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Bacterial Communities from the Arsenic Mine in Złoty Stok, Sudety Mountains, Poland

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Abstract

Investigations of bacterial communities and characterization of mineralogy of the environment in the Złoty Stok As-Au deposit were carried out. PXRD analysis revealed the presence of picropharmacolite as the most common secondary arsenic mineral in the mine. Total DNA was extracted from slime streams or slime biofilms samples to investigate the bacterial communities. PCR amplification of 16S rDNA was performed followed by subcloning of its products. Over 170 clones were analyzed by means of RFLP method. Eight group of clones representing different restriction patterns were identified. The nucleotide sequences of their inserts suggest that bacteria present in the mine environment belong to: *Flavobacteria*, *Sphingobacteriia*, *Bacteroides*, *Proteobacteria*, *Mollicutes* and *Firmicutes*. The metagenomic approach allows to demonstrate a higher diversity of microbiota than classical microbiological studies of cultivable isolates.

Key words: arsenic mine ecosystem, metagenomic approach, microbial community, supergene minerals

Introduction

Bacteria are able to grow and live as single (planktonic) cells but it is known that interactions between cells and cell-to-cell signalling exist. All processes in which a respective number of bacterial cells are involved and carried out is referred to as quorum sensing (Hammer and Bassler, 2003; Leggett *et al.*, 2014). This effect is controlled by chemical signal molecules termed autoinductors that are produced by bacteria and released to the environment. The concentration of those chemicals increases as a function of increasing cell-population density and after gaining minimal stimulatory concentration it leads to an alteration in gene expression (Miller and Bassler, 2001; Waters and Bassler, 2005). Thus, bacteria are able to form intricate multicellular communities, referred to as biofilms, which are the most ancient multicellular life forms on Earth (Pamp *et al.*, 2009; Römling and Balsalobre, 2012). These communities can form various sizes and shapes. Biofilms can be formed by cells surrounded by extracellular polymeric substances (EPS) (Wu and Xi, 2009), which are one of the components of the so-called matrix or extracellular matrix. Apart from that, proteins, extracellular DNA (eDNA), cell lysis products, water and organic matter from the surrounding environment are also involved in composition of the matrix (Narváez *et al.*, 2005; Pamp *et al.*, 2009; Wu and Xi, 2009). The matrix provides structural stability of the biofilm and allows cells to live as a community, protecting them from harmful physical and chemical factors like osmotic shock, UV radiation, predators or heavy metals and allows cells to interact with each other (Gonzales-Toril *et al.*, 2003; Jiao *et al.*, 2011). The structure of biofilms allows the bacteria living inside to function in an extreme environment. It is known that such communities can be found in mines, where nutrients are limited and pH is low (Drewniak *et al.*, 2008; Wu and Xi, 2009). Biofilms are very common in the environment and play a key role in many complex biochemical processes, which require cooperation. This microbial community also provides an ideal environment for horizontal gene transfer, which leads to genetic diversity as well as microbial evolution (Elias

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and Banin, 2012). The aim of this study was to initially characterize the ecosystem of Złoty Stok mine including the studies of its mineralogy and identification of bacterial communities (biofilm).

Experimental

Materials and Methods

Geology and mining history of the sample collection site. The Złoty Stok As-Au deposit is the largest gold deposit in the Polish part of the Western Sudetes. This deposit is located in the northern part of the Złoty Stok-Skrzynka shear zone. This zone is composed of mica schists, amphibolites, amphibole schists, myllonites and gneisses. These rocks are intercalated by marbles, serpentinites and coarse-grained gneisses. Main ore minerals, *i.e.* löllingite, arsenopyrite, pyrite, pyrrhotite and magnetite occur in black and green serpentinites and in diopside-tremolite rocks, which are connected with marble lenses. Impregnations of iron sulphides and arsenides also occur in mica schists, amphibolites and calc-silicate rocks. The precipitation of ore minerals is caused by regional metamorphic processes and migration of hydrothermal fluids from Kłodzko-Złoty Stok granitoid. The mining tradition in this area is very long. The first document confirming gold mining dates back to 1273. In 1709 the production of arsenic oxide started in Złoty Stok. The adit was built between 1916 and 1918. Gold mining and arsenic trioxide production were discontinued in 1962.

Sampling. Samples of supergene minerals and microbial communities were collected from a small drainage in the Gertruda adit. Microbial communities forming slime streamers or slime biofilms were found only in wet or completely submerged sites (Fig. 1). The occurrence of these microbial colonies is limited to the forefront of the mine. Five separate samples of slime streamers from different parts of the sampling area completely filled with water from the biofilm habitat were collected into sterile 50 ml polyethylene flasks. Supergene minerals coexisting with the microbial colonies were collected separately into small polyethylene containers.

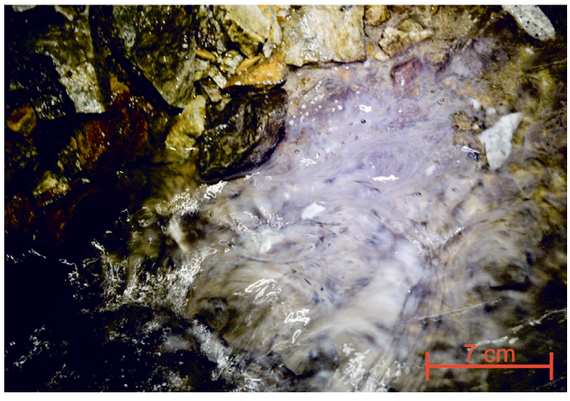

Fig. 1. Slime biofilm occurred only in wet places.

Surface morphology analyses of slime streamers and XRD analyses. Details of the surface morphology were studied with Sigma scanning electron microscopes (Carl Zeiss Microscopy GmbH, Jena, Germany) with an EDS detector (Bruker). We used an energy dispersion analyser (EDS) for qualitative chemical analysis. Powder X-Ray Diffraction (PXRD) analyses were made with an X'Pert Pro diffractometer in the Institute of Geochemistry, Mineralogy and Petrology, Faculty of Geology, University of Warsaw. The radiation was CoKα (1.73425, step of measurement = 0.02, 2.5131–75.9891°2Th).

Genomic DNA isolation. The DNA was isolated from environmental samples using a modified method developed by Ausubel and co-authors (Ausubel *et al.*, 2003). One gram of the sample material was placed into a sterile Eppendorf tube and centrifuged at 14.000 rpm at 4°C for 7 min. The supernatant was discarded and the pellet was suspended in 450 µl of TE buffer containing 4 mg/ml lysozyme and incubated for 1 h at 37°C on a horizontal shaker at 100 rpm/min. The lysate was supplemented with 50 µl 10% SDS and 10 µl of 20 mg/ml proteinase K and incubated for 1 h at 37°C. In order to remove polysaccharides 100 µl of 5 M NaCl solution was added and vortexed, followed by 80 µl of 10% CTAB and 0.7 M NaCl vortexing and incubation for 10 min at 65°C. Samples were extracted two times with an equal volume of chloroform (500 µl) and centrifuged at 14.500 rpm for 5 min at 4°C. The aqueous phases were transferred into fresh Eppendorf tubes and DNA was precipitated by adding 0.7 volume of isopropanol. After gentle mixing the samples were left for 20 min at –20°C. Precipitates were collected by centrifugation at 7.000 rpm for 10 min at 4°C. The pellets were washed with 70% ethanol, dried and resuspended in 40 µl of autoclaved double distilled water. The quality of genomic DNA was analysed by agarose gel electrophoresis.

Amplification and cloning of 16S rDNA sequences. The nearly complete 16S rDNA gene sequence was amplified by PCR with primers 16S f 5'-AGG CAG CAG TGG GGA ATA TT-3' and 16S r 5'-ACT TGA CGT CAT CCC CAC CT-3' (GeneAmp® PCR System 9700 – Applied Biosystems). PCR reactions were carried in a final volume of 25 µl. The reaction mixture contained 10 pmoles of each primer, 0.1 mmole of each dNTP, 1.0 U of *Taq* DNA polymerase, 2.5 µl of 10× reaction buffer (Novazym) and 100 ng of template genomic DNA. After initial denaturation at 94°C for 2 min. 30 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, polymerisation at 72°C

Table I
Similarities of the 16S rDNA clone sequences to sequences retrieved from databases.

Clone	Closest relative (accession no.)	Percent of homology	Phylogenetic classification
UPR_1 (KP772348)	Uncultured *Cytophagales* bacterium (FJ517047)	96.4	*Sphingobacteriia*
UPR_2 (KP772349)	*Pedobacter* sp. (KC252876)	97.5	*Sphingobacteriia*
UPR_3 (KP772350)	*Flavobacterium* sp. (KC969641)	97.1	*Flavobacteria*
UPR_4 (KP772351)	*Pseudomonas* sp. (JQ977323)	99.0	γ-*Proteobacteria*
UPR_5 (KP772352)	*Methylotenera versatilis* (NR074693)	96.8	β-*Proteobacteria*
UPR_6 (KP772353)	*Flavobacterium* sp. (JQ687101)	97.3	*Flavobacteria*
UPR_7 (KP772354)	*Mollicutes* bacterium (AY297808)	96.8	*Mollicutes*
UPR_8 (KP772355)	*Flavobacterium* sp. (KF499997)	95.9	*Flavobacteria*
UPR_9 (KP772356)	*Flavobacterium* sp. (KC969642)	96.3	*Flavobacteria*
UPR_10 (KP772357)	*Flavobacterium* sp. (JF694002)	98.5	*Flavobacteria*
UPR_11 (KP772358)	Uncultured *Hyphomicrobiaceae* bacterium (EU266801)	94.3	α-*Proteobacteria*
UPR_12 (KP772359)	Uncultured *Bacteroidetes* bacterium (JN656899)	97.0	*Bacteroides*
UPR_13 (KP772360)	*Fusibacter* sp.(AF491333)	95.2	*Firmicutes*
UPR_14 (KP772361)	*Pseudomonas frederiksbergensis* (KF424295)	97.5	γ-*Proteobacteria*
UPR_15 (KP772362)	*Flavobacterium* sp. (JF693993)	95.8	*Flavobacteria*
UPR_16 (KP772363)	*Fusibacter* sp. (KJ420408)	95.9	*Firmicutes*
UPR_17 (KP772364)	*Flavobacterium* sp. (JQ778313)	94.9	*Flavobacteria*
UPR_18 (KP772365)	*Aeromonas* sp. (KF278599)	98.8	γ-*Proteobacteria*

for 1 min followed by final polymerisation at 72°C for 4 min were applied. The PCR products were analysed on 1% agarose gels and cloned into p-GEM T Easy vector using a cloning kit (Promega – pGEM®-T Easy Vector Systems), following the manufacturer's protocol, and introduced into competent *Escherichia coli* cells, (supplied with the cloning kit). Transformants were selected by α-complementation test and grown overnight on plates with LB medium containing ampicillin (50 μg/ml), IPTG (200 mg/ml) and X-Gal (20 mg/ml). A total number of 170 white colonies were collected and plasmid DNA was isolated. The recombinant plasmids containing 16S rDNA gene fragments were amplified using vector primers: M13F (–47) 5'-CGC CAG GGT TTT CCC AGT CAC GAC-3' and M13R 5'-TCA CAC AGG AAA CAG CTA TGA C-3'. The reaction mixture was prepared as described above.

Analysis of 16S rDNA sequences. The amplified 16S rDNA products were characterised by hydrolysis with endonuclease *Cfo*I at 37°C for 2 h. The digestion products were separated in 1.7% agarose gel electrophoresis for 90 min at 80 V. Bands were visualized by staining with ethidium bromide and UV illumination. RFLP patterns were grouped visually into OTUs (operational taxonomic units), and representatives of each OTU were selected for insert sequencing and analysis (Schloss and Handelsman, 2006; Schloss and Westcott, 2011; Römling and Balsalobre, 2012). Clones description was given as follows: UPR_X, where X is the clone number. They were sequenced with a BigDye® terminator v3.1 cycle sequencing kit on an ABI 3100 automated capillary DNA sequencer (Applied Biosystems, USA). The accession numbers of sequences generated in this study are listed in Table I. The sequences were analysed with the BLAST program in the NCBI Genbank database. For phylogenetic analysis, the sequences were aligned using ClustalW. The phylogenetic tree was constructed with MEGA 6 after initial analyses in Neighbour-Joining distance program.

Results

Supergene minerals present at the site of occurrence of microbial communities. The presence of picropharmacolite was confirmed by PXRD analysis. It is the most common secondary arsenic mineral in the Gertruda adit. It forms tiny spheroidal aggregates, up to 0.4 cm in diameter, comprising thin-acicular crystals up to 2 mm in length. Elongated crystals parallel to the *c* axis are the most common. This mineral is colourless or white, transparent to translucent with silky lustre. Aggregates of picropharmacolite growth can be seen separately on the surfaces of fissures inside of calc-silicate rocks, which contain weathered löllingite and arsenopyrite. Sometimes picropharmacolite forms white coatings up to several square centimetres. Qualitative EDS analysis corresponds to pure picropharmacolite without other elements (*e.g.* Co, Ni and Zn) which may substitute magnesium in the structure of this mineral. Hörnesite coexists with picropharmacolite. It forms white, ball-like aggregates composed of

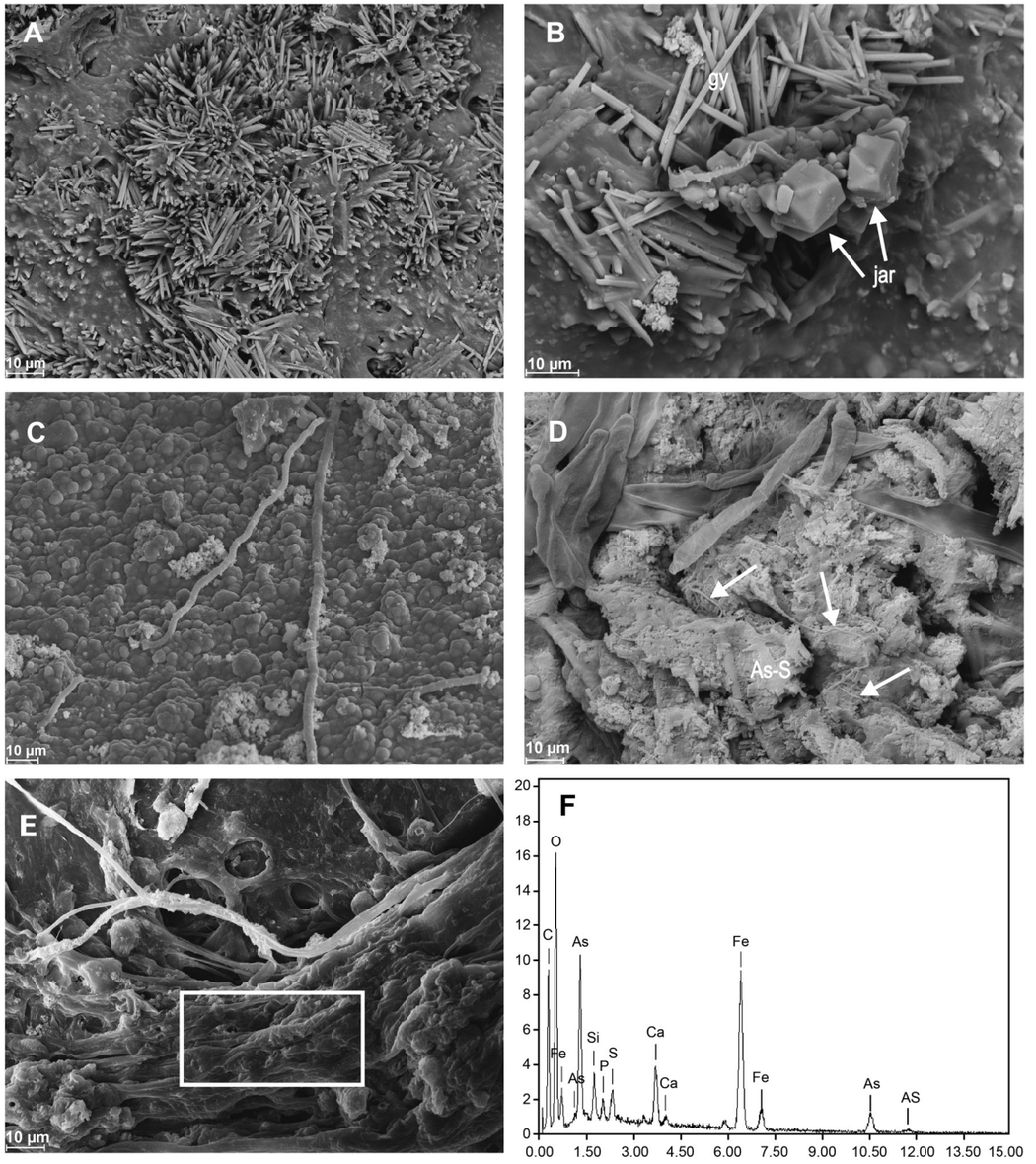

Fig. 2. A – Hörnesite crystals surrounded by a microbial mat; B – hydroniumjarosite (jar) and gypsum (gy) on the surface of a microbial mat; C – bacteria mineralised by pitticite, D – cryptocrystalline arsenic sulphide (As-S) on an old wooden mining construction; arrows indicate microbial colonies partially mineralised by As sulphide; E – a microbial mat mineralised by an unidentified Fe-Ca-As-S phase (the rectangle indicates the EDS analysed area); F – EDS spectra indicating the chemical composition of microbial structures visible in Fig. 2.

acicular crystals up to 0.2 cm long. Individual crystals are colourless or white and have perfect monoclinic morphology. EDS analysis confirmed high purity of the mineral. Accumulations of hörnesite are very often coated by microbial mats (Fig. 2A). Inside myllonites, which are connected with fault zones intersecting Złoty Stok, a hydroniumjarosite deposit is present. It forms yellow, dusty coatings on a mylonite surface. At high magnifications small pseudoregular crystals of this mineral are visible (Fig. 2B). EDS analysis shows only small amounts of potassium in cation positions. In the Gertruda adit scorodite formed two morphologically distinct types. The first type formed grey and green, dusty, cryptocrystalline masses. This scorodite replaced weathered löllingite-pyrite aggregates. The biggest accumulations of this type of scorodite are 10 cm in diameter. The other type is represented by spherical, green aggregates, which form botryoidal coatings. This type of scorodite always occurs in marginal parts of dusty scorodite accumulations. Both morphological types of scorodite were identified upon PXRD data. Kaňkite was identified in samples related with scorodite and pitticite.

This mineral forms light green botryoidal aggregates, with coatings of several square centimetres. Aggregates of kaňkite are composed of thin tabular crystals, which are visible at high magnifications. Pitticite is a product of primary arsenic ore weathering. This phase is amorphous hydrated Fe-arsenate-sulphate. In the Gertruda adit it forms compact masses with a botryoidal surface and resinous lustre. Pitticite accumulations are connected with microbial structures. We often observed mineralisation of microbial mats by this mineral phase (Fig. 2C). In the Gertuda adit ferrihydrite is the most common secondary iron mineral formed by weathering processes in primary ore minerals. It is usually present in sinters, less than 20 cm in length. Ferrihydrite is accompanied by goethite. The presence of both minerals was confirmed with the PXRD method. Peaks on X-ray powder diffraction patterns of ferrihydrite are often diffuse and of low intensity. It indicates low crystallinity of the mineral. Amorphous and cryptocrystalline arsenic sulphide precipitated from underground mine waters occupied by microbial colonies. This phase formed yellow dusty coatings on old wooden mining constructions. Coatings are composed of thin irregular crystals, which are visible at high magnifications (Fig. 2D). Microbial communities are very often mineralised by an unidentified Fe-Ca-As-S phase. It formed a thin film on the surface of colonies. The EDS analysis revealed the presence of iron, calcium, arsenic and sulphur as major elements (Fig. 2F). Small amounts of phosphorus were probably related to the bioactivity of microorganisms. Silicon was probably related to amorphous silica, which precipitated on the surface of the colony.

The analysis of 16S rDNA sequences. A total number of 170 cloned 16S rDNA sequences were analysed with the RFLP (restriction fragment length polymorphism) method. Eighteen clones revealing different from each other digestion patterns (Fig. 3) were reamplified and sequenced. Nucleotide sequences were deposited in GenBank. Seven sequences were closely related to the sequences of a division that belonged to *Flavobacteria*. Others were apparently related to seven other divisions: two clones to *Sphingobacteriia*, one clone to *Bacteroides*, three clones to γ-*Proteobacteria*, one to α-*Proteobacteria* and one to β-*Proteobacteria*. Another clone belonged to *Mollicutes* and two clones were classified as *Firmicutes*. The richer division fell into the *Flavobacteria*, which were closely related to *Flavobacterium* sp. (Table I). A phylogenetic tree was constructed using the neighbour-joining method with the sequences representing all known divisions (Fig. 4). The evolutionary history was inferred by means of the neighbour-joining method (Saitou and Nei, 1987). The optimal tree is shown, where the total branch length is 1.66576087. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches (Felsesnstein, 1985). The evolutionary distances were computed with the p-distance method (Nei and Kumar, 2000) and are expressed as units of the number of amino acid differences per site. The analysis involved 37 amino acid

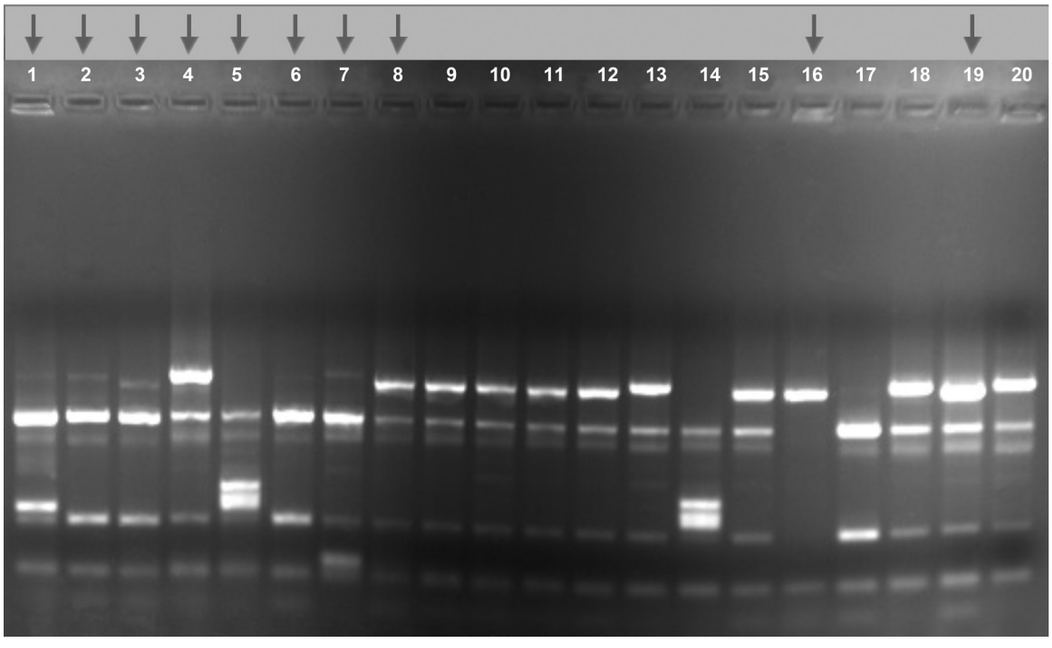

Fig. 3. Example of one gel sample with the restriction patterns of a 16S rDNA gene amplified by PCR and digested with endonuclease *Cfo*I. Each path refers to different microorganism clones that were analyzed. Clones with unique patterns (marked by arrows at the top) were collected and sequenced.

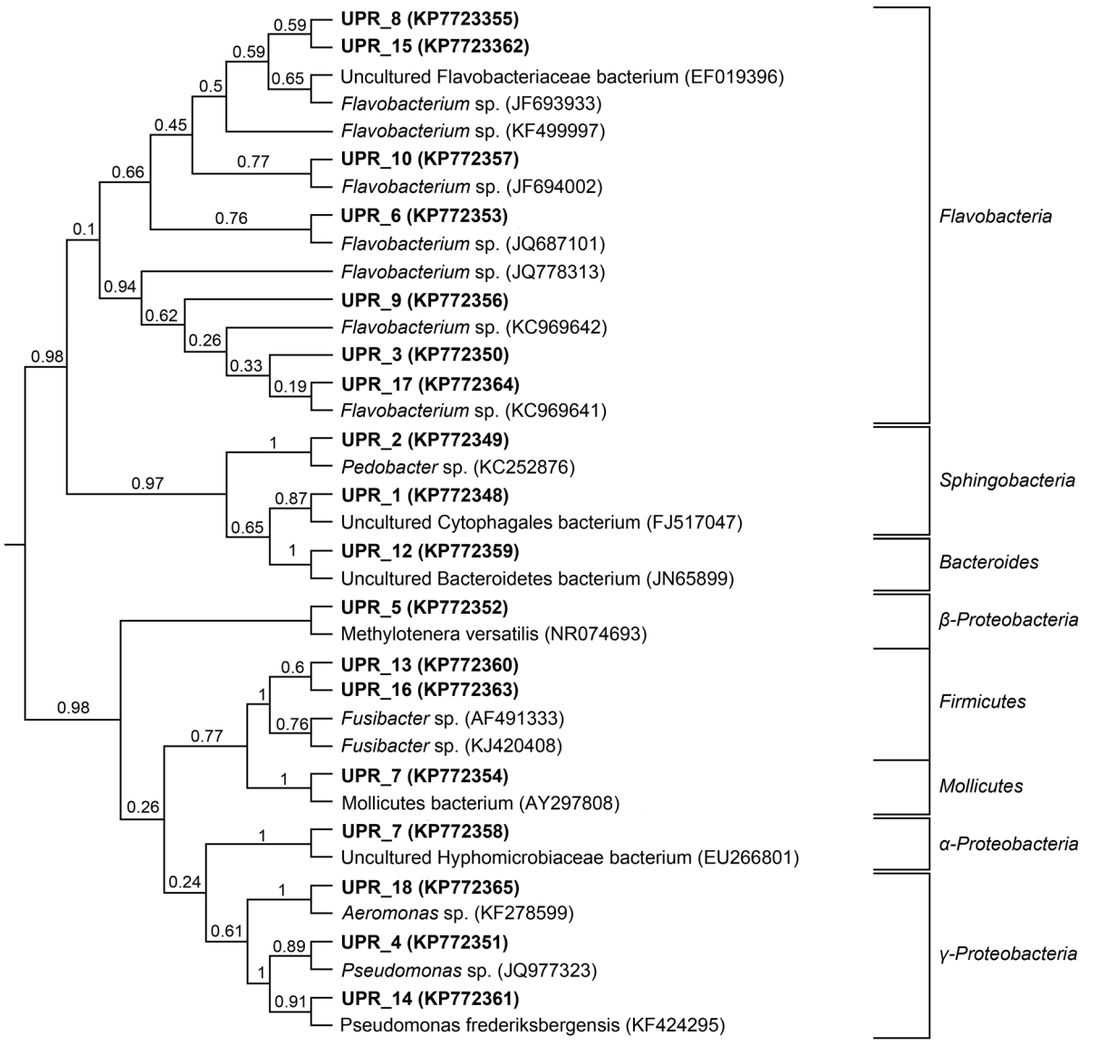

Fig. 4. A phylogenetic tree based on analyses of 16S rDNA sequence. The clone numbers of uncultured microorganisms are indicated in uppercase boldface type (UPR).

sequences. The coding data was translated assuming a standard genetic code table. All positions with gaps and missing data were eliminated. In total there were 92 positions in the final dataset. Evolutionary analyses were conducted with the MEGA6 (Tamura *et al*., 2013).

Discussion

An approach based on the cloning of 16S rDNA fragments obtained by the amplification of DNA isolated directly from environmental samples allows to determine the diversity of bacteria which can survive in the harsh environment and, perhaps, may influence its geochemistry. Our results are different from those presented by Drewniak *et al*. (2008; 2010; 2012) which were limited to cultured strains capable of utilizing inorganic arsenic species. The data obtained by the Authors clearly demonstrate the presence and cultivability of bacteria contributing to arsenic geochemistry. The sequence analysis revealed the presence of a wide range of bacterial phylogenetic groups, including *Flavobacteria*, *Sphingobacteriia*, *Bacteroides*, *Proteobacteria*, *Mollicutes* and *Firmicutes*. The presence of picropharmacolite was confirmed by PXRD analysis as the most common secondary arsenic mineral in the mine. The microbial colonies occupied amorphous and cryptocrystalline arsenic sulphide precipitated from underground mine waters. They were able to form a yellow dusty coating on old wooden mining construction. The presence of iron, calcium, arsenic and sulphur as major elements revealed by the EDS analyses. The activity of microorganisms is also related with the presence of a small amount of phosphorus in that site. To present

a broad picture of these processes and the microbial mat community larger areas of the mine should be analysed. Therefore, it is necessary to conduct further research on the isolation of these bacteria.

The results of sequencing of 16S rDNA fragments obtained from isolation of total genomic DNA from biofilm shows richer composition of bacterial community in the environmental sample than in an approach based on cultivation methods. A metagenomic approach clearly shows that some bacteria cannot be cultivated, even by using selective medium. Thus, analyses of environmental samples should be based on metagenomic and molecular biology techniques. Analyses of environmental samples, to see a 'bigger picture', of all processes that take place in the environment should not be limited only to one type of analysis. To understand how such different groups of microorganisms are able to live together, especially during extreme conditions, it is necessary to conduct complex analyses of the environment.

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