

## Genetic variability in *Acidithiobacillus* spp. – a working horse of environmental biotechnologies

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### Abstract

The genus *Acidithiobacillus* comprises 7 species of Gram-negative obligatory acidophilic chemolithotrophic bacteria that derive energy mainly from the oxidation of reduced sulphur compounds. Four of the species also catalyse the dissimilatory oxidation of ferrous iron while three (*A. thiooxidans*, *A. albertensis*, and *A. caldus*) do not. Bacteria from the genus *Acidithiobacillus* are often associated with mineral biotechnologies (biomining) and acid mine drainage. While acceleration of mineral solubilisation is a positive aspect in environmental biotechnologies, it is undesirable in acid mine drainage with strong negative ecological impact and there is profound interest in genetics and genomics of these bacteria. Representatives of *Acidithiobacillus* genus occur world-wide, however there are limited data on *Acidithiobacillus* spp. variability from Slovakia. In our work the variability of *Acidithiobacillus* spp., from Slovakia was analysed and the presence of *A. ferrooxidans* was detected. In addition, for the first time we report here on the occurrence of *A. albertensis* as well. Comparative analyses confirmed pronounced genetic and genomic diversity within the genus, especially within *A. ferrooxidans* and *A. thiooxidans* complexes. Based on data presented, several *Acidithiobacillus* species could be considered as a complex species and the description of several new species is very probable in the near future.

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## Introduction

The genus *Acidithiobacillus* comprises a group of Gram-negative obligatory acidophilic chemolithotrophic bacteria that derive energy mainly from the oxidation of reduced sulphur compounds. The members of the genus are motile by one or more flagella and comprise both mesophiles and moderate thermophiles (Kelly and Wood 2000). *Acidithiobacilli* use various sulphur compounds as electron donor and therefore, are often found in metal sulphide deposits all over the world (Karavaiko *et al.* 2003), fresh waters

associated with sulphide deposits (Gonzalez-Toril *et al.* 2003) and seawater (Kamimura *et al.* 2003). The final electron acceptor is usually oxygen (Kelly and Wood 2000) however, Fe<sup>3+</sup> and S<sup>0</sup> were reported to be alternative electron acceptors for some species (Ohmura *et al.* 2002). The ability to fix CO<sub>2</sub> makes *acidithiobacilli* independent of an organic carbon source and allows them to thrive in mine waste ecosystems where the organic carbon concentration is extremely low (Dold *et al.* 2005).

The *Acidithiobacillus* genus was formed when the former *Thiobacillus* genus was split into the genera

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*Acidithiobacillus*, *Halothiobacillus*, and *Thermithiobacillus* (Kelly and Wood 2000). The type species of the genus, *A. thiooxidans* was described nearly 100 years ago as *Thiobacillus thiooxydans* (Waksmann and Joffe 1922) and up to now 7 species within the genus were validly described. All species are autotrophs capable of growth utilising inorganic sulphur compounds as sole energy substrate. Four of the species (*A. ferriphilus*, *A. ferrivorans*, *A. ferrooxidans*, and *A. ferridurans*) also catalyse the dissimilatory oxidation of ferrous iron while three (*A. thiooxidans*, *A. albertensis*, and *A. caldus*) do not. Representatives of *Acidithiobacillus* genus occur world-wide in a diverse range of natural (acid rock drainage, sulphur springs, etc.) and industrial settings (ore concentrates, leaching solutions of the mining industry, etc.), with varying physicochemical characteristics.

Bacteria from the genus *Acidithiobacillus* are often associated with mineral biotechnologies (biomining) and acid mine drainage. Biomining utilises these bacteria for recovery of metals from sulphidic low grade ores and concentrates. Acid mine drainage results in acidification and metal contamination of soil and water emanating from the dissolution of metal sulphides from deposits and mine waste storage (Johnson 2014). Acidophilic microorganisms play a central role in these processes by catalysing aerobic oxidation of sulphides. While acceleration of mineral solubilisation is a positive aspect in mineral biotechnologies, it is undesirable in acid mine drainage with strong negative ecological impact and there is profound interest in genetics and genomics of these bacteria.

DNA based analyses have already revealed pronounced genetic diversity within *Acidithiobacillus* genus, especially within *A. ferrooxidans* complex. The diversity probably could be not attributed to the geographic distribution of acidithiobacilli as practically all known species were detected worldwide (Nunez *et al.* 2017). More than 50 % of all *Acidithiobacillus* isolates characterised at 16S sequence level can be mapped to Asia, followed by Europe (20 %), South (9 %), and North America (7 %), and Africa and Oceania (less than 2 % each). From the Slovakia territory two isolates were characterised, both identified as *A. ferrooxidans*

(Nunez *et al.* 2017). The aim of our study was to understand the variability within acidithiobacilli in Slovakia and worldwide based on genetic and genomic analyses.

## Experimental

### *Molecular identification of Acidithiobacillus spp. isolates from the Slovakia*

Molecular identification of *Acidithiobacillus* spp. isolated recently from Smolnik (East Slovakia) acid mine drainage water – LC isolate (Luptakova *et al.* 2012) and *A. albertensis* TS isolate from Hodruša-Hámre (Central Slovakia) gold mine (Pristas *et al.*; manuscript in preparation) were performed using widely accepted 16S based methodology (Yarza *et al.* 2014). The sequences obtained were deposited into GenBank database under accession numbers MH740926 and MH796351 for LC and TS isolate, respectively. The sequences were compared against database of 16S sequences of type strains of bacteria and archaea using BLASTN analysis (Altschul *et al.* 1990). Phylogenetic placement of isolates was confirmed by multiple sequence comparison against 528 type strains of *Acidithiobacillus* spp. downloaded from RDP database (available at <https://rdp.cme.msu.edu/>) using the Neighbor-Joining method implemented in MEGA 7 software (Kumar *et al.* 2016) with 500 bootstrap replications.

### *Analysis of genetic variability within Acidithiobacillus genus*

To analyse genetic variability in *Acidithiobacillus* genus 16S rRNA sequences of LC and TS isolates obtained in this study were added to the pre-aligned 16S *Acidithiobacillus* spp. sequences and analysed as described above. For genome comparison, available genome sequences of *Acidithiobacillus* spp. were downloaded from the GenBank database (<https://www.ncbi.nlm.nih.gov/genome>). Final dataset was composed of 8 sequences - GCA\_001931655 for *A. albertensis*, GCA\_000214095.3 for *A. ferrivorans*, GCA\_000175575.2 for *A. caldus*, GCA\_000227215.2 for *A. thiooxidans*,

GCA\_000021485 for *A. ferrooxidans*, GCA\_000179815.2 for *Acidithiobacillus* sp. GGI-221, GCA\_002341825.1 for *Acidithiobacillus* sp. UBA2486, and GCA\_002733395.1 for *Acidithiobacillus* sp. NORP59. For each pair of sequences ANI value (Average Nucleotide Identity) was calculated using EzTaxon server (<https://www.ezbiocloud.net/tools/ani>; Yoon *et al.* 2017). Based on ANI values obtained a similarity dendrogram was constructed using UPGMA algorithm implemented in MEGA 7. For functional genome comparisons the RAST server pipeline (Rapid Annotation by Subsystem Technology) available at <https://rast.theseed.org/> was used (Overbeek *et al.* 2014).

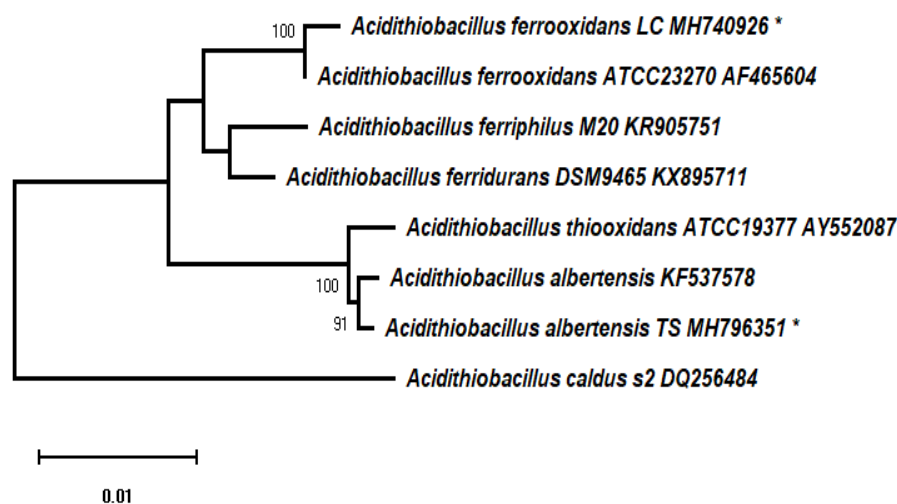
## Results and Discussion

Two recently isolated *Acidithiobacillus* spp. isolates were identified using 16S rRNA sequencing followed by BLASTN analysis of sequences obtained. The 16S rRNA sequence of LC isolate from Smolnik acid mine drainage water showed the highest similarity (99.7 %) to the 16S rRNA sequences of type species of *A. ferrooxidans* strain ATCC 23270. The 16S rRNA sequence of TS isolate from Hodruša-Hámre gold mine has shown the highest similarity (99.7 %) to the *A. albertensis* DSM 14366 sequence. Multiple sequence alignments confirmed identification of both isolates resulted from BLASTN analysis (Fig.1). The LC isolate sequence grouped with *A. ferrooxidans* sequence, while this of TS isolate grouped with *A. albertensis* sequence.

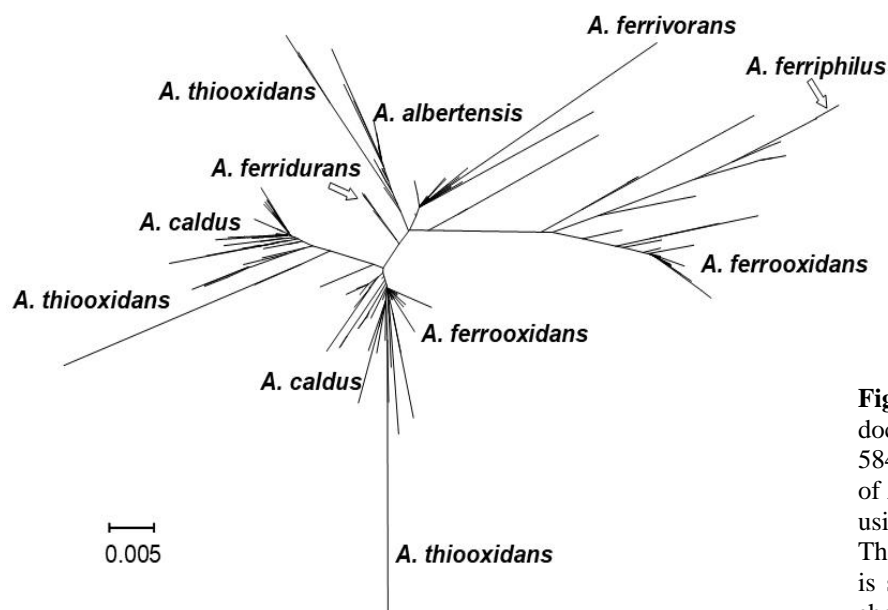
The tree indicates that the ability to obtain energy from the dissimilatory oxidation of ferrous iron is probably of old evolutionary origin as all four species able to utilise ferrous ions (*A. ferrophilus*, *A. ferrivorans*, *A. ferrooxidans*, and *A. ferridurans*) formed well supported group phylogenetically distant from the species unable to utilize ferrous ions. The analysis indicates that *A. caldus* is the most distant species of *Acidithiobacillus* genus, forming a separate branch non-related to the other members of the genus (Fig. 1).

Up to now, up to 120 *Acidithiobacillus* spp. isolates or environmental 16S rRNA sequences were identified from the European territory, most of them identified as *A. ferrooxidans* and *A. thiooxidans* and the occurrence of *A. ferrooxidans* related bacteria have already been reported from Slovakia as well (Nunez *et al.* 2017). However, no *A. albertensis* related sequences have been reported neither from the Slovak nor European territory yet. *A. albertensis* species was described in 1983 (Bryant *et al.* 1983) and it is not studied very frequently. *A. albertensis* related isolates were reported up to now mainly from China and Canada (Nunez *et al.* 2017), while our observation is the first report on *A. albertensis* from Europe.

Biotechnology and environmental protection interest led to the characterization of multiple *Acidithiobacillus* spp. isolates mainly from industrial environments. Most of the isolates were identified as *A. ferrooxidans* or *A. thiooxidans* as for many years these two species were the only validly described species of the *Acidithiobacillus*



**Fig. 1.** Unrooted phylogenetic tree documenting phylogenetic placement of *Acidithiobacillus* spp. from Slovakia (*A. ferrooxidans* LC and *A. albertensis* TS; indicated with asterisks) inferred using the Neighbor-Joining method. The bar represents genetic distance shown in number of base substitutions per site. Numbers at nodes are bootstrap values after 500 repetitions.



**Fig. 2.** Unrooted phylogenetic tree documenting phylogenetic diversity within 584 available 16S rRNA sequences of *Acidithiobacillus* spp. The tree was inferred using the Neighbor-Joining method. The placement of validly described species is shown. The bar represents genetic distance shown in number of base substitutions per site.

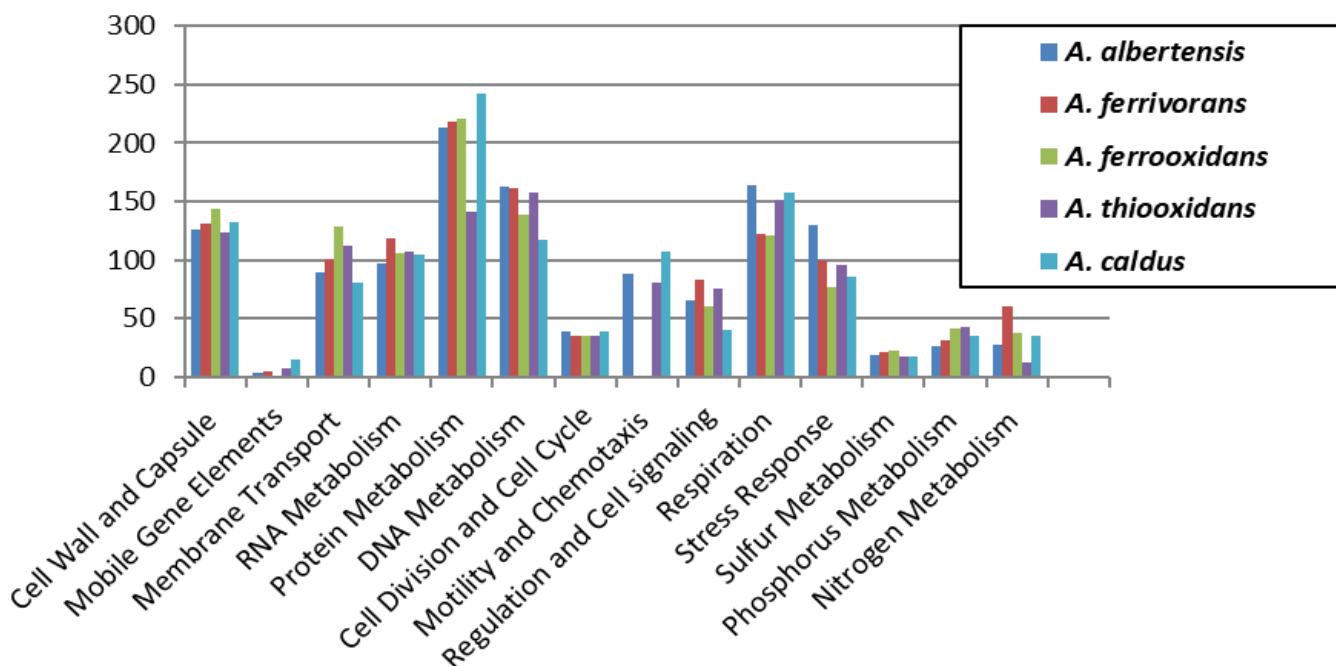
genus (Jones *et al.* 2016). In the recent years great diversity within the genus was recognised and several new species of acidithiobacilli were described (Hallberg *et al.* 2010; Hedrich and Johnson 2013; Falagan and Johnson 2016). To understand genetic variability in *Acidithiobacillus* genus all available 16S rRNA sequence of *Acidithiobacillus*-related bacteria were downloaded from RDP database (Cole *et al.* 2014) and compared using phylogenetic approach. The resulted phylogenetic tree (Fig. 2) clearly documents extraordinary variability within *Acidithiobacillus* genus. While the recently described species e.g. *A. ferrivorans* and *A. ferridurans* form monophyletic, well supported clades in the tree, other species are spread over the tree and could be found in multiple branches, indicating genetic variability within the genus. The diversity within *Acidithiobacillus* genus have already been reported (Karavaiko *et al.* 2003; Ni *et al.* 2008) and based on our data the existence of at least 20 species could be proposed solely on 16s rRNA analysis.

Recent advances in genomics allowed us to better understand the common and distinctive features some of *Acidithiobacillus* species isolated from similar habitats and provided data to understand potential adaptation mechanisms of these bacteria to the harsh environments. Complete or draft genome sequences of several *Acidithiobacillus* species have become available in the recent few

years (Cárdenas *et al.* 2016). Comparative genomic analyses indicate that genomes of acidithiobacilli are relatively similar. The genome sizes vary from 2.78 to 3.78 Mbp, G + C content vary from 52.5 to 67.5 %, and numbers of predicted genes vary from 2648 to 4007. In all these parameters *A. caldus* is the most distant species, showing the highest G + C content, the smallest genome size, and the smallest number of genes (data not shown). Based on the functional comparative genomic analyses of *Acidithiobacillus* species, it was shown that different metabolic pathways might allow these microorganisms to acquire energy, carbon sources, and nitrogen sources from acidic environments, ensuring their survival and proliferation in these environments (Fig. 3). Using RAST server pipeline (Overbeek *et al.* 2014) very similar numbers of genes were found e.g. those of involved in RNA metabolism or cell wall and capsule subsystems in all *Acidithiobacillus* species analysed. The species, however, differ significantly in number of genes classified to the Nitrogen or Phosphorus metabolism, and Protein metabolism, as well as stress response (Fig. 3). The comparisons indicate that non-motile species of the genus (*A. ferrooxidans* and *A. thiooxidans*) completely lack the genes for motility and chemotaxis. The phenotypic diversity observed within the genus is thus a consequence of genetic and genomic diversity.

To evaluate the genomic diversity in more detail,



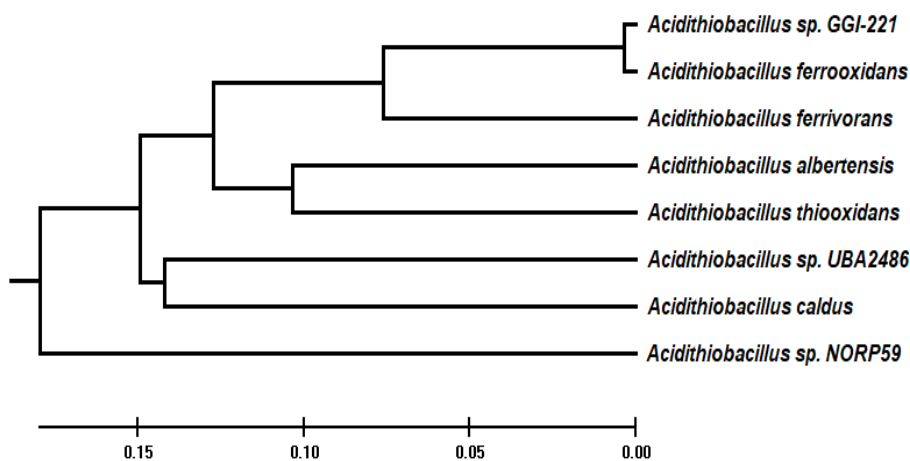


**Fig. 3.** Comparative genomics of selected *Acidithiobacillus* species. Number of genes classified to the selected subsystems as identified by RAST server is shown.

average nucleotide identity (ANI) values were calculated for every pair of genomic sequences and compared. ANI was proposed recently as a means to compare genetic relatedness among prokaryotic strains. The method compares nucleotide-level genomic similarity between the coding regions of two genomes and is considered the best measure of genetic relatedness among bacterial strains (Figueras *et al.* 2014; Jain *et al.* 2018). ANI values in range from 0.993 to 0.640 were observed among analysed set of sequences. Based on ANI values relatedness tree of *Acidithiobacillus* spp. was constructed (Fig. 4). The tree shows congruent topology with 16S rRNA based tree (Fig. 1).

The ferrous iron-oxidising species form a separate group distant from the species that are unable to utilize ferrous ions, and (like in 16S rRNA based tree) *A. caldus* is the most divergent species of *Acidithiobacillus* genus related distantly only to any other species. The tree also documents diversity within the genus when the yet uncharacterised isolates UBA2486 and NORP59 have produced significantly low ANI values and therefore could be assigned as a new species within *Acidithiobacillus* genus. Similarity values higher than 0.95 are necessary to place the isolates to the same species (Figueras *et al.* 2014).

The first species of the *Acidithiobacillus* genus was



**Fig. 4.** UPGMA (unweighted pair group method with arithmetic mean) generated dendrogram showing relatedness of *Acidithiobacillus* sp. based on ANI (average nucleotide identity) values.

described already 100 years ago (Waksmann and Joffe 1922) and up to now 7 species of acidithiobacilli were validly described, most of them in the recent 20 years. However, application of modern genetic and genomic methods indicates that variability within the genus is highly underestimated and several species, especially *A. ferrooxidans* and *A. thiooxidans*, have to be considered a complex species and the description of multiple new species of acidithiobacilli is very probable in the near future.

## Conclusions

Our data indicate that genetic variability of *Acidithiobacillus* genus is highly underestimated. Several species, especially *A. ferrooxidans* and *A. thiooxidans*, have to be considered a complex species and the description of multiple new species of acidithiobacilli is very probable in the near future. From the Slovakia territory *A. ferrooxidans* species is reported predominantly, but for the first time we report here on the occurrence of *A. albertensis* as well.

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