidation

The most extensively characterized mechanism of iron oxidation has been predicted only in *At. ferrooxidans* strains (*rus* operon) (Carlos et al., 2008). *In silico* predictions indicated that

At. ferrooxidans Wenelen, *L. ferriphilum* Pañiwe and *Sb. thermosulfidooxidans* Cutipay also contain components of the *rus* operon. It has been demonstrated that *leptospirilli* species have a greater affinity for ferrous iron and a lower sensitivity to inhibition by ferric iron (Rawlings et al., 1999). The principal component rusticyanin was not detected in *L. ferriphilum* Pañiwe and *Sb. thermosulfidooxidans* Cutipay genomes. This suggests that an uncharacterized functional homolog of *At. ferrooxidans* rusticyanin would be present and that molecular mechanisms of iron oxidation in these bacteria evolved independently (Orengo and Thornton, 2005). In addition, through the bc1-NDH complex, the molecules NAD(P) can receive electrons from the *rus* system. One of the secondary metabolites produced during the synthesis pathway I for NAD(P) is L-glutamate, an amino acid required during biosynthesis of HEM molecules and the principal co-factor of cytochromes (Quatrini et al., 2009). While these components are present in other species of the consortium, *L. ferriphilum* Pañiwe showed a higher number of these proteins. This findings support the idea that this species is the dominant iron-oxidizing bacterium inside the mining consortium; thus, increased participation in the bioleaching process might be expected (Rawlings, 2005).

3.2.2. Reduced inorganic sulfur compounds (RISC)

Classical sulfur dioxygenase and sulfite acceptor oxidoreductase were predicted in the genome of *At. ferrooxidans* Wenelen, *At. thiooxidans* Licanantay and *Sb. thermosulfidooxidans* Cutipay strains, indicating the presence of an active RISC mechanism inside the consortium. *At. thiooxidans* Licanantay contains an elevated number of HDR gene copies (one of the principal protein complex involved in RISC) (Bobadilla Fazzini et al., 2013), two copies for the sulfur oxidizing gene pathway (*sox*) and one archaeal type sulfur oxygenase reductase gene system (*sor*). Unlike *At. thiooxidans* strains previously reported (Quatrini et al., 2009), *At. thiooxidans* Licanantay contains the complete *cys* operon involved in sulfate assimilation and cysteine biosynthesis. The presence of this system provides a higher possibility to generate cysteines. Together, glutamate and cysteines are the amino acid precursors of glutathione, a metabolite that plays a catalytic role in elemental sulfur activation. In addition, cysteine produced by the bacterium can be used to generate Fe-S cluster. This cluster is the principal co-factor of the HDR complex, which utilizes the reduced glutathione to assimilate sulfate, completing the assimilation cycle. *A. multivorum* Yenapatur, unlike the other species of the consortium, contains a higher number of unique proteins classified as components of carbohydrate transport and protein metabolism. Thus supporting the fact that this bacterium plays a role degrading organic metabolites, highly toxic for chemolithoautotrophs, which in turn oxidizes thiosulfate and prevents damage against *Acidiphilium*. This process establish a mutualistic relationship inside the consortium (Okabe et al., 2007; Okibe and Johnson, 2004).

3.2.3. Basal metabolism and biofilm formation

Based on the integrated model of metabolic pathways in the consortium, were able to link basal metabolic routes with the bioleaching process. Circular pathways in which the function and synthesis of the components directly depended on the capacity of the bacteria to reduce inorganic sulfur compounds and oxidize iron were found. Glutathione appears to play a crucial role in metabolic processes directly or indirectly related with iron and RISC oxidation. It was recently demonstrated that *At. thiooxidans* Licanantay produces a high concentration of glutathione under different growth conditions (Martinez et al., 2013). The resulting acidification of the cytoplasm can be controlled by glutamate, one of the principal mechanisms of acid resistance in different bacteria (Foster, 2001). The high concentration of hydrogen can also be used for consortium species to generate ATP. This occurs through the ATP-driven proton pumping activity of the F0F1-ATP synthase, used as an energy source during oxidation of reduced copper ores. Moreover, glutathione precursors such as NAD(P) were also detected as one of the most synthesized molecule by *Acidithiobacilli*, indicating that these molecules have a key putative role in optimizing the bioleaching process.

The bacterial attach capacity to the mineral and the biofilm formation plays a crucial role during the bioleaching process (Yang et al., 2014). All studied species present classical proteins involved in the production of extracellular polymeric substances and biofilm-associated proteins, highlighting LuxR and VpsS-R transcriptional factor family members as putative regulators of these processes. Previous work indicates that elevated production of the second messenger c-di-GMP is directly correlated with biofilm formation (Ruiz et al., 2012). The high number of *ydeH* copies (at least five genes per bacterium) involved in the synthesis pathway of c-di-GMP suggests that the consortium possess an elevated potential to generate biofilm.

4. Environmental resistance mechanisms

Biomining bacteria resist high levels of metals via different systems such as active efflux or trapping of metal ions (Navarro et al., 2013; Orell et al., 2010). During the bioleaching process, the increase of soluble oxidized metals can induce the formation of reactive oxygen species (ROS). Although all bacteria contain the classic ROS response genes (SOD, catalases, peroxidases, reductases, and thioredoxines), the number of these components does not differ from other microorganisms (Imlay, 2013). Thus, high environmental resistance may relate to the ability to handle high ionic strength, osmotic pressure and concentrations of metal and other inhibitory ions and avoiding intracellular toxic effects (Fig. 6). The analysis indicated that all five bacteria contain a high amount of metal resistant determinants, principally ATPases type P and chaperons, classical from copper tolerant organism. First, the high number and duplication of copper efflux proteins is directly correlated with the environment where these bacteria grow (copper sulfide ores). *At. ferrooxidans* Wenelen showed the highest tolerance to copper versus all the tested strains, which also corresponded with the highest number of copper resistant elements encoded in its genome.

In the case of the gram-positive *Sb. thermosulfidooxidans* Cutipay, the lack of outer membrane prevents the existence of RND metal proteins. To compensate for this absence, a high number of CopA (copper ATPase) are encoded in this bacterium. Secondly, arsenic-resistant mining strains show a more efficient leach activity (Hallberg et al., 1996). *At. thiooxidans* Licanantay exhibited the highest tolerance against arsenic (more than 10 times compared with the others species), a property that might be conferred by the unique presence of ArsM, an arsenic methyltransferase involved in metal resistance (Morgante et al., 2015).

Finally, high concentrations of chloride ions released during the process impact yields in the biomining of copper (Chang-Li et al., 2012), attributed to the loss of the outer membrane integrity and the complete inhibition of biooxidation (Bobadilla-Fazzini et al., 2014). Most of the native strains showed an elevated tolerance to chloride ions (Table 1). A comparative genomics strategy identifies a particular set of ion transporters potentially involved in chloride resistance, which are only encoded in the resistant strains of the consortium (Supplementary Table S4).

5. Conclusions

The genome analysis of these five bacteria provides specific information about the role of each species in the consortium,

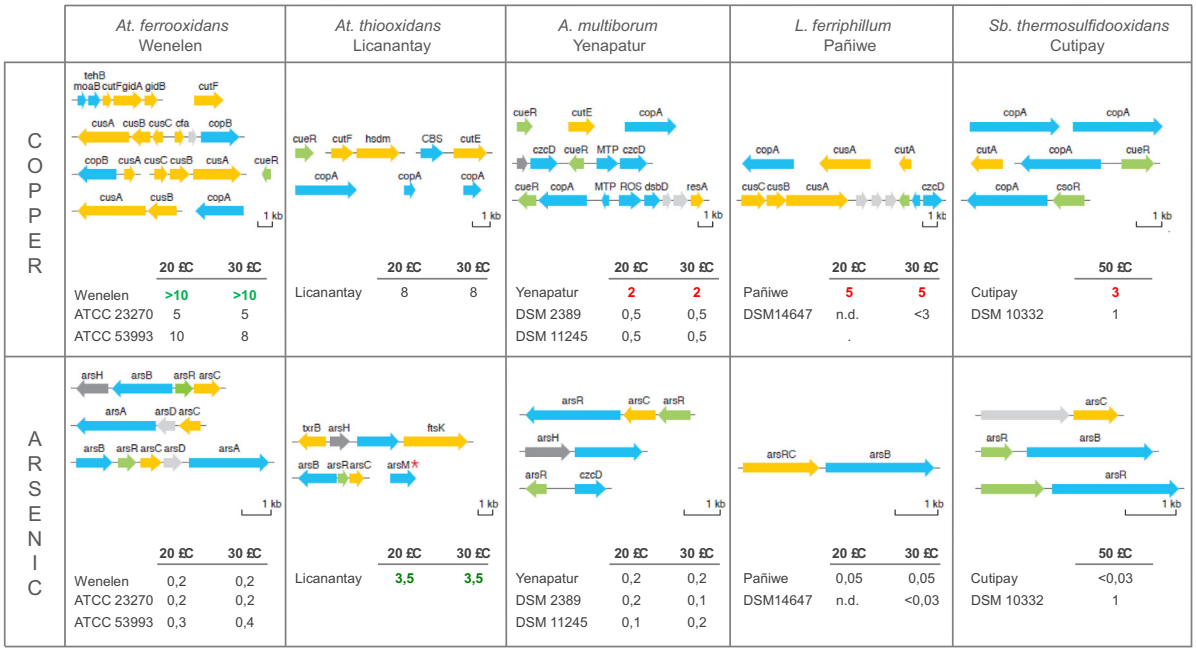

Fig. 6. Copper and arsenic gene resistant clusters and ion tolerance. Definition of colored arrows (COG super-class) are as follows: orange, cellular process and signaling; light blue, metabolism; green, storage and processing; light grey, poorly characterized; dark grey, NoCOG. A red asterisk denotes a unique component. Tables indicate minimum inhibitory concentration results. A red value indicates the highest resistance between the same genus species. A green value denotes the highest tolerance between all the tested strains. Gene Id codes in Supplementary Table S3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Minimum inhibitory concentration (MIC) to chloride ions.

	Chloride ion (KCl) [g/L]		Chloride ion (NaCl) [g/L]	
	20 °C	30 °C	20 °C	30 °C
At. ferrooxidans Wenelen	5	6	5	5
At. ferrooxidans ATCC 23270	7	>10	3	5
At. ferrooxidans ATCC 53993	8	8	7	7
A. multivorum Yenapatur	>10	>10	>10	>10
A. cryptum DSM 2389 (JF5)	4	4	5	5
A. multivorum DSM 11245 (AIU301)	4	4	5	5
L. ferriphilum Pañiwe	2	5	1	3
L. ferriphilum DSM14647 (ML-04)	n.d.	2	n.d.	>1
At. thiooxidans Licanantay	>10	>10	>10	>10
Sb. thermosulfidooxidans Cutipay**	10		5	
*Sb. acidophilus DSM 10332***	3		1	

** Experiment temperature 50 °C.

an important advance useful for designing of heap bioleaching plant (Panda et al., 2015). These bacteria represent the first native mining consortium isolated directly from copper mines. In addition, this information can be used to intervene directly the composition of the consortium inside the bioreactor (Feng et al., 2015a,b). Finally, these five bacteria constitute the industrial bioleaching consortium best described to date and represent the culmination of a decade of research in genomics of mining bacteria.

Author contributions

M.L., R.B., P.P., V.C., M.G. and A.M. analyzed the results and wrote the manuscript. M.P.C., D.T., A.D. and M.B. performed all the bioinformatics analysis. M.L., A.R. and C.H. participated in protein manual annotation. gDNA bacterial extraction was done by R. B.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2016.07.012.

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