



## Comparative genomics unravels metabolic differences at the species and/or strain level and extremely acidic environmental adaptation of ten bacteria belonging to the genus *Acidithiobacillus*

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### ABSTRACT

Members of the *Acidithiobacillus* genus are widely found in extreme environments characterized by low pH and high concentrations of toxic substances, thus it is necessary to identify the cellular mechanisms needed to cope with these harsh conditions. Pan-genome analysis of ten bacteria belonging to the genus *Acidithiobacillus* suggested the existence of core genome, most of which were assigned to the metabolism-associated genes. Additionally, the unique genes of *Acidithiobacillus ferrooxidans* were much less than those of other species. A large proportion of *Acidithiobacillus ferrivorans*-specific genes were mapped especially to metabolism-related genes, indicating that diverse metabolic pathways might confer an advantage for adaptation to local environmental conditions. Analyses of functional metabolisms revealed the differences of carbon metabolism, nitrogen metabolism, and sulfur metabolism at the species and/or strain level. The findings also showed that *Acidithiobacillus* spp. harbored specific adaptive mechanisms for thriving under extreme environments. The genus *Acidithiobacillus* had the genetic potential to resist and metabolize toxic substances such as heavy metals and organic solvents. Comparison across species and/or strains of *Acidithiobacillus* populations provided a deeper appreciation of metabolic differences and environmental adaptation, as well as highlighting the importance of cellular mechanisms that maintain the basal physiological functions under complex acidic environmental conditions.

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### Introduction

Both natural and man-made acidic environments with low pH and high concentrations of toxic substances are harmful to most prokaryotic and eukaryotic organisms [13,68]. However, acidophiles that have a pH optimum of less than pH 3 are endowed with the peculiar adaptive and survival mechanisms needed to thrive in these extreme environments. The

*Acidithiobacillus* genus, the Gram-negative, extremely acidophilic and chemolithoautotrophic *Gammaproteobacteria*, currently contains several recognized species, including *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans*, *Acidithiobacillus caldus*, and *Acidithiobacillus ferrivorans* [24,60,71,73]. These four autotrophic microorganisms isolated from extremely acidic environments are remarkably adaptable to the particular circumstances subjected to sudden stress changes or long-term stress conditions. They have the capability to acquire energy by oxidizing elemental sulfur and a wide range of reduced inorganic sulfur compounds (RISCs) [14]. Additionally, *A. ferrooxidans* and *A. ferrivorans* have also been shown to utilize energy and electrons derived from the oxidation of ferrous iron for CO<sub>2</sub> and nitrogen fixation, as well as other anabolic pathways [14,40].

For some time, the considerably limited information concerning the cellular mechanisms of acidophilic prokaryotes used to

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**Table 1**  
Summary of the *Acidithiobacillus* species and their genomes analyzed in this study.

Species	Strain	Habitat	Temp. optimum	pH range	Accession no.	Size (Mb)	Status	Reference
<i>A. caldus</i>	SM-1	Pilot bioleaching reactor	Moderate thermophilic (45 °C optimum)	2.0–2.5	NC.015850-54	3.24	Complete	[73]
	ATCC 51756	Coal spoil at the Kingsbury mine, UK			NZ.CP005986-89	2.99	Complete	[59]
<i>A. thiooxidans</i>	A01	Wastewater of coal dump, China	Mesophilic (30 °C optimum)	2.0–2.5	AZM000000000	3.82	Draft	[71]
	ATCC 19377	Kimmeridge clay, UK			AFOH000000000	3.02	Draft	[58]
	Licanantay	Copper mine, Atacama, Chile			JMEB000000000	3.94	Draft	[55]
<i>A. ferrivorans</i>	SS3	Norilsk, Russia	Psychrotolerant	1.8–2.0	NC.015942	3.21	Complete	[24]
	CF27	Cobalt/copper mine, Idaho, USA			CCCS000000000	3.43	Draft	[53]
<i>A. ferrooxidans</i>	ATCC 53993	NG <sup>a</sup>	Mesophilic (30 °C optimum)	1.8–2.0	NC.011206	2.89	Complete	NG <sup>a</sup>
	ATCC 23270	Bituminous effluent of coal mine			NC.011761	2.98	Complete	[60]
	YQH-1	River in Wudalianchi volcano, China			LJBT000000000	3.11	Draft	[67]

<sup>a</sup> NG means that the relevant information is not given.

survive and proliferate in such extreme environments has been generated by conventional methods, such as genetic manipulation, since the limitation of these approaches has greatly restricted large-scale studies involving the molecular biology and physiology of extreme acidophiles [60]. Fortunately, with the rapid improvement of genome sequencing technologies and the continuous development of bioinformatics-based analytical methods, effective tools have revolutionized the exploration of microorganisms and have provided a very valuable resource for investigating several integrative metabolic and regulatory models [61].

The *Acidithiobacillus* genus, which belongs to the new class *Acidithiobacillia* reported by Williams and Kelly [63], is known for its ubiquity in many acidic environments [65,75,76]. In recent decades, numerous studies dependent on genome sequencing have focused on important issues mainly related to key metabolic pathways [23,28,60,61,70], including carbon and nitrogen assimilation, reduced inorganic sulfur compounds (RISCs) and iron oxidation, as well as hydrogen and formate utilization. For instance, *A. ferrooxidans* and *A. ferrivorans* are believed to play key roles in the biogeochemical cycle of iron. In these microbes, the Fe(II) oxidation model, which mainly involves two types of rusticyanins as well as two forms of high-potential iron–sulfur proteins, has been well studied [4,53,60,75]. In addition, a highlight of this current study was the microbial response to environmental stresses, which has always been a critical issue in ecological fields [69]. Recently, many studies based on metagenomic data from extremely acidic environments have shown that some acid and heavy metal resistance genes were identified and assigned to the *Acidithiobacillus*-like populations [12,32,76]. In particular, there are several papers that discuss issues associated with environmental adaptation of *A. ferrooxidans* [60,77], including heavy metal resistance, extrusion of toxic organic compounds, flagella formation and chemotaxis, as well as adhesion and biofilm formation. However, relatively little information is available at present concerning the adaptive mechanisms that allow this and another three species to survive under acidic conditions.

Recent acquisition of genome sequences provides details for the discovery of additional genes, and supports a new field called comparative genomics [17]. By comparative analysis, a substantial body of information, such as gene function and gene/genome evolution, can be acquired, thus improving our understanding of bacterial evolutionary relationships [26,44,54]. Therefore, in order to explore the integrated metabolic diversity and adaptive mechanisms in extremely acidic environments, on the basis of previous studies, the genomes of ten typical acidophiles belonging to four species of *Acidithiobacillus* were selected for a comparative survey. All bacterial strains used in this study were able to withstand low pH (approximately 2.0), as well as acquire energy from extremely acidic environments and use them as carbon and nitrogen sources,

while maintaining their basal metabolic activities. Therefore, this study used the comparative genomics of ten bioleaching bacteria in order to reveal and elucidate their metabolic differences and environmental adaptation, as well as provide information to advance our understanding of the adaptive mechanisms of microorganisms subjected to different extreme conditions.

## Materials and methods

### Availability of supporting data

The genome sequences of ten *Acidithiobacillus* strains (Table 1) were obtained from the GenBank database. In-house Perl scripts were then used to extract the protein sequences of each strain for subsequent analysis.

### Phylogenomic tree and pan-genome analysis

CVTree3, an automated web server for the construction of phylogenomic trees, was used to assess the evolutionary relationship of ten bacteria based on whole genome sequences (K-tuple length 9) [66]. Compared to traditional phylogenetic trees, the resulting trees are justified directly by taxonomic comparison, instead of performing statistical re-sampling tests, such as bootstrap or jack-knife analyses. For pan-genome analysis, an all-versus-all BLASTP ( $E$ -value  $\leq 1e^{-5}$ ) was performed on the extracted protein sequences from each strain using the FASTA format. The BLAST output was then used as the input file to identify single-copy orthologs using the software PanOCT (sequence identity  $\geq 50\%$ ;  $E$ -value  $\leq 1e^{-5}$ ) [9]. Subsequently, both the shared and strain-specific protein sequences were searched against the extended cluster of orthologous genes (COG) database [10] using the BLASTP algorithm.

### Identification of genes associated with key metabolisms and environmental adaptation

Pan-genome analyses showed the core genomes and dispensable/accessory genomes among the *Acidithiobacillus* spp., thereby facilitating the confirmation of their shared and/or unique genes across all strains used in this study. In particular, we focused on the detection of protein-coding sequences (CDSs) associated with key metabolisms, including carbon metabolism, nitrogen metabolism and sulfur metabolism. The relevant CDSs assigned to core and dispensable genomes were identified and extracted based on the BLAST algorithm against the generalist databank (NCBI-NR). Additionally, CDSs involving environmental adaptation, such as bacterial adhesion and biofilm formation, were functionally annotated via homology searching against the previously reported gene or protein sequences. Subsequently, the entire annotations

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