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The first evidence of *Acidithiobacillus albertensis* in weathered ore samples from active gold mine Hodruša-Hámre (Slovakia)

Jana Sedláková-Kaduková[1,✉], Jana Kisková[1], Lenka Maliničová[1], Ivana Timková[1], Stanislav Jeleň[2] and Peter Pristaš[1]

[1] *Department of Microbiology, Institute of Biology and Ecology, Faculty of Science, Pavol Jozef Šafarik University in Košice, Šrobárova 2, SK-041 54 Košice, Slovak Republic*

[2] *Earth Science Institute, Slovak Academy of Sciences, Ďumbierska 1, SK-974 01, Banská Bystrica, Slovak Republic*

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Abstract

Sulphur-oxidising autotrophic bacterial communities in deep biosphere from weathered ore samples from active gold mine Hodruša-Hámre, Slovakia were analysed using cultivation approach followed by DNA extraction, PCR amplification and 16S rRNA gene analyses. Indirect measurement of pH changes in cultivation media evidenced the presence of acidophilic bacteria with active production of acids. The decrease of pH was observed at the beginning of isolation and later pH in range of 1.5 – 2 was maintained in both, sulphuric acid and thiosulphate, media. The presence of homogenous population of gram-negative rods was proved by Gram staining. Molecular analyses have revealed that the population of sulphur-oxidising bacteria in gold mine is dominated by a single species of *Aciditiobacillus* genus, identified as *A. albertensis*, suggesting the low level of autotrophic bacteria diversity in deep deposits. For the first time this species was isolated from weathered rocks of a gold mine subsurface environment.

Introduction

In the past years, our perspective on the Earth's biosphere has expanded from just the terrestrial and oceanic realms to include deep subterranean and subseafloor environments (Inagaki *et al.* 2003). The deep terrestrial subsurface environments such as those exemplified by deep mines represent an emerging area for exploring microbial populations with bewildering arrays of metabolic capabilities (Rastogi *et al.* 2010). Majority of these environments are characterised as extreme because of their hostile life conditions such as extreme temperature, pH, pressure, low oxygen content, no light and toxic metals presence. The existence of microorganisms in such environment is of increasing scientific and practical interest because subsurface microorganisms with novel metabolic properties may be of potential value to industry for applications in bioremediation and biotechnology (Takai *et al.* 2001). Microbes in the deep terrestrial subsurface environment are considered to play a key role in deposition and weathering the minerals being a part of geochemical processes and are the only life forms that have been encountered in the deeper regions of the Earth´s crust (Li *et al.* 2017).

Microbial communities existing in deep subsurface soil especially in gold mines have been studied very rarely and therefore they remain largely uncharacterized. The Banská Hodruša Au + Ag, Pb, Zn, Cu deposit at the Rozália mine (48°27´N,

✉ *Corresponding author:* jana.sedlakova@upjs.sk

18°51´E) is the last operating gold mine in Slovakia and it offers a unique opportunity for direct exploration of the mining-impacted deep subsurface environment. It occurs in the central zone of the large Middle Miocene Banská Štiavnica stratovolcano (15.0 – 10.7 mil. years) within the Banská Štiavnica – Hodruša ore district, which belongs to the largest ore districts in the Carpathian arc, famous for Ag-Au mining since the Middle Ages. The subhorizontal vein system occurs in 400 – 650 m depth, it is about 1.2 km long, hosted by andesite, near to the flat roof of the pre-mineralization subvolcanic granodiorite pluton. The deposit consists of two parts, separated by a thick sill of quartz-diorite porphyry. The eastern part is currently mined, and the western part has already been depleted (Kubač *et al.* 2018; Koděra *et al.* 2018).

Gold-sulphide ore from epithermal vein deposit Banská Hodruša consists of gold, electrum, galena, sphalerite, chalcopyrite, and pyrite. Minor amounts of tellurides are present in the form of hessite, petzite and other rare Au sulphides and tellurides. Gangue minerals are represented by abundant quartz and adularia, clay minerals and carbonates. The ore has excellent metallurgical properties, content of gold is in range of 5 – 600 $g.t^{-1}$, with average 20 – 30 $g.t^{-1}$ and maximum achievable gold recovery by flotation is 96 – 98 % (Chovan *et al.* 2016).

Microbial diversity present in Hodruša-Hámre mine have not been reported yet. In the present study our objective was to elucidate the existence and composition of sulphur-oxidising autotrophic bacterial population in the weathered ore samples of Hodruša-Hámre gold mine, Slovakia, by culture-based molecular methods. Sulphur oxidising TS strain of *Acidithiobacillus albertensis* species was isolated for the first time in Europe and basic microbiological characteristics and phylogenetic relatedness of the strain were determined.

Experimental

Sample collection

The samples (500 g each) were collected along the junction of the drift wall and the floor in vein Blanka. Yellow mats were covering the rock surface around the sample colleting site. The area was not disturbed by any type of activities including human trafficking from the end of 2000. The weathered ore was collected 10 cm below the surface with sterile spatula. The temperature at the time of sampling was 19 °C, it was measured using VWR EU 620-1259 thermometer. The samples were transported to the laboratory in sterile zip lock sacks on ice and in refrigerator.

Isolation of autotrophic bacteria

Six grams of homogenized soil sample was resuspended into 100 mL of sulphuric acid with pH 1.5 and 100 mL of thiosulphate medium. The composition of thiosulphate medium was 90 mL of mineral medium and 10 mL of 10 % (w/v) $Na_2S_2O_3$, mineral medium was composed of 1.2 g KH_2PO_4, 0.2 g K_2HPO_4, 0.75 g $MgCl_2 \cdot 6H_2O$, 0.15 g $CaCl_2 \cdot 2H_2O$, 0.5 g NH_4Cl, 0.5 g Na_2CO_3 into 1,000 mL of deionised water. Few drops of concentrated HCl were added into sterilised mineral media to dissolve precipitates. Both cultures were cultivated aerobically at 25 °C and pH values of media were measured once a week for 237 d using VWR pH 110 pH meter Fresh medium (20 mL) was added into culture flask after 29 d of cultivation.

DNA isolation, 16S rRNA gene amplification, RFLP analysis and sequence analysis

Genomic DNA was extracted from bacterial cultures as described by Chen *et al*. (2009). Amplification of 16S rDNA gene was performed using fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') universal bacterial primers according to Vandžurová *et al*. (2013). The amplification products (0.5 µg of DNA) were analysed by restriction fragment length polymorphism (RFLP) method using AluI, HpaII, HaeIII, HhaI, EcoRI, and PstI (Thermo Scientific, USA) restriction endonucleases according to the manufacturer's instruction. The restriction fragments were separated by electrophoresis in 1 % (w/v) agarose gel.

The 16S rDNA amplicon of the culture from thiosulphate medium was cloned into the

pTZ57R/T vector using an InsTAclone PCR Cloning Kit (Thermo Fisher Scientific, USA) following manufacturer's instructions. Recombinant plasmids were isolated with GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich, USA), checked on 1 % (w/v) agarose gel and purified using Wizard® SV Gel and PCR Clean-Up kit (Promega, USA). Sequencing was performed using Sanger dideoxy sequencing method using plasmid specific primers by GATC Biotech sequencing facility (GATC Biotech AG, Germany). Sequences from both primers were assembled in CAP3 Sequence Assembly Program (http://doua.prabi.fr/software/cap3) (Huang and Madan 1999) and submitted to the GenBank database under accession number MH796351.

The sequences were taxonomically classified using BLASTN analysis against a database of 16S rDNA sequences of the type strains of bacteria and archaea (http://www.ncbi.nlm.nih.gov/blast) (Altschul *et al.* 1990). To elucidate phylogenetic relatedness of TS isolate the 16S rRNA sequences of *Acidithiobacillus* spp. were downloaded from GenBank database and aligned using clustalW algorithm. The phylogenetic tree was constructed using Neighbor Joining method with 1000 bootstrap replicates. For all phylogenetic analyses MEGA software ver. 7 (Kumar *et al.* 2016) was used.

Results and Discussion

The presence and activity of sulphur-oxidizing bacteria in both media was indirectly observed by changes of the medium pH values (Fig. 1). In medium containing sulphuric acid only a slight increase of pH was observed, the pH value increased from 1.5 up to 2.6 at the 35th day followed by the decrease to 1.6 later on. After that time no significant changes in pH were observed till the end of monitoring period at day 237. In thiosulphate medium with initial pH 4 the significant decrease of pH was observed to 2.1 after 17th days of cultivation followed by the decrease to 1.54 after 94 d. This pH value remained unchanged until the end of the observation period. Values of pH from 5.8 to 6.4 were measured in control experiments without bacteria added so it is evident that bacterial activity was responsible for the pH decrease.

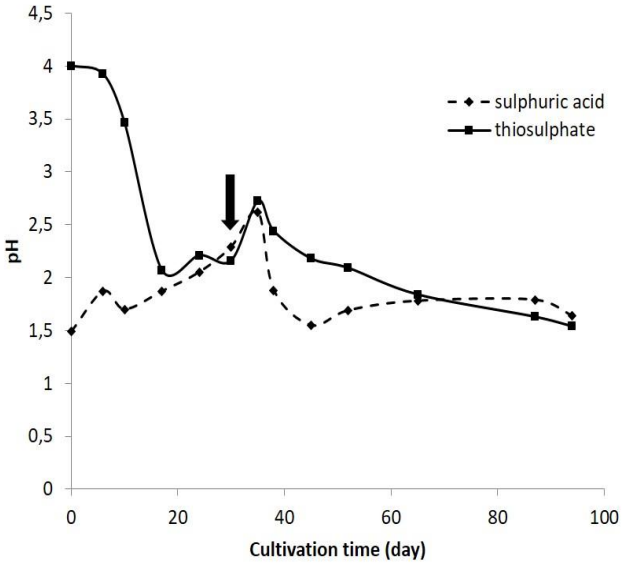

Fig. 1. Changes of pH during cultivation of bacteria from weathered ore samples from Hodruša-Hámre gold mine in two cultivation media (the arrow indicates the addition of 20 mL of fresh media into cultivation flasks at 29 d of cultivation).

Changes of pH in both cultivation media suggested acidifying activity of bacteria indirectly confirming presence of the bacteria. As no carbon source was added to the cultivation media bacterial growth and pH changes observed have to be accounted for the presence of autotrophic bacteria.

Under direct microscopic observation a morphologically homogenous population of small (approximately 1x0.5 μm) gram-negative rods were observed only in thiosulphate medium (Fig. 2).

Bacterial DNA was, however, isolated from both media. RFLP analysis of the amplified 16S rRNA gene revealed that the identical RFLP banding pattern were generated for both, H_2SO_4 and thiosulphate media (data not shown). Lack of diversity observed in this experiment indicated the presence of highly genetic related autotrophic sulphur-oxidising bacteria in both media. The culture growing in thiosulphate medium designated as TS strain was used for further analyses. No heterotrophic growth of TS strain on LB medium with added glucose nor iron – oxidation activity on 9K medium (Silverman *et al.* 1959) were observed. The amplification and sequence analysis of 16S rRNA gene was used for molecular identification of TS isolate. Analysis of obtained 16S rRNA sequence against the prokaryotic_16S_ribosomal_RNA database

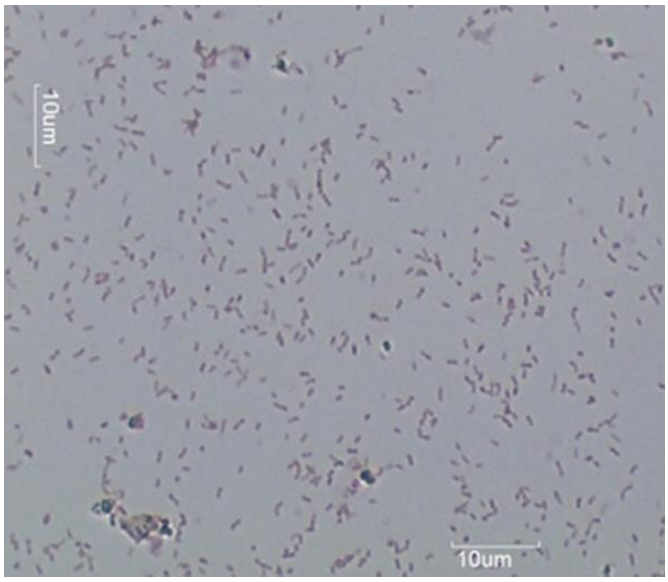

Fig. 2. Bright field photomicrograph of Gram-stained *A. albertensis* TS isolate growing in thiosulphate medium (magnification 640x). The image was taken using Motic BA310 microscope with digital camera attached. Scale bar 10 μm.

available at NCBI site (http://www.ncbi.nlm.nih.gov/blast) showed that 16S rRNA sequence of TS isolate is highly similar to the 16S rRNA sequences of multiple *Acidithiobacillus* spp. with highest similarity to the *A. albertensis* DSM 14366/ATCC 35403 strain with similarity as high as 99.7 %. Similarity to other well established species of *Acidithiobacillus* genus were lower e.g. *A. thiooxidans* (98.7 %), *A. ferrooxidans* (97.7 %) or *A. ferridurans* (97.6 %), respectively. Multiple sequence alignment placed 16S rRNA sequence of TS isolate to the large well supported clade (bootstrap support 100, see Fig. 3) of *A. albertensis* sequences. Based on the morphological, metabolic and phylogenetic analyses the autotrophic sulphur-oxidising TS isolate cultivated from weathered ore samples from active gold mine Hodruša-Hámre could be classified as *A. albertensis*.

The bacteria of *Acidithiobacillus* genus are Gram-negative obligatory acidophilic chemolithotrophic autotrophs capable of growth utilising inorganic sulphur compounds as sole energy source (Karavaiko *et al.* 2003). Due to increasing industrial and environmental impact of acidithiobacilli there is profound interest in biology, genetics, and genomics of these bacteria (Pristas *et al.* 2018). The genus comprises 7 species which occur world-wide in a range of natural

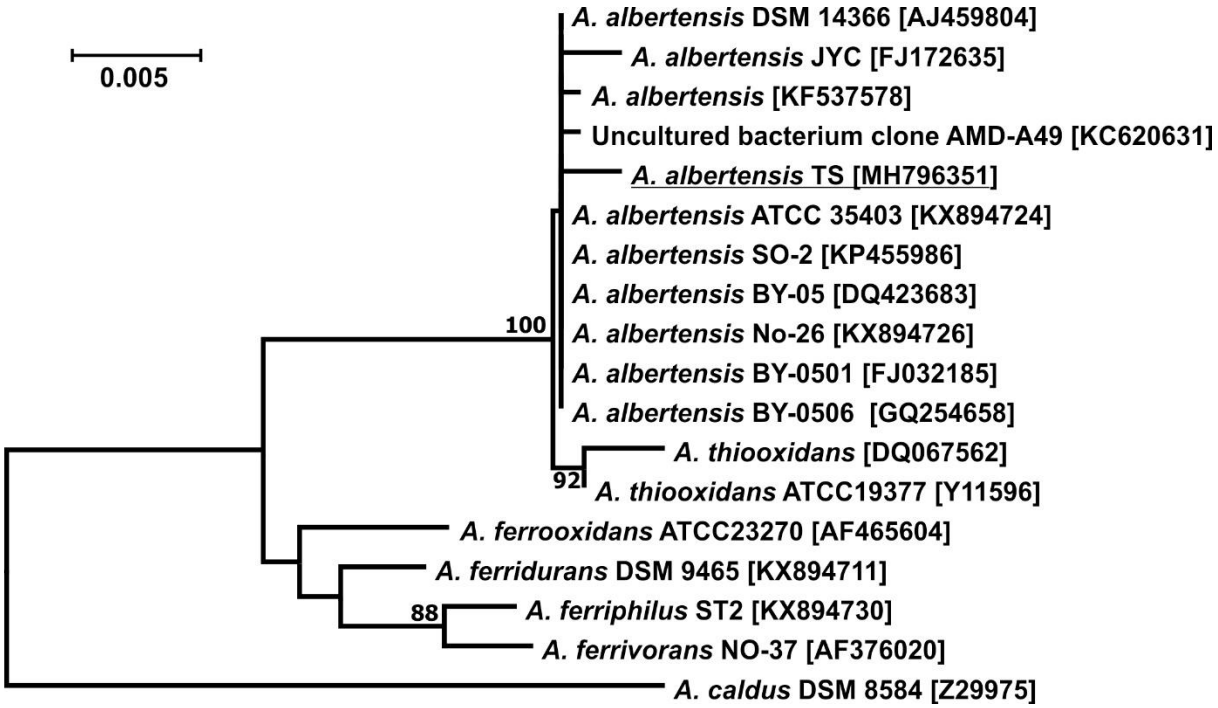

Fig. 3. Unrooted phylogenetic tree documenting relatedness of *A. albertensis* TS isolate (underlined) to other *Acidithiobacillus* spp. 16S rRNA gene sequences. GenBank accession numbers are shown in parentheses. The tree was constructed using Neighbor Joining algorithm implemented in MEGA software ver. 7 (Kumar *et al.* 2016). Number at nodes are bootstrap values after 1000 repetition, only values over 80 are shown.

Table 1. List of *Acidithiobacillus* sp. isolated from European mines (modified from Nuñez *et al.* 2017). Data on *A. albertensis* characterised in our study are shown bold.

Country	Species	Sample Type	Ore Type
Austria	*A. ferrooxidans*	water	uranium
Finland	*A. ferriphilus*	NA	zinc
	A. ferrooxidans	NA	copper
France	*A. ferridurans*	water	zinc, lead
	A. ferrooxidans	water	zinc, lead
	A. ferrooxidans	NA	uranium
Germany	*A. ferrooxidans*	soil	NA
	A. ferrooxidans	water	copper
	A. ferrooxidans	slurry	copper
	A. ferrooxidans	soil	coal
	A. ferrooxidans	soil	uranium
Norway	*A. ferrivorans*	water	copper
Romania	*A. ferrooxidans*	soil	sulphide
	A. ferrooxidans	soil	zinc
Slovakia	*A. ferrooxidans*	water	copper
	A. albertensis	**soil**	**gold**
Spain	*A. ferrooxidans*	water	nickel
	A. ferrivorans	soil	copper
	A. caldus	soil	coal
United Kingdom	*A. ferrooxidans*	soil	coal
	A. ferrooxidans	soil	copper
	A. ferrooxidans	water	tin
	A. ferrooxidans	NA	pyrite
	A. thiooxidans	soil	coal

and industrial settings (Kelly and Wood 2000), six members of the genus, except *A. albertensis*, were already isolated in European industrial areas (Table 1).

Although *A. albertensis* species was for the first time described by Bryant et al. already in 1983 (Bryant *et al.* 1983), it is not frequently studied species and there are very limited number of strains available, mainly from China territory (Liu *et al.* 2008). In previous studies this species was isolated from acidic soil adjacent to a sulphur stockpile in Canada (Bryant *et al.* 1983), from acid-mine drainage of copper ore rich of sulphur in China (Xia *et al.* 2007), from copper ore sludge in China mine (Xingyu *et al.* 2010), from acidic river Rio Agrio in Argentina (Urbieta *et al.* 2012) and from operating zinc sulphide heap processing the ore from Red Dog Mine in Alaska (Lizama *et al.* 2012). For the first time *A. albertensis* bacterium was isolated from gold mine subsurface environment and it is for the first time when this bacterium was isolated from Europe territory. Preliminary report on TS isolate characterization appeared in Pristas *et al.* (2018). TS isolate of *A. albertensis*, characterised in our study, shows typical features of the species. It is acidophilic, mesophilic, obligatory chemoautotrophic, aerobic, gram-negative rods, not producing spores. The strain is unable neither to grow heterotrophically nor to catalyse the dissimilatory oxidation of ferrous iron. The sequence analysis placed 16S rRNA sequence of TS isolate to the large cluster of *A. albertensis* sequences with similarity values as high as 99.7 %. To the cluster some environmental sequences fall as well (uncultured bacterium clone AMD-A49, GenBank accession No. KC620631) from acid mine drainage sample in China. The sequence comparison confirmed close relationship of *A. albertensis* and *A. thiooxidans* species (Fig. 3) forming a cluster well separated from ferrous ions oxidizers.

The BY-05 strain of *A. albertensis* was employed for bioleaching of metal sulphides ores (Xia *et al.* 2007) so probably biotechnological utilisation of the species might be oriented more to sulphur dissolution or as a part of bioleaching consortium with iron-oxidising bacteria to metal dissolution.

Conclusions

The subsurface biosphere in gold deposits was very rarely studied, however, according to the newest findings become evident that bacteria actively contribute and regulate biogeochemical cycles in this environment. The diversity of culturable microorganisms involved in sulphur oxidation was investigated in the weathered rocks of gold mine Hodruša-Hámre, Slovakia. Just the single species of *Acidithiobacillus albertensis* was isolated from the samples suggesting the low level of biodiversity of autotrophic bacteria in deep subsurface deposits. In this study we for the first time reported autotrophic bacterial diversity and isolation of *A. albertensis* from solid sample of weathered rocks in deep gold mine. Although *A. albertensis* is not an unknown species, there is still very little known about these bacteria and their potential

in bioleaching of metals from ores or metal-bearing waste. Further studies of functional bacteria and potential new functional species need to be carried out to explore their specific contributions to gold biogeochemical cycling.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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