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THERMOACIDOPHILIC ARCHAEA, OXIDIZING FERROUS IRON, ELEMENTAL SULFUR AND REDUCED SULFUR COMPOUNDS ISOLATED FROM DIFFERENT SULFIDE DEPOSITS IN BULGARIA

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Abstract: Acidophilic chemolithotrophs oxidizing sulfide minerals, ferrous iron, elemental sulfur and reduced sulfur compounds are the typical microbiota of the sulfide ore deposits. At the same time, the thermoacidophilic archaea can also be found in such habitats because of their specific growth requirements. These archaea belong mainly to genera *Sulfolobus, Metallosphaera* and *Ferroplasma*. They are an excellent candidate for different industrial metal extraction applications, based on their oxidative potential and metabolic activity.

In this study the diversity of thermoacidophilic archaea from some Bulgarian sulfide ore deposits was investigated. Elective media and specific conditions of cultivation were used to obtain enriched and pure cultures and their identification by the methods of classical taxonomy. The results showed that archaea from genera S*ulfolobus, Metallosphaera* and *Ferroplasma* are present in some of the sulfide deposits investigated. The isolates are the most closed to *Sulfolobus metallicus, Metallosphaera cuprina* and *Ferroplasma acidiphilum*. The isolates oxidizing ferrous iron, elemental sulfur and metal sulfides are suitable for industrial extraction of metals.

INTRODUCTION

Sulfide minerals, in terms of their physical, chemical and structural characteristics, represent a highly diverse and industrially significant group of minerals, with a tremendous industrial significance in modern industry (Hochella et al., 1990). The most prominent and effective for practical applications are the processes of oxidation of sulfide minerals and reduced sulfur compounds, related to acidophilic chemolithotrophic bacteria and archaea (Muravyov et al., 2015). The metabolic activity of the autochthonous microflora, inhabiting sulphide deposits is rapidly developing contemporary trend in industrial extraction of metals (Ilyas S. et al., 2007; Zhuang et al., 2015; Barmettler et al., 2016). Some of the most intensively studied representatives of this group are *Acidithiobacillus ferrooxidans* and related bacterial species from genus *Acidithiobacillus* as well as genus *Leptospirillum* (Christel et al, 2018) due to their widespread prevalence, a dominant role in the microbial communities, naturally inhabiting the sulfide deposits and in particular for their pronounced oxidative capacity, which is extensively used in various biological leaching applications (Groudev et al., 1982; Rohwerder et al., 2003).

However, thermoacidophilic archaea are also one of the components of the indigenous biota of the sulfide ore deposits, depending on the specific oxidizing activates. They are also an excellent candidate for different industrial metal extraction applications. The investigations concerning the taxonomy and abundance of thermoacidophilic archaea are extremely important for understanding the nature of their complex relationships with the other members of the microbial communities from sulfide ore deposits (syntropy between archaea and eubacteria, mutualism between iron-oxidizing acidophiles using Fe^{2+} as an electron donor and iron-reducing heterotrophs using Fe^{3+} as an electron acceptor, mutualism between obligate lithotrophs and mixotrophs and others (Rawlings et al, 2007, Yan Jia et al, 2019).

Up to now, the information about taxonomical profiles and the share of archaea as a part of natural biota in sulfide deposits is scarce and insufficient. Thermophilic sulfur/iron oxidizers, which thrive above 60ºC, usually belong to the Archaea domain, genera *Sulfolobus, Acidianus, Metallosphaera* and *Ferroplasma*. Genus Sulfolobus (Brock et al, 1972) includes thermoacidophilic archaea characterized with obligate or facultative autotrophic growth on sulfur or on a variety of simple organic compounds, typical acidophiles (pH optimum of 2–3 and range from 0.9–5.8) and temperature optimum of 70–75°C and range from 55–90°C. Few species are described up to now. S*ulfolobus islandicus, S. solfataricus,* and *S. acidocaldarius* are the best described members of the genus because of significant difference in their metabolism (Liu et al, 2011).

Archaea of the genus *Ferroplasma* are typically acidophillic, pleomorphic, irregularly shaped cocci. Up to day very few species of *Ferroplasma* have been isolated and characterized: *Ferroplasma acidiphilum* (Golyshina et al, 2000). *Ferroplasma acidiphilum* obtains energy for life by oxidation of ferrous iron available as pyrite (FeS2) or ferrous sulfate ($FeSO_4$). Thus, it could be appropriately termed as a chemolithoautotroph but with mixotrophic abilities (growth in yeast extract). It grows at temperature range of 15-45˚C (optimum between 35-36˚C) and pH range of 1.3 to 2.2.(optimum 1.7). This archaeon is generally found in acidic mine tailings, primarily those containing pyrite (FeS_2). It is especially abundant in cases of severe acid mine drainage, where chemolithotrophic bacteria as *Acidithiobacillus caldus* and *Leptospirillum ferriphilum* are presented (Golyshina et al, 2017).

The species of genus *Metallosphaera* (Huber et al, 1989) are highly thermoacidophilic archaea, oxidizing pyrite and chalcopyrite. *Metallosphaera sedula*

(Huber et al, 1989) is a coccus, obligate aerobe growing best at 75°C and pH 2.0. It is capable of heterotrophic, mixotrophic and autotrophic growth, oxidation of iron, sulfur and chalcopyrite. In acid mine drainage (AMD) communities it is usually in symbiosis with F*erroplasma* spp. and *Leptospirillum* spp.

Based on their physiological and biochemical features, it could be assumed that the members of the above-mentioned genera must be the dominant archaea taxons in the habitats under consideration. One of the main difficulties for obtaining enriched and pure cultures and their identification is connected to similarity between their physiological and biochemical characteristics and dependence of ecological factors of each habitat (Groudev et al., 1982). For this reason evaluation of the diversity of microbiota and archaea of the sulfide deposits is achieved mainly by molecular methods (Selenska-Pobell et al., 2002, Demergasso et al., 2005, Baker et al., 2003; Bergamo et al., 2004; Akbar et al., 2005, Johnson et al., 2007, Williams et al., 2010).

This study focused on enumeration, isolation and identification of acidophilic archaea, oxidizing iron and/or reduced sulfur compounds based on constructed selective cultivation scheme and classical taxonomy and evaluation of their oxidizing potential for practical application.

MATERIALS AND METHODS

Four sulfide ore deposits in Bulgaria have been investigated. Different types of sulfide deposits were selected as follow: copper sulfide deposits Elshitsa (samples E-1 and E-2), Tsar Asen (sample T-1) Vlaikov vrah (sample V-1 and V-2); uranium sulfide deposit Kurilo (samples C-1 and C-2) and gold-containing sulfide deposit Chelopech (sample Ch-1). The samples were taken from ore mass (samples E-1, V-1, C-1, Ch-1) and mine drainage (samples E-2, T-1, V-2 and C-2). The most probable number method, modification of Brown (Brown & Braddock, 1990) and 9K medium (Silverman and Lundgren 1959) with some modifications were used for investigation of microbial and archaeal growth.

Enriched cultures and isolates of pure cultures were obtained by using elective media (Golyshina et al, 2000, Zhou H et al, 2008) with trace elements (Dopson et al, 1999).

The elective cultivation scheme was constructed, as follow: a) inoculation of samples in 9K medium + Fe^{2+} (10 mM ferrous iron) and cultivation at temperature of 37 °C, 55 °C, and 70 °C; b) inoculation of samples in 9K medium + Fe^{2+}+ yeast extract (0.02% (w/v) and cultivation at temperature of 37 °C, 55 °C, and 70 °C; c) inoculation of samples in 9K medium + S° (0.5% (w/v) of finely ground elemental sulfur) and cultivation at temperature of 37 °C, 55 °C, and 70 °C; d) inoculation of samples in 9K medium + S° + yeast extract and cultivation at temperature 30 °C, 55 °C, and 70 °C . The initial pH of each variant of the media was between 2.5 - 3.0. The cultivation was achieved in 250 ml flasks with 100 ml medium, inoculated with 10% (w/v or v/v) of each sample on a rotary shaker for 7-10 days. Sulfur and ferrous oxidation were monitored by measuring changes in culture pH (oxidation of sulfur to sulfate is an acid-generating reaction), and changes in concentrations of total soluble iron.

The isolation of pure cultures was obtained by serial dilutions and cultivation for 7-10 days in liquid media as well as on solid media (21 days cultivation) (Golyshina et al, 2000). The purity of the cultures was estimated by phase-contrast microscopy.

The morphology of the cells was observed by light microscopy and scanning electron microscope (SEM).

Identification of the isolates was done according to Bergey's Manual of Systematics of Archaea and Bacteria (BMSAB), 2015. Morphological characteristics (shape, size of the cells, motility, spores, Gram status, and capsule formation), cultural (aerobic and anaerobic growth, autotrophic and mixotrophic growth), physiological (optimal temperature for growth, optimal pH, tolerance to heavy metals) and biochemical (utilization of different energy substrates) parameters have been investigated.

RESULTS AND DISCUSSION

Microscopic observations were carried out before selective cultivation of the samples. Cells of different shape were observed including long filamentous structures (Fig. 1). However, after the cultivation on elective media and conditions, these filamentous structures were not detected.

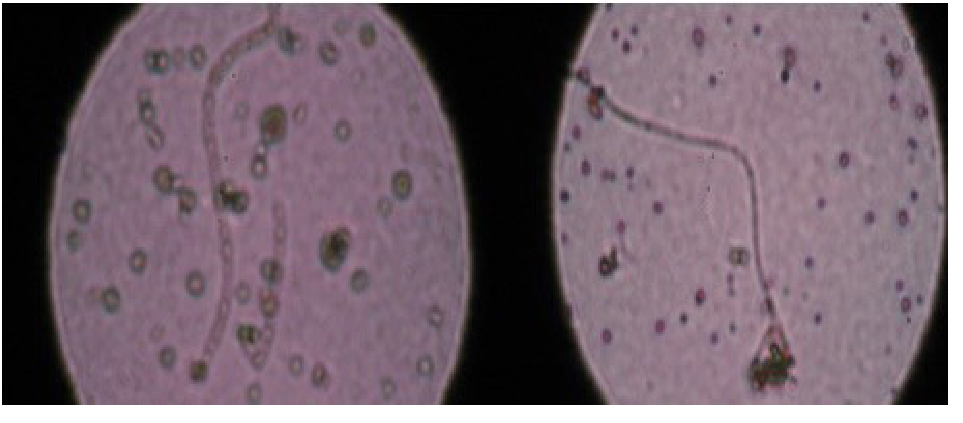

Fig. 1 Light microscopy of acid drainage samples before starting the selective procedure.

During the cultivation of the samples on elective media and the corresponding temperature, the presence of bacterial growth was monitored. Initially, after inoculation of the flasks containing 9K medium with ferrous sulfate added, the color was not significantly affected by the inoculum and remained greenish-yellow. Samples were cultured for 5-7 days. In the presence of bacteria, oxidizing iron, clear changes in the color of the medium was observed, formed with sludge from jarosite (Fig. 2) as well as changing of the pH. During the cultivation on sulfur-containing media, a slight opalescence of the medium was observed as well as increasing of the concentration of the SO_4^{2-} and change of pH.

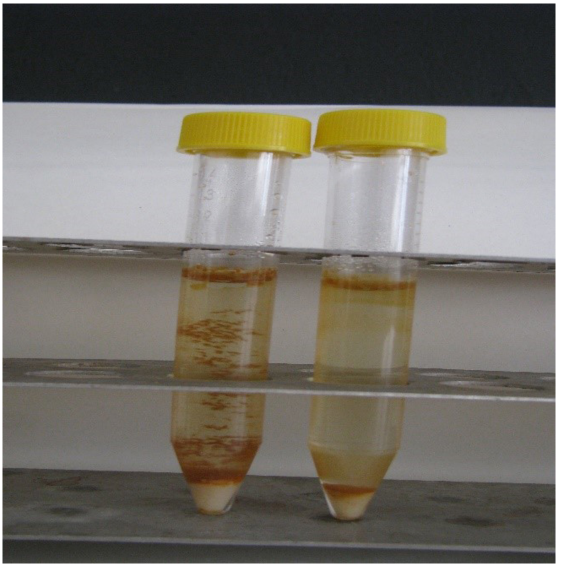

Fig. 2 Typical view of samples during the cultivation on iron-containing media

The results of the cultivation of the samples according the selective cultivation scheme constructed are presented in Table. 1.

Table 1: Selective cultivation of the tested samples

Medium	Sample №							
	R-1	R-2	R-3	L-1	L-2	L-3	M-1	M-2
9K + Fe^{2+}, , 37° C	-	-		-	-	-	-	-
9K + Fe^{2+}, 55° C	+	-	+	-	-	+	-	-
9K + Fe^{2+}, 70° C	+	+	-	+	+	-	+	-
9K + Fe^{2+}, yeast extract ,37°C	-	-	-	-	-	-	-	-
9K + Fe^{2+}, yeast extract ,55° C	+	-	+	-	+	-	-	+
9K + Fe^{2+} , yeast extract ,70° C	+	+	+	+	+	+	+	+
9K + S^0, 37° C	-	-	-	-	-	-	-	-
9K + S^0,, 55° C	+	-	+	-	+	-	+	-
9K + S^0, 70° C	+	+	+	+	+	+	+	+
9K + S^0, yeast extract 37° C	-	-	-	-	-	-	-	-
9K + S^0, yeast extract 55° C	+	-	-	+	-	-	-	+
9K + S^0, yeast extract 70° C	+	+	+	+	+	+	+	+

It is noteworthy that in all samples grown at 37°C (ferrous iron and sulfur as energy substrates) there is intensive growth connected to mesophilic chemolithotrophic eubacteria (mainly genera *Acidithiobacillus* and *Leptospirillum*). The genera mentioned are usually dominant in microbial communities in sulfide ores and acid mine drainage formed. Only one species of the archaeal genus *Ferroplasma* is possible to grow in medium 9K + Fe^2 under these conditions. Growth in the same media, but at 55°C was similar with few exceptions (no growth in 3 of the samples tested). The temperature 55°C is suitable for growth of few archaea and moderate eubacteria like *Acidithiobacillus caldus, Leptospirillm ferrifulum* as well as genera *Acidimicrobium, Ferromicrobium, Sulphobacillus, Ferrithrix, Alicyclobacillus*. Most of the bacteria from above mentioned genera are mixotrophs so they can grow also in the media with yeast extract at the same temperature. All of them differ from moderate archaea by other characteristics.

In all samples grown at 70° C there is intensive growth. Based on energy and physiological requirements, it could be assumed that in samples (70° C, with and without yeast extract) archaea from genera *Sulfolobus* and *Metallosphaera,* but not *Ferroplasma* (temperature range 22° C to 63° C) are presented.

Eleven pure cultures were obtained after dilutions method and cultivation on solid media. After checking of the purity of the cultures few of them were mixed ones and were included. Eigth isolates were subjected to identification as follow : isolates R-3, L-2 and M-2 (9K + Fe^{2+}, 70 °C) ; isolates R-1(9K + Fe^{2+}, yeast extract, 70 °C) ; isolates R-2 , L-3-(9K + S°, 55 °C) ; isolates L-1, M-1 (9K + Fe2+, 37 °C). The characteristics of the morphological (shape, size, motility, spores, Gram status, capsule formation) and cultural (pH optimum, temperature optimum, autotrophic or mixotrophic growth, obligate or facultative aerobic growth) parameters are given in the Table 2.

Table 2: Morphological, cultural and physiological characteristics of the isolates

Parameter	Isolate №							
	R-1	R-2	R-3	L-1	L-2	L-3	M-1	M-2
Cell shape	cocci	cocci	cocci	cocci	cocci	cocci	cocci	cocci
Length (μm)	< 2	< 2	~ 3	< 2	~ 3	~ 2	< 2	~ 3
Gram status	+?	+?	-	+?	+?	-	+?	-
Spores	-	-	-	-	-	-	-	-
Capsule	+	+	+	-	+	-+	-	+-
Motility	+	+	+	-	-	+	-	-

The most commonly observed microscopic picture is coccoid shape, typical cocci, irregular cocci, arranged individually or in short chains (Fig. 3).

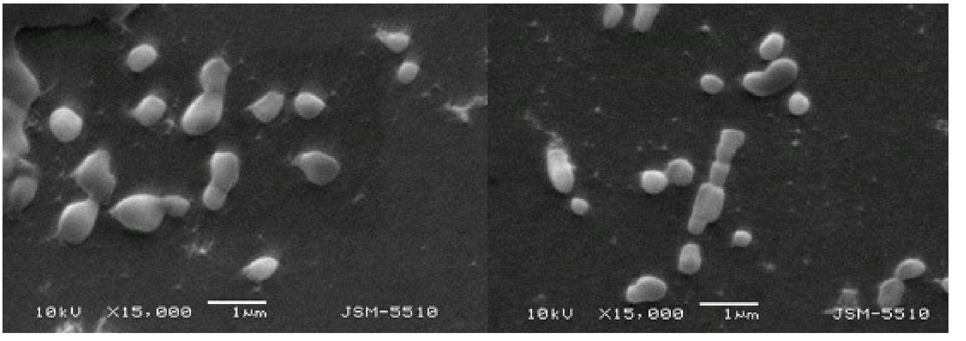

Fig. 3 SEM of typical cell shape of some of the isolates

All isolates are with size less or up to 2 μm), not spore forming, some are motile (long flagellum usually). All of them are obligate aerophiles, acidophiles, differ from each other's by temperature range and optimum as well as pH. Most of the isolates are able to grow mixotrophically.

For identification of the isolates it is important to have information about their ability to oxidize different substrates as a source of energy. Ferrous iron (10 mM), elemental sulfur (0.5%, wt/v), pyrite concentrate (1%, wt/v) and chalcopyrite concentrate (1%, wt/v) were tested. The oxidation was monitored with or without supplementation of yeast extract (0.02% (w/v) at optimal temperature for each isolate.

Table 2: Morphological, cultural and physiological characteristics of the isolates

Medium	Sample №							
	R-1	R-2	R-3	L-1	L-2	L-3	M-1	M-2
9K + Fe^{2+}	+	+	+	+	+	+	+	+
9K + Fe^{2+}, yeast extract	-	-	+	+	-	+	-	-
9K + S^0,	+	+	+	+	-	+	-	-
9K + S^0, yeast extract	+	+	-	+	-	-	-	+
9K + chalcopyrite concentrate	+	+	+	+	+	+	+	+
9K + pyrite concentrate	+	+	+	+	+	+	+	+

On the basis of the results obtained it can be concluded that the isolates L-1 and M-1 are close to *Ferroplasma acidophilum*. Application of culture-dependent and especially culture- independent approaches led to recognition of few members of genus *Ferroplasma* from a bioleaching plants, mines and acid drainage. They differ from each other mainly by temperature and pH requirements. *Ferroplasma acidarmanus* (Dopson et al, 2004), *Ferroplasma thermophilum* (Zhou et al, 2008). *Ferroplasma cupricumulans* (Hawkes et al, 2006). F*erroplasma cupricumulans* is non-motile pleomorphic cocci, capable of chemomixotrophic growth with ferrous sulphate, pyrite and yeast extract. Growth was achieved between 22 °C and 63 °C (optimum 53.6 °C). The optimal pH for growth was 1.0–1.2. *Ferroplasma thermophilum* is non motile coccus with temperature optimum of 45°C and the pH optimum pH 1, capable of chemomixotrophic growth on ferrous iron, pyrite and chalcopyrite and yeast extract. *Ferroplasma acidophilum* is pleomorphic (more often irregular coccus), with temperature range between 15-45 °C and optimum 37°C and pH optimum 1.7 (Golyshina et al, 2000).

Isolates R-3, M-2 and R-1 are close to *Metallosphaera cuprina*. This archaeon was originally isolated from ores near hot springs (Liu et al, 2011) and differs from other species because it can grow in ranges of 55-75° C (optimum at 65°C) and pH between 2.5-5.5 (optimum 3.5). Like *M. prunae* (Fuchs et al, 1995), but not *M. hakonensis* or *M. sedula* (Kurosawa, et al, 2003), *M. cuprina* has long and curved flagella. *Metallosphaera cuprina* is an aerobic and facultative chemolithoautotrophic archaeon, oxidizing metal sulphides (mainly pyrite and chalcopyrite), elemental sulphur, tetrathionate and FeSO4.

Isolates R-2, L-2 and L-3 were referred to *Sulfolobus metallicus*. Few species are described up to now belonging to genus *Sulfolobus*. *Sulfolobus islandicus, S. solfataricus,* and *S. acidocaldarius* are the best described members of the genus. *Sulfolobus metallicus* (Huber et al, 1991) is a strict chemolithoautotroph gaining energy by oxidation of sulfur and sulfidic ores (pyrite, chalcopyrite, sphalerite), or ferrous iron (Morales et al, 2011). It can grow between 50°C and 75°C in acidic environments between pH 1.0 and 4.5. This archaeon is very often found in sulfide ore deposits and bioleaching reactors (Bode et al, 2008, Astudillo et al, 2008).

The isolates investigated are of different origin. Some of them are isolated from ores of the sulfide deposits (R-3, M-1, R-1, R-2) others from acid mine waters (L-1, M-1).

The analysis of the results confirmed the difficulty in the isolation and identification of these archaea by the methods of classical taxonomy. This is connected with variability of the archaeal characteristics used because physiological adaptation to the specific environmental conditions. Due to that molecular taxonomy must be used obligatory for confirmation of the taxonomic status of the isolates. They possess oxidizing potential for application in different bioleaching operations as single or mixed cultures because the adaptation to specific conditions of the deposits.

CONCLUSION

In all analyzed samples, the presence of iron, sulfur and sulfides-oxidizing acidophilic archaea were observed. The presence of these archaea depends on the type of the deposits but in any case, they are permanent component of acidophilic communities formed. The elective cultivation scheme constructed allows to obtain enriched cultures and isolation of pure ones. The characteristics (morphological, cultural and physiological) according to classical taxonomy allowed to refer the isolates to known species of the archaeal genera, but the confirmation of this must be achieved by PCR methodology and sequencing. After that the isolates can be used for practical application.

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Declaration of interest statement: All authors declare no conflict of interest.

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