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MICROBIAL DIVERSITY AND ISOLATION OF MULTIPLE METAL-TOLERANT BACTERIA FROM SURFACE AND UNDERGROUND PITS WITHIN THE COPPER MINING AND SMELTING COMPLEX BOR

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Abstract - The bacterial diversity of the surface and deep sediment of the Copper Mining and Smelting Complex Bor, Serbia, was investigated using culture-dependent and culture-independent approaches. Sequencing analysis of 16S rDNA libraries revealed greater bacterial diversity in the surface sediment of the mining complex (MS) in comparison to deeper mine sediment (MU). While in the MS sample members of seven different phylogenetic groups were detected, in the MU sample library representatives of only three different groups were detected. The use of a culture-dependent approach revealed the presence of only three bacterial groups in both samples: *Actinobacteria*, *Firmicutes* and *Proteobacteria*, while six isolates exhibiting the highest metal tolerance were members of *Arthrobacter* and *Staphylococcus* genera. The most promising isolate, MSI08, was able to grow in the presence of high concentrations of Cd^{2+} (535 μM), Ni^{2+}(17 mM) and Cr^{6+} (38.5 mM) and as such this indigenous strain has potential in the bioremediation of the contaminated surrounds of the city of Bor.

Key words: Copper mine, bacterial diversity, heavy metal tolerance, *Staphylococcus*

INTRODUCTION

Hazardous heavy metal pollution, originating from both natural and anthropogenic activities, is one of the most important environmental problems (Guo et al., 2009). Mining presents one of the major anthropogenic activities resulting in the release of heavy metals into the environment (Passariello et al., 2002). Heavy metals, such as Cd, Hg, Cr, Ni, Zn and Cu, often pose a significant threat to the environment and public health due to their toxicity, accumulation in the food chain, and persistence in nature (Evanko and Dzombak, 1997).

When present in excessive concentrations, heavy metals are highly toxic to microorganisms and affect their community structure by reducing their number, diversity, and biochemical activity. At the same time, metal exposure results in the establishment of metal-tolerant/resistant microbial populations (Piotrowska-Seget et al., 2005). Due to selective pressure from the metals in the growth environment, indigenous microorganisms have evolved various mechanisms to resist heavy metal stress and may play a significant role in the restoration of contaminated environments (Rathnayake et al., 2009). Since it is not possible to degrade heavy metals by any means, the only way to remove them from the environment is to use a bioremediation strategy, which is the most efficient and least costly method for treating metal-contaminated environments (Wei et al., 2009). Due to the heterogeneity of soil, the presence of different contaminant

mixtures, variability in pH, and oxidation-reduction potential of the contaminated site, already adapted indigenous microorganisms are the bioremediators of choice (Colin et al., 2012; Halter et al., 2011; Tabak et al., 2005). Therefore, it is of great importance to isolate novel microorganisms capable of tolerating high metal concentrations or dealing with the presence of multiple metals simultaneously.

The Bor metallogenic basin represents a type of deep surface mining and it is one of the largest copper mining and smelting complexes in Europe (about 80 km long and 20 km wide). On the other hand, Bor also represents a potential regional risk spot, since the failure of its tailing dams could release high amounts of toxic materials that would reach the Danube River through the Borska River and other effluents. In this study, we were set to examine the microbial diversity of two soil samples exposed to copper and other heavy metals collected from Copper Mining and Smelting Complex Bor. Collected samples were from the surface and deep sediment that contain different metal concentrations. We used culture-independent (metagenomic) and culture-dependent approaches to enable the isolation of microbial strains able to tolerate elevated concentrations of heavy metals, which could potentially be applied in the bioremediation efforts at the mining site and wider.

MATERIALS AND METHODS

Sample collection and chemical analysis

Two samples were collected from two different sites at the copper mine Bor, Serbia, on June 2010 (44°04'53.09``N, 22°05`54.90``E). One sample was taken from the surface within the Copper Mining and Smelting Complex (MS) while the other was taken from the underground mine pit (MU). The aseptically collected samples were brought to the laboratory and stored at 4°C prior to analysis. Metal contents were determined using an inductively coupled plasma/optical emission spectrometer (ICP/OES) as described by Relic et al. (Relic et al., 2011). Total organic nitrogen (TN), carbon (TC), hydrogen (TH) and sulfur (TS) were assessed using a Vario EL III elemental analyzer (Elementar Analysensysteme GmbH, Hanau-Germany).

Metagenomic DNA extraction and 16S rDNA PCR amplification

The total metagenome was extracted from the sediment samples (0.25 g) using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, California, USA), carrying out sodium pyrophosphate pretreatments described by Rösch et al. (Rösch et al., 2002). The quality and concentration of the extracted DNA were analysed by NanoVue Plus (GE Healthcare, Waukesha, Wisconsin, USA) and visualized using agarose (0.6%, w/v) gel electrophoresis. Amplification of 16S rDNA was performed using 30 ng of template DNA per reaction, bacteria-specific primers: 27f and 1492r (Lane, 1991) and KAPA Hifi PCR Kit (Kapa Biosystems, Inc., Woburn, Massachusetts, USA) according to the manufacturer's manual, with the following cycling conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of 98°C for 20 s, 60°C for 15 s, and 72°C for 1 min, and completed with extension period of 5 min at 72°C.

Clone library construction and sequencing

To obtain two clone libraries (MS and MU), amplified PCR products (~1500 bp) were purified with a QIAquick PCR Purification Kit (QIAGEN GmbH, Hilden, Germany), cloned into a pCR®-Blunt vector (Invitrogen GmbH, Darmstadt, Germany) and electroporated into *E. coli* DH5α cells. The libraries were screened by PCR using M13 universal sequencing forward and reverse primers (Messing, 1983) and selected clones containing correct size inserts were sequenced using the Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, Foster City, USA). Sequences were analyzed and assembled in DNA Star Homologs (DNASTAR, Inc., Madison, Wisconsin, USA) and identified by the BLASTN algorithm (Altschul et al., 1997). The BLASTN program was used to search for similarity in the GenBank database services provided by the NCBI, and the Seqmatch tool was used to search for similar sequences compiled by

the Ribosomal Database Project-II Release 9.4 (RDP; http://rdp.cme.msu.edu; (Cole et al., 2009)).

Isolation of bacteria from soil

The soil samples (1 g) were resuspended in sterile potassium phosphate buffer (10 ml; 50 mM, pH 7.4). The serial dilutions of suspension were spread plated (300 µl) on minimal TG agar medium containing soil extract and glucose as carbon source (Sørheim et al., 1989) and supplemented with antifungal cycloheximide (75 mg l^{-1}). Plates were incubated at 30°C for 5 days. Single colonies were transferred to fresh TG broth and glycerol stocks (20%, v/v in TG medium) were made for the maintenance.

Identification of bacterial isolates

Genomic DNA (gDNA) from the bacterial isolates was extracted using the protocol described by Sambrook et al. (1989). Identification of bacterial isolates was performed by 16S rDNA amplification followed by fragment sequencing and sequence analysis (described above). In this case, 50 ng of gDNA was used as a template in PCR reactions.

The nucleotide sequences of metal-tolerant bacterial isolates determined in this study have been deposited in the GenBank data library under the accession numbers JX036476-JX036481. A phylogenetic tree was constructed by the neighbor-joining (NJ) algorithm contained in the PHYLIP program package (http://bioweb2.pasteur.fr/ (Felsenstein, 1989)).

Biochemical characterization of the isolate MSI08 was performed using a BBL Crystal GP ID Kit (Becton Dickinson, Sparks, MD, USA). This isolate was deposited at the Institute of Soil Science, Belgrade, Serbia, under ISS 616 (Culture collection ISS WDCM 375).

Metal and halogen tolerance of bacterial isolates

To test the ability of the isolates to grow in the presence of higher concentrations of heavy metals, strains were transferred onto metal toxicity (MT) medium plates (Sani et al., 2001) supplemented with metal ions in the form of the following salts: Cu^{2+} ($CuSO_4 \cdot 6H_2O$), Cd^{2+} ($3CdSO_4 \cdot 8H_2O$), Cr^{6+} ($K_2Cr_2O_7$), Ni^{2+} ($NiCl_2 \cdot 6H2O$), Hg^{2+} ($HgCl_2$), Fe^{3+} ($FeCl_3 \cdot 6H_2O$) and Zn^{2+} ($ZnCl_2$). Metal concentrations were 20 times higher than allowed by the regulation of Serbian Ministry of Environment, Mining and Spatial Planning (EPA/Serbia, 1994), which corresponds to the following concentrations of salts: $CuSO_4$, 5 g l^{-1}; $CdSO_4$, 111.3 mg l^{-1}; $K_2Cr_2O_7$ 11 g l^{-1}; $NiCl_2$, 2.2 g l^{-1}; $HgCl_2$, 73 mg l^{-1}; $FeCl_3$, 114 mg l^{-1} and $ZnCl_2$, 12.6 g l^{-1}. Growth of isolates was also tested in the presence of higher amounts of salts ($CuSO_4$, 15.9 g l^{-1}; $CdSO_4$, 20.9 g l^{-1}; $K_2Cr_2O_7$, 28.5 g l^{-1}; $NiCl_2$, 12.9 g l^{-1}; $HgCl_2$, 27 g l^{-1}; $FeCl_3$, 16.2 g l^{-1}; $ZnCl_2$, 13.6 g l^{-1}), which constitute 100 mM concentrations of the corresponding metal ions. All plates were incubated at 30°C for 5 days and checked every 24 h for growth.

As the importance of microbial halotolerance in bioremediation has been reported previously (Margesin and Schinner, 2001), the ability of isolates to grow in the presence of halogen ions (Cl^- and F^-) was assessed using liquid MT medium supplemented with NaCl (10%, w/v corresponding to 1.7 M) and NaF (1%, w/v corresponding to 0.2 M).

To study the effects of heavy metals on the growth of bacterial strains in liquid culture, strains were grown in MT broth supplemented with the above metals. After 48 h incubation (30°C, 180 rpm), serial dilutions of the cultures were made and spread plated on MT agar containing no metal ions (100 µl per each plate) and colonies were enumerated after 48 h growth on 30°C. Appropriate controls of cultures grown in MT without the addition of metal ions were also included.

Electron microscopy of Staphylococcus sp. MSI08

The isolate identified as *Staphylococcus* sp. MSI08 by 16S rDNA sequencing was further characterized morphologically and biochemically. Samples of bacterial cells for transmission electron microscopy (TEM) were taken after 48 h incubation (30°C, 180 rpm) in liquid MT medium – control sample, and in

MT medium supplemented with 3 mM Cr6+. TEM (model CM12; Philips) was performed following the procedure of O'Donnell et al. (O'Donnell et al., 1993).

RESULTS AND DISCUSSION

The Copper Mining and Smelting Complex Bor is of great industrial importance to the region; however, environmental concerns in respect to heavy metal pollution have been raised more recently (Dimitrijevic et al., 2009; Milijasevic et al., 2011). The aim of this study was to investigate microbial diversity at two sites exposed to heavy metal presence (one surface and one underground) and to isolate indigenous metal-adapted microorganisms that could potentially be applied in bioremediation efforts.

Physicochemical properties of the soil samples

Both soil samples collected from the mining site Bor were acidic (Table 1). The sample taken from the surface ground of the mining area (MS) was pH 5.2 and contained 40% (w/w) H_2O, while the sample collected inside the mine (MU) was pH 3.9 and contained 76% (w/w) H_2O.

Table 1. Metal contents[a] (mg kg^{-1}) and elemental composition[b] (g kg^{-1}) of mine soil samples

Characteristics	Sample	
	MS	MU
pH	5.2	3.9
Al[a]	4661	510
Cd	$75x10^{-6}$	$140x10^{-6}$
Co	3	1
Cr	3	0.035
Cu	23	222
Fe	3650	1336
Mn	140	49
Ni	$1643x10^{-6}$	$722x10^{-6}$
Zn	11	19
As	24	0.009
TN[b,c]	2.3	1
TC	25.6	7.7
TH	11.5	15.2
TS	2.2	100.5

[c] TN, total nitrogen; TC, total carbon; TH, total hydrogen; TS, total sulfur

Both samples had similar levels of Co and Zn, while the amounts of other metals varied between these two samples. In the MS sample there were 2000 and 85 times more As and Cr, respectively, in comparison to the MU sample. While total carbon, total nitrogen and total hydrogen levels were 3.3, 2.3 and 1.3 times higher in the MS sample in comparison to MU sample, there was 45 times more sulfur in the MU sample, indicating its inorganic origin (Table 1). The ratio of total nitrogen to carbon was 11.1 and 7.7 in the MS and MU samples, respectively (Table 1).

Although originating from the copper mining site, concentrations of the metals detected in the MS and MU samples were within the values allowed by the regulation of Serbian Ministry of Environment, Mining and Spatial Planning, except for copper, which was 2.5 times higher in comparison to the regulation (EPA/Serbia, 1994). Expectedly, the copper concentration was two-fold higher in the MU sample in respect to the MS sample (Table 1). While the concentration of Cu reported by He et al. in the Zhong Tiaoshan copper mine in China was similar to the concentration of Cu in the MU sample, the amounts of Co, As and Ni were 16, 43 and 20000 times higher than those detected in the MU sample (He et al., 2007). On the other hand, in the copper mine sample from Shen-bu copper mine in China, Cu concentration was found to be 19 times higher, alongside Co, Zn, Cd and Cr concentrations that were 10, 91, 38500 and 46 times higher than those detected in the MU sample (Yang et al., 2008). Previously reported metal concentrations (Co, Cd, Zn, Ni, As) found in polluted mine sites and surrounding environments were generally higher in comparison to the surface sample of copper mine Bor (Mendez et al., 2008; Passariello et al., 2002; Rodríguez et al.,

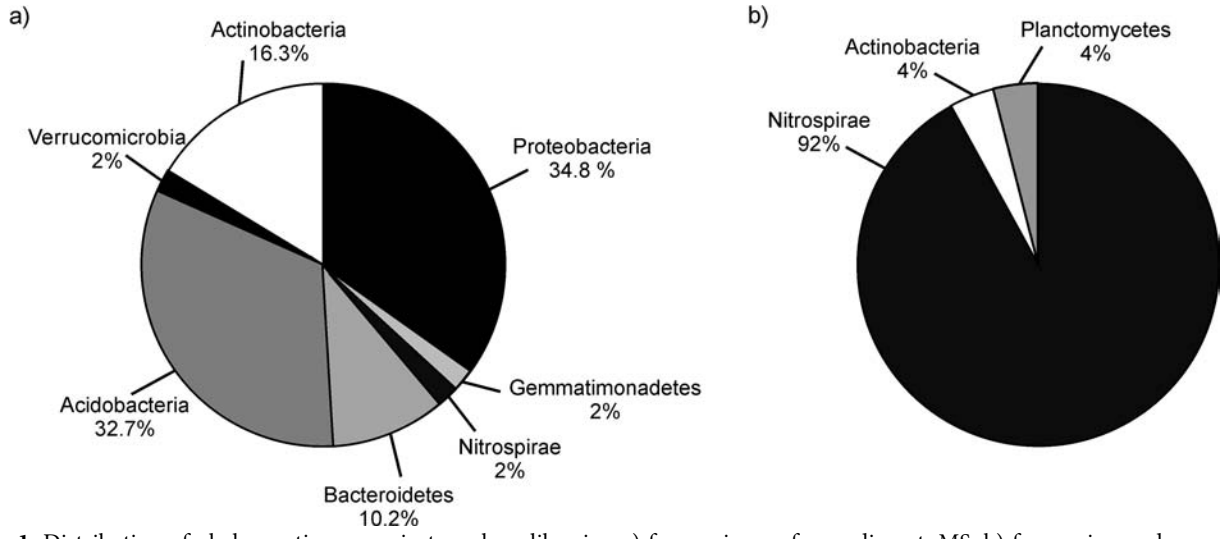

Fig. 1. Distribution of phylogenetic groups in two clone libraries: a) from mine surface sediment, MS; b) from mine underground, MU.

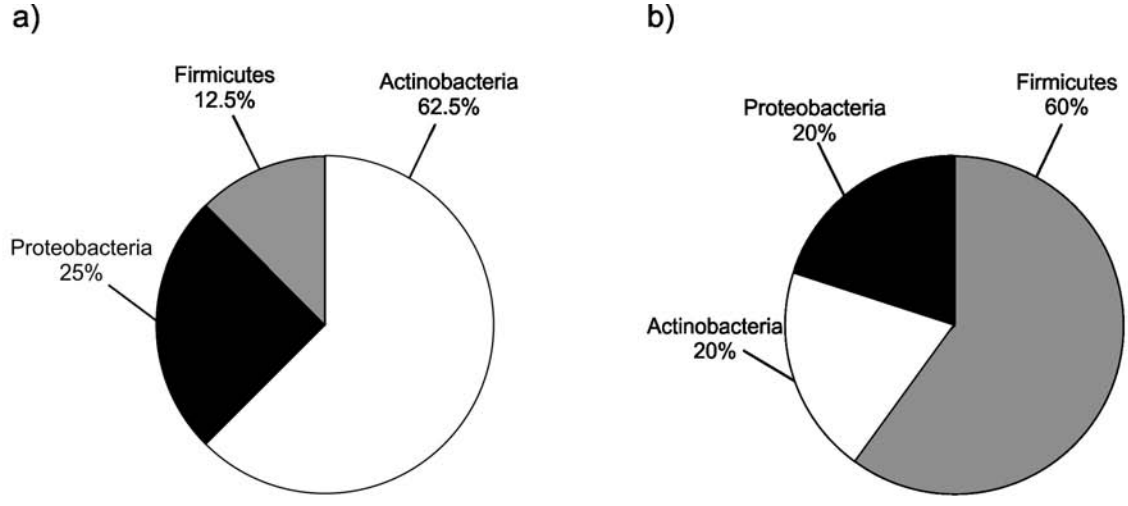

Fig. 2. Phylogenetic distribution of isolated bacteria: a) from mine surface sediment, MSI; b) from mine underground, MUI.

2009). For example, Passariello et al. evaluated the environmental contamination of an abandoned mining site exploited for stibnite ore and found concentrations of Cd and Zn 15600 and 21 times higher, respectively, than those detected in the MS sample (Passariello et al., 2002).

Microbial diversity

The culture-independent (metagenomic) and culture-dependent methods were applied in assessing the microbial diversity of the surface and deep sediment of the Bor Mining and Smelting Complex in order to obtain a more complete overview of the microbial community structures of these two sites.

Two clone libraries, MS and MU, containing 124 and 194 clones, respectively, with the appropriate size inserts, were generated. Sequencing random samples of clones from both libraries indicated greater diver-

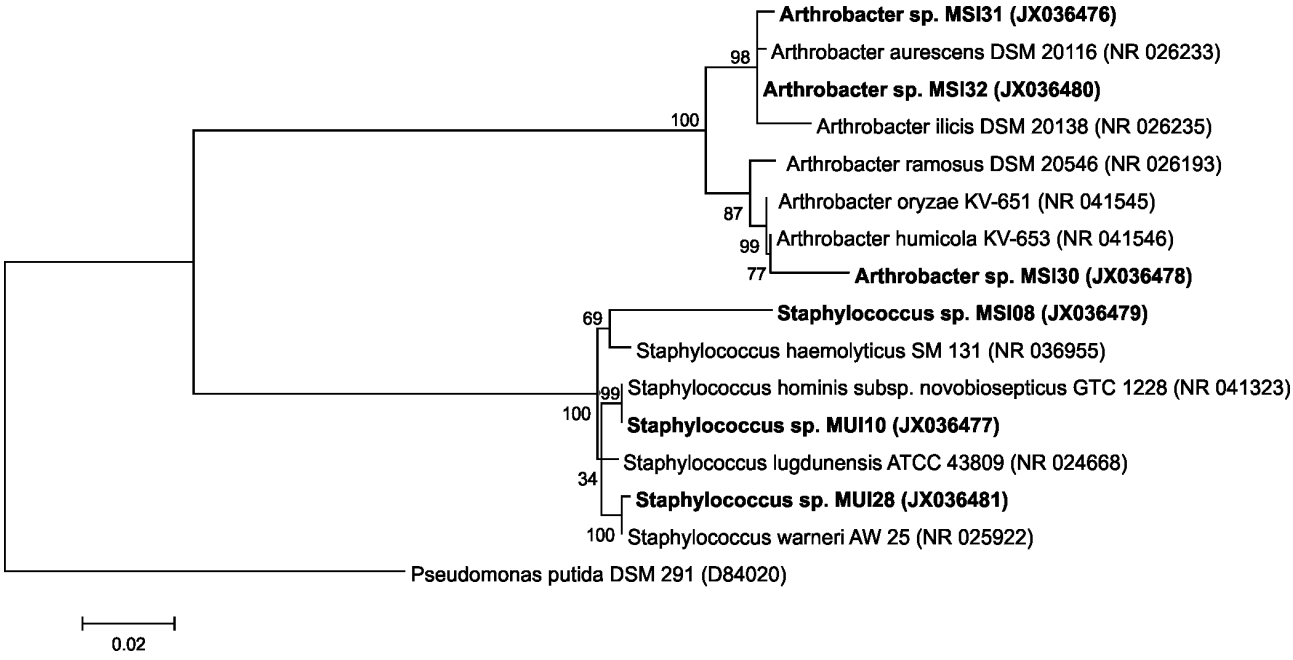

Fig. 3. Phylogenetic tree of 16S rDNA gene sequences based on neighbor-joining analyses showing the relationship of MSI and MUI isolates and related species. Bootstrap values at branch points are expressed as a percentage of 1000 replications. Isolated MSI and MUI strains are designated in bold. GenBank accession numbers are in brackets.

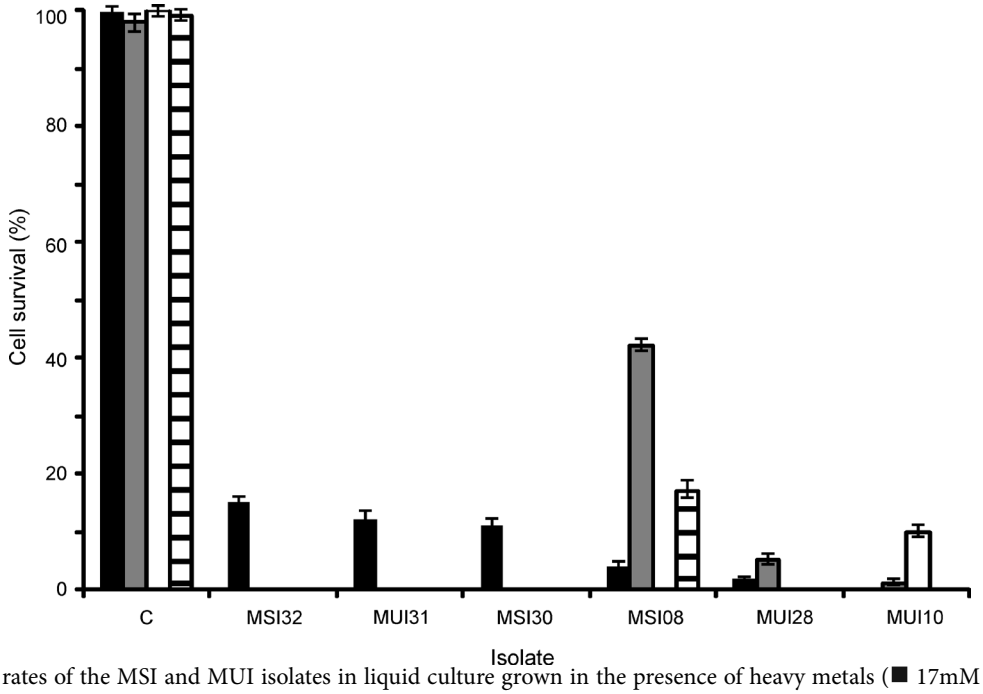

Fig. 4. Survival rates of the MSI and MUI isolates in liquid culture grown in the presence of heavy metals (■ 17mM Ni^{2+}, ▩ 535μM Cd^{2+}, □ 31.5mM Cu^{2+}, ⬓ 38.5mM Cr^{6+}; C - bacterial growth without heavy metal presence). All data are the average of at least three independent experiments.

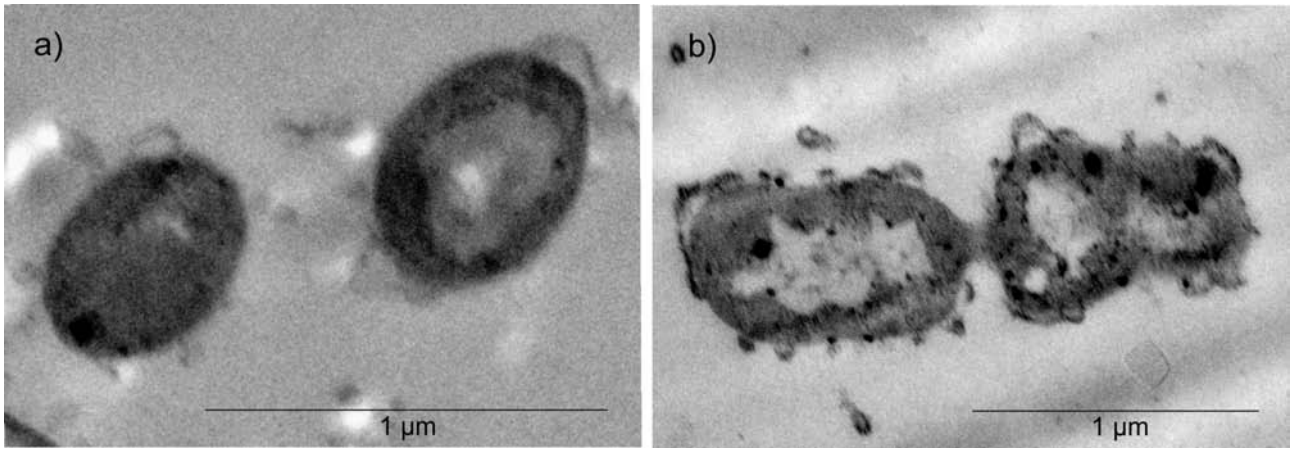

Fig. 5. Transmission electron micrograph of *Staphylococcus* sp. MSI08 isolate cultivated in MT medium: a) control (no Cr^{6+} present); b) in presence of Cr^{6+}(3mM).

Table 2. Isolated strains from the Copper mine Bor area sediment samples growing in the presence of elevated metal and halogen concentrations.

Isolate	17 mM Ni^{2+}	535 μM Cd^{2+}	38.5 mM Cr^{6+}	31.5 mM Cu^{2+}	1.7 M NaCl	0.2 M NaF
MSI08	+	+	+		+	
MSI30	+				+	+
MSI31	+					+
MSI32	+				+	+
MUI10		+		+		
MUI28	+	+			+	

sity among the MS clones (data not shown). Thus, to broadly assess the diversity of the cloned inserts, 58 clones from the MS and 30 from the MU library were identified to genus level using the first 600 bp of the 16S rDNA sequence. In the MS library, the predominant phylum was *Proteobacteria* (34.8 %), and in the MU library the most abundant phylum was *Nitrospirae* (92%) (Fig. 1). Overall, greater microbial diversity was in the library generated from the mine surface sediment with members of seven different phylogenetic groups detected, while in the library from the deeper mine sediment representatives of only three different groups were detected (Fig. 1).

The distribution of phylotypes in the MS clone library corresponds to the results of other similar studies, indicating that the most abundant phylogenetic groups in terrestrial systems, even when affected with heavy metal pollution, are *Proteobacteria*, *Actinobacteria*, and *Acidobacteria* (Gremion et al., 2003; Jansen, 2006; Lin et al., 2011). *Actinobacteria* and *Nitrospirae* were detected in both libraries, except that *Actinobacteria* was less abundant in MU while *Nitrospirae*, with *Leptospirillum* sp. as only representative identified, was less abundant in the MS clone library (Fig. 1). The lower diversity in the MU clone library was partially expected due to the more extreme physical conditions present in the mine underground (Table 1). Furthermore, the presence of *Leptospirillum* sp. in a relatively large percentage is not unusual since they are often found in environments with lower pH, such as the deep sediments of mines (Schrenk et al., 1998). This suggests that the surface sediment of the mining complex not only has a more diverse bacterial community compared to underground, but may also be

Table 3. 16S rDNA identification of isolates.

	the best GenBank match		
Isolate	classification	accession number	% identity
MSI08	*Staphylococcus haemolyticus*	EU867340	95%
MSI30	*Arthrobacter oryzae*	AB648969	98%
MSI31	*Arthrobacter nitroguajacolicus nitroguajacolicus nitroguajacolicus*	FN908762	99%
MSI32	*Arthrobacter aurescens*	HQ597008	99%
MUI10	*Staphylococcus hominis*	JQ660295	99%
MUI28	*Staphylococcus aureus*	JF431908	99%

Table 4. Characteristics of metal tolerant *Staphylococcus* sp. MSI08 isolate

Characteristic	Result [a]
Utilisation of carbon source	
Trechalose	+
Lactose	+
Sucrose	+
Mannitol	+
Maltotriose	+
Arabinose	-
Glycerol	-
Fructose	+
Utilisation of nitrogen source	
L-arginine	-
L-valine	-
L-phenylalanin	-
L-pyroglutamic acid	+
L-tryptophan	-
L-isoleucine	-
Presence of hydrolyzing enzymes	
Urea	-
Esculin	+
ß-D-cellobioside	-
ß-D-glucoside	+
α-D-maltoside	+
α-D-galactoside	-
Phosphate	+

[a] +, good growth or activity; -, absence of growth or activity

able to maintain some populations associated with healthier soil.

Thirty-two strains isolated from the mine surface sediment (MSI isolates) and 30 strains isolated from the mine underground (MUI isolates) were identified by 16S rDNA sequencing. With respect to the bacterial community composition, the bacterial groups detected in both samples were affiliated to *Actinobacteria*, *Firmicutes* and *Proteobacteria* (Fig. 2). While the identified MSI isolates mostly belong to the phylum *Actinobacteria*, which was 5 and 2.5 times more represented than *Firmicutes* and *Proteobacteria,* respectively (Fig. 2a), among the MUI, members of *Firmicutes* were 3 times more represented than *Actinobacteria* and *Proteobacteria* (Fig. 2b). Although with a different distribution, the same three phyla were detected among pure culture isolates in the study performed by Islam and Sar, that involved samples originated from uranium ore (Islam and Sar, 2011).

The higher degree of diversity within the analyzed mine samples was obtained using the metagenomic approach (Fig. 1). On the other hand, analysis of the cultivated bacteria revealed species that were not obtained using this method (Fig. 2). Researchers generally use the culture-independent method when exploring bacterial diversity, since it is known that only less than 1% of microbial species are culturable and the use of a metagenomic approach provides a far more complete picture of the organisms involved in a community (Mocali and Benedetti, 2010; Rondon et al., 2000). However, the results of this and some more recent studies emphasize the importance of employing both methods in order to avoid gaps in microbial community diversity data (Islam and Sar, 2011).

Resistance of bacterial isolates to heavy metals and halogens

All isolated strains (32 MSI and 30 MUI) were screened for their ability to grow in the presence of metal concentrations that were 20-fold higher than the maximum concentrations recommended by the regulation of Serbian Ministry of Environment, Mining and Spatial Planning (EPA/Serbia, 1994) on the solid medium. Five isolates were able to grow in the presence of 17 mM Ni^{2+}, three in the presence of 535 μM Cd^{2+}, one in the presence of 38.5 mM Cr^{6+} and one in the presence of 31.5 mM Cu^{2+} (Table 2). None of the isolates was able to grow in the presence of Hg^{2+}, Fe^{3+}, Zn^{2+} (data not shown). When much higher salt concentrations (corresponding to 100 mM concentrations of metal ions) were used, none of the isolated strains grew. Among the tested strains, four isolates were able to grow in the presence of 1.7 M (10%) NaCl, while three grew in the presence of 0.2 M (1%) NaF.

Isolates that were capable of growing in the presence of one or more elevated metal concentrations were further identified by 16S rDNA sequencing (1300 bp) to belong to the *Arthrobacter* and *Staphylococcus* genera (Table 3). A phylogenetic tree of the MSI and MUI isolates was generated using related sequences from GenBank and it consists of two independent clades: Actinomycetales and Bacillales (Fig. 3). 16S rDNA sequences of all metal-tolerant isolates shared 95-99% 16S rRNA gene sequence identity with their closest matching entries in GenBank. Sequence similarity among MSI08, MUI10 and MUI28 isolates belonging to the *Staphylococcus* genus was between 93% and 95%.

Among the five isolates able to grow on Ni^{2+}, three were annotated as *Arthrobacter* sp., namely MSI30, MSI31and MSI32 with 16S rRNA sequence similarity between 93% and 97% (Table 3 and Fig. 3). *Arthrobacter* species resistant to extremely high Ni^{2+} concentrations of up to 25mM were previously isolated from strongly polluted industrial areas (Margesin and Schinner, 1996). Islam and Sar isolated bacterial strains from uranium ore and cultivated them in the presence of similar metal ions range, but at 8.5-, 77- and 21-times lower concentrations of Ni^{2}, Cr^{6+} and Cu^{2+}, respectively (Islam and Sar, 2011). Similarly, indigenous copper-resistant bacteria isolated by Albarracín et al. (2005) were resistant up to 1000 mg l^{-1} of $CuSO_4$, which is 5 times lower than the Cu^{2+} concentration on which MUI10 were able to grow (Table 2).

Three isolates were identified as belonging to the *Staphylococcus* genus, namely MSI08, MUI10 and MUI28 (Table 3). Members of the *Staphylococcus* genus were also previously reported to be resistant to metal ions. A *Staphylococcus* sp. isolate from tannery effluent in India (Sagar et al., 2012) was capable of growing on a similar concentration of Ni^{2+} as the *Staphylococcus* strains isolated in this study, while the Cr^{6+} concentration was twice lower than the concentration MSI08, MUI10 and MUI28 were able to tolerate.

Although the MSI08 isolate, identified as *Staphylococcus* sp., had the highest sequence identity with the *Staphylococcus haemolyticus* strain CCGE3068 of 95% coupled with 99% coverage (Table 3), it differed from the known *S. haemolyticus* strains in the ability to hydrolyze esculin and inability to utilize arginine and glycerol (Table 4). Thus, it was identified as a possibly novel species of *Staphylococcus* genus.

It is common that some metal-resistant strains exhibit higher tolerance to the presence of metal ions on solid medium (Hassen et al., 1998; Yilmaz, 2003). For this reason so we tested the resistance of six MUI and MSI isolates in liquid medium by measuring the survival percent by the enumeration of colony-forming units (Fig. 4). From the strains that were able to grow on 17 mM Ni^{2+} on solid medium, the survival rate when grown in liquid medium in the presence of the same Ni^{2+} concentration was between 2 and 12% (Fig. 4). MSI30, MSI31 and MSI32 strains showed similar growth capacities, while only 2% of the MUI28 population could survive these growing conditions (Fig. 4). When grown in the presence of Cd^{2+} (535 μM), the survival rate was highest for the MSI08 isolate that exhibited 42% survival, which was 20 to 40 times higher in comparison to the survival rates of MUI20 and MUI28 isolates, respectively (Fig. 4). MIU08 survival rate in the presence of Cr^{6+} was four times better than that in the presence of Ni^{2+} and Cr^{6+} (Fig. 4). Generally, the survival rates of the isolates that could grow well in the presence of the same metal concentrations on the solid medium, were poor after growth in the presence of the same metal concentrations in liquid medium.

Characterization of Staphylococcus sp. MSI08 isolate

As the MSI08 isolate was identified to be the best performing in terms of metal tolerance, and was a possibly novel *Staphylococcus* species that could utilize a wide range of sugars as carbon sources with a clear preference for inorganic salts as nitrogen source (Table 4), TEM was also performed with this isolate grown in liquid MT medium and MT medium supplemented with 3mM Cr^{6+}. Cells grown under both conditions were similar in size and shape, while some morphological differences were observed (Fig. 5). Cells grown in the presence of Cr^{6+} exhibited an extracellular polymeric matrix (Fig. 5b). This is often the case with bacteria grown under metal stress conditions as this is a way to increase cell surface volume (Nithya et al., 2011). However, the presence of dividing bacteria under Cr^{6+} presence conditions indicated their ability to grow and divide (Fig. 5b).

In conclusion, combining metagenomic and direct cultivation approaches we have assessed the microbial diversity of two mildly heavy-metal-contaminated sediment samples taken from inside the copper mining and smelting area. Despite having a lower content of metals present, the isolate of *Staphylococcus* sp. MSI08, identified as possessing the greatest bioremediation potential in terms of metal resistance pattern, was found in the surface sediment of the mining area.

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