Novel arsenic hyper-resistant bacteria from an extreme environment, Crven Dol mine, Allchar, North Macedonia

Vladimir Bermanec[1]†, Tina Paradžik[2]†, Snježana P. Kazazić[2], Chantelle Venter[3], Jasna Hrenović[1*], Dušica Vujaklija[2*], Robert Duran[4], Ivan Boev[5], Blažo Boev[5]

[1] University of Zagreb, Faculty of Science, Zagreb, Croatia (vladimir.bermanec@public.carnet.hr, jasna.hrenovic@biol.pmf.hr)

[2] Ruđer Bošković Institute, Bijenička 54, 10000 Zagreb, Croatia (Dusica.Vujaklija@irb.hr; Tina.Paradzik@irb.hr; Snjezana.Kazazic@irb.hr)

[3] Stellenbosch University, Department of Physiological Sciences, Faculty of Science, Stellenbosch, South Africa (chantellev@sun.ac.za)

[4] Université de Pau et des Pays de l'Adour/E2S UPPA, IPREM UMR CNRS 5254, Pau, France (rober.duran@univ-pau.fr)

[5] Goce Delčev University of Štip, Štip, North Macedonia (blazo.boev@ugd.edu.mk; ivan.boev@ugd.edu.mk)

† Both authors contributed equally to this manuscript

[*] Corresponding authors: +3851 4571 258 (DV); +3851 6189 700 (JH)

Keywords: biogeochemistry, arsenate, arsenite, hyper As-resistance, As-transporters, 16S rRNA

Highlights:

- As-rich mixture of pharmacolite, hornesite, and talmessite from Crven Dol mine
- *Stenotrophomonas* sp., *Microbacterium* spp. and bacterial consortium were isolated
- Novel hyper-resistant bacteria survive 32 g/L arsenite and 176 g/L arsenate
- As-tolerance involve efflux systems including ArsB, Acr3(1) and Acr3(2) proteins
- SEM/EDS analyses confirmed intracellular accumulation of arsenic

Abstract

Novel hyper-resistant bacteria were isolated from the Crven Dol mine (Allchar, North Macedonia), arsenic-rich extreme environment. Bacteria were recovered from a secondary mineral mixture, an alteration of hydrothermal realgar rich in arsenates (pharmacolite, hornesite, and talmessite). The sample was recovered from the dark part of the mine at 28 m depth. Three bacterial strains and a bacterial consortium were isolated for their capacity to survive exposure to 32g/L (209 mM) of arsenite, and 176 g/L (564 mM) of arsenate. The 16S rRNA gene analysis identified bacterial isolates as *Stenotrophomonas* sp. and two *Microbacterium* spp. This analysis also revealed that bacterial consortium comprise two Bacteriodetes exhibiting similarity to *Olivibacter ginsengisoli* and to uncultured bacterium, and one γ-proteobacteria with similarity to *Luteimonas* sp. Among all isolates *Stenotrophomonas* sp. exhibited the highest tolerance to As compound as well as the capacity to accumulate As inside the cells. Analysis of genes involved in As-resistance showed that recovered isolates possess the genes encoding the ArsB, Acr3(1) and Acr3(2) proteins, indicating that at least a part of their resistance could be ascribed to As-efflux systems described in isolates obtained from human-polluted environments.

1. Introduction

Arsenic has been recognized as one of the most toxic chemical which represent a major public health concern (Hughes et al., 2011). This study focused on the isolation and identification of bacterial community present in the Crven Dol mine, a unique ore body extremely As rich environment, located in Allchar, North Macedonia. The complex geological characteristic of the ore body has been described previously (Palinkas et al., 2018). The most significant mineralization processes occur close to the entrance of adit 21 at the level -823 m on the eastern side and close to the vertical shaft (Fig. 1A). Crven Dol minerals are composed of

carbonate rocks (dolomite and minor limestone/marble) intruded by a subvolcanic magmatic body highly hydrothermally altered. The chemical composition and rocks were reported in previous studies (Frantz et al., 1994; Janković, 1997). Secondary minerals found in the Crven Dol are weathering products developed through oxidation processes of primary ore minerals, realgar and other sulphides in deposit and its reaction with carbonate mother rock – dolomite. After mining activity in Crven Dol massive realgar body was additionally exposed to oxidizing atmosphere and realgar was weathered to mixture of secondary arsenate minerals – pharmacolite, hornesite and picropharmacolite (Boev, 2002).

Microorganisms living in such As-rich environments are well adapted to elevated concentrations of As and other toxic compounds (Bruneel et al., 2008; Giloteaux et al., 2010; Volant et al., 2014). As-transforming bacteria, both aerobes and anaerobes, are phylogenetically and physiologically diverse (Suhadolnik et al., 2017). Among them the highly As-resistant bacterial species such as *Thiomonas* sp., *Acidithiobacillus ferrooxidans*, *Herminiimonas arsenicoxydans*, *Leptothrix* sp. and *Stenotrophomonas* sp. are well known (Ben Fekih et al., 2018; Ghosh et al., 2018). Many studies revealed that microorganisms have evolved a variety of strategies to survive toxic effects of As compounds. Namely, As typically exists in one of the four oxidation states: As(V), As(III), As(-III), and As(0), among which the most dominant forms are arsenite - As(III), and arsenate - As(V) (Oremland and Stolz, 2003). Trivalent As is generally more toxic than pentavalent As since it binds strongly to vicinal sulfhydryl groups in proteins (Hughes et al., 2011). In contrast, arsenate has the ability to compete with phosphate oxyanions for transport and energetics functions, but its primary toxic effects is due to its transformation to arsenite (Ben Fekih et al., 2018). The widespread resistance mechanisms include the reduction of As(V) into As(III) and the efflux of the later from the cell (Fahy et al., 2015). Arsenite transporters are reported for Proteobacteria, Firmicutes and Actinobacteria, while the presence of putative As-resistance genes are

78 confirmed in many sequenced genomes (Fahy et al., 2015). These bacterial efflux systems
79 include members of the two protein families, ArsB and Acr3 which has two subsets
80 designated Acr3(1) and Acr3(2) (Achour et al., 2007). Although these transporters share same
81 function, they exhibit different mechanisms and metalloid specificity (Yang et al., 2015).
82 ArsB proteins are found only in prokaryotes, whereas Acr3 proteins are widespread in
83 bacteria, archaea, fungi and some plants (Castillo and Saier, 2010; Yang et al., 2015). In
84 bacteria, the genes encoding for these proteins are found on chromosome, plasmids and
85 transposons, suggesting important role of horizontal gene transfer in bacterial adaptation to
86 As-rich environments. Multiple copies of As-resistant genes contribute to a higher level of
87 resistance to As compound (Ben Fekih et al., 2018).
88 Since Crven Dol mine is extremely rich in As, Sb, Hg and Tl representing very hostile
89 environment we expected biological activity to be limited only to microorganisms well
90 adapted to elevated concentrations of As and other toxic compounds. Observed white mineral
91 powder deposited on the rock (Fig. 1B) as a mixture of secondary mineral suggested a
92 possible mineral transformation due to microbial metabolic activity. Taking this into account,
93 the objective of this study was: (**i**) a mineralogical and geochemical characterization of this
94 secondary minerals rich in As, (**ii**) isolation and identification of microorganisms which can
95 survive in such harsh environment and (**iii**) investigation of the As-related genes in order to
96 gain a better insight into the resistance mechanism involved.

97

98

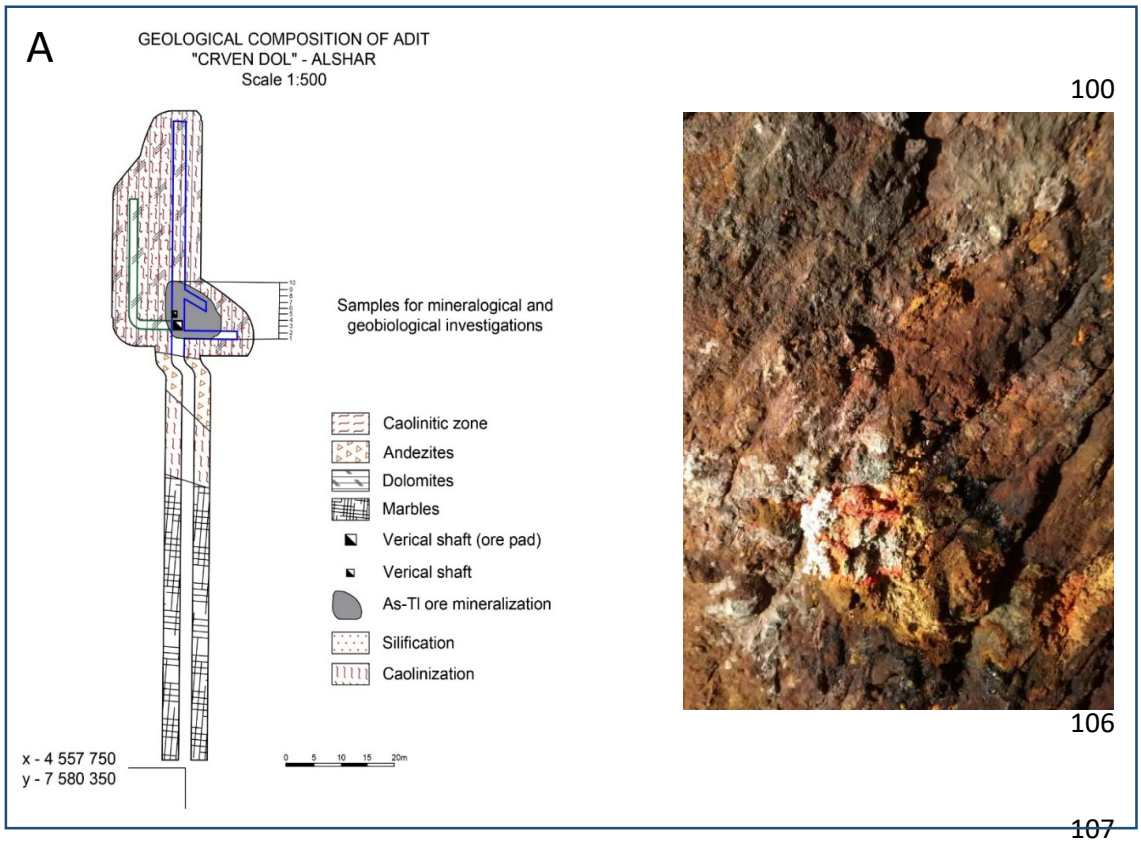


Fig. 1. A) Sampling position in Crven Dol mine and simplified geological setting of ore body. GPS coordinates of the entrance to the mine are shown. B) Alteration zone around realgar mineralization: the wet white mineral mixture powder (the secondary mineral) was sampled for chemical and microbiological characterization in this study.

2. Experimental

2.1 Site description, sampling, and characterization of a rock sample

Sample of secondary mineral - a wet white mineral mixture powder, was collected before collapse of Crven Dol mine in 2016 at approximately 28 m depth (GPS coordinates of the entrance are x – 4 557 750, y – 7 580 350, Fig. 1A). Sample was aseptically placed in a sterile plastic tube, and transferred to the laboratory within 24 h. The pH of sample was determined after its dilution (1:2.5) in distilled water. The mineral composition of sample was determined

applying Philips X'Pert diffractometer equipped with spinner using 40 mA current and 45 kV. Anode material was Cu. Start position was 4.01°2Θ, end position 64.99°2Θ with step size 0.02°2Θ and scan time 0.5 s.

2.2 Isolation and phenotypic characterization of As-resistant bacteria

One gram of a wet white mineral mixture powder was suspended in peptone water (Biolife, Italy) up to 10 mL. The original and diluted suspensions (up to 10^{-2}) were directly plated (0.1 mL) on nonselective nutrient agar (Biolife, Italy). Plates were incubated aerobically at 22 °C and colony appearances were inspected daily during seven days. Based on the colony size, surface, color and slime-layer production, different morphological variants were selected for further analyses. Selected isolates were Gram-stained and characterized by routine bacteriological techniques. Bacterial growth was tested at various temperatures and fermentation ability was checked on Kligler iron agar (Biolife, Italy). Note that all isolates were maintained on the nutrient agar supplemented with As-compounds to prevent loss of As-resistance in case that resistance genes are associated with mobile genetic elements.

2.3 MALDI-TOF MS analysis

Matrix-assisted laser desorption ionization-time of flight mass spectrometry - MALDI-TOF MS (Microflex LT mass spectrometer and software MALDI Biotyper 3.0, Bruker Daltonics, Germany) and MALDI Biotyper database were used for isolate identifications or to further differentiate between closely related species. Species were identified according to the manufacturer's score classification. Procedure based on the direct transfer-formic acid method identification was applied as follows. Small amount of isolate biomass was smeared onto a 96-spot MALDI steel target plate and 1 μL of 70% formic acid (Fisher Chemical, Spain) was

144 added. After drying each spot was overlaid with 1 µL of MALDI matrix (saturated solution of
145 α-cyano-4-hydroxycinnamic acid (α-HCCA) in 50% acetonitrile and 2.5% trifluoroacetic
146 acid), dried and submitted for Biotyper analysis.
147
148 *2.4 Bacterial genomic DNA extraction*
149 Isolates and bacterial consortium obtained in this study were cultured in peptone water for
150 five days at 22 °C for genomic DNA extraction. DNA was extracted from the obtained
151 biomass using Wizard Genomic DNA Purification Kit (Promega Corporation, USA),
152 following the recommendations of the manufacturer. The extracted genomic DNA samples
153 were stored at -20 ºC until further processing.
154
155 *2.5 PCR amplification, TA cloning and sequencing*
156 PCR amplification of 16S rRNA gene for all bacterial isolates were performed using 63F
157 (5'-CAGGCCTAACACATGCAAGTC-3') and 1387 R (5'-GGGCGGWGTGTACAAGGC-3')
158 primers, and cycling conditions as described previously (Giloteaux et al., 2010). In addition,
159 degenerate primers for genes involved in bacterial As detoxification (Fahy et al., 2015), i.e.
160 for arsenite transporters, *arsB* (AarsB1F /AarsB1R), and for two subclasses of gene *acr3*
161 (*acr3(1)* - Aacr1F/Aacr2R, and *acr3(2)* - dacr5F/dacr4R) as well as primers targeting arsenite
162 oxidase gene *aioA* (aoxBM1-2F/ aoxBM3-2R) were used to investigate the presence of
163 targeted genes in the genome of the isolates and consortium. PCR conditions applied for each
164 set of primers were described earlier (Fahy et al., 2015). In order to determine the
165 composition of the bacterial consortium, 16S rRNA PCR products of expected size were
166 cloned using a pGEM®-T Easy Vector Systems (Promega Corporation, USA) and PCR were
167 performed on recombinant colonies. All PCR amplicons were evaluated using agarose gel
168 electrophoresis and gel slices containing PCR products (~1300 bp 16S rRNA gene, ~750 bp

of *arsB* and of *acr3*) were purified using Qiagen agarose purification kit. Purified DNA sequences were submitted for sequencing to Macrogen service (South Korea). The 16S rRNA gene sequences and translated coding sequences of targeted genes were subjected to a BLAST search (Altschul et al., 1997).

2.6 Phylogenetic analysis

The evolutionary relationships of all isolates were performed using partial sequence (1260 nt) of 16S rRNA gene. Multiple sequence alignment (MSA) of 16S rRNA gene sequences was performed using Clustal Omega (Sievers et al., 2011). Phylogenetic trees of 16S rRNA gene sequences were constructed using maximum likelihood method in PhyML (Guindon et al., 2010). aLRT values (approximate likelihood ratio test) were used to infer branch support. The branches with aLRT values over 0.9 were considered well supported. FigTree software (http://tree.bio.ed.ac.uk/software/figtree/) was used for statistical report and graphic presentation of the results. The nucleotide sequences of 16S rRNA genes, as well as transporter genes, *arsB*, *acr3(1)* and *acr3(2)* were deposited in the NCBI GeneBank under accession number (to be added in proof).

2.7 Analysis of As-tolerance of bacterial isolates

The As-tolerance was determined for bacterial consortium and the three pure bacterial cultures. The tested concentration of sodium arsenite ($NaAsO_2$) were 0.25-32 g/L, and for sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$) 2.75-176 g/L in Luria-Bertani (LB) broth after aerobic incubation for five days at 22°C (Sunita et al., 2012). The tolerance of all isolates to As was determined as MTC, maximum tolerable concentration resulting in undisturbed colony formation; as MIC, minimum inhibitory concentration causing the visible inhibition of bacterial growth; and as MBC, minimum bactericidal concentration resulting in lack of colony

194 formation. The MTC, MIC and MBC were determined by inoculation of 10 μL from each
195 experimental tube on the nutrient agar following the incubation of plates at 22 °C for five
196 days using as a positive control bacteria grown in LB broth without addition of As salts.
197
198 *2.8 Scanning electron microscopy* / energy dispersive spectroscopy *analyses*
199 Pure culture of *Stenotrophomonas* sp. and the Consortium growing at MTC of As-compounds
200 were further examined by scanning electron microscopy / energy dispersive spectroscopy
201 (SEM/EDS) analysis. Glutaraldehyde-fixed bacterial cells were processed according to
202 standard procedures for scanning electron microscopy (SEM). Briefly, the samples were
203 washed in phosphate buffer, dehydrated through a graded series of ethanol, dried in
204 hexamethyldisilizane (HMDS) and transferred onto coverslips. Dry samples were
205 subsequently placed onto aluminium stubs and carbon coated before imaging the samples
206 with the Zeiss Crossbeam 540 FEG SEM using the InLens detector at 0.5 kV. The EDS
207 analysis (Oxford Instruments, United Kingdom) was done using 20kV and Backscatter
208 detector on at least three areas on the sample. The As content in cells of *Stenotrophomonas*
209 sp. and Consortium exposed to arsenite and arsenate was compared. Statistical analyses were
210 carried out using Statistica 13.3 software (TIBCO Software Inc.). For pairwise comparisons
211 ordinary t-test for independent variables was used. Decisions regarding statistical significance
212 were made at $p < 0.05$.
213
214 **3. Results and Discussion**
215 *3.1 Characterization of the white mineral mixture*
216 The white mineral mixture powder collected in Crven Dol mine (Fig. 1) and suspended in
217 water had pH 6.9. The X-ray diffraction XRD analysis showed a mixture of gypsum

218 ($CaSO4 \cdot 2H_2O$) and arsenates $(AsO_4)^{3-}$ including: pharmacolite ($CaH(AsO_4) \cdot 2H_2O$), hornesite
219 ($Mg_3(AsO_4)_2 \cdot 8H_2O$), and talmessite ($Ca_2Mg(AsO_4)_2 \cdot 2H_2O$) which are common in carbonate
220 host rock. These minerals are formed under supergene oxidation after exposition of primary
221 ore minerals (realgar and orpiment) to weathering processes (Boev, 2002). It has been shown
222 that the process of transforming realgar (AsS) to As-rich secondary minerals is accelerated by
223 microbial metabolism resulting in a mixture of arsenate minerals and gypsum (Drewniak and
224 Sklodowska, 2013). Thus, we hypothesized that As-rich white mineral mixture sample (Fig.
225 1) collected at Crven Dol mine possesses bacterial community highly resistant to As.
226

227 *3.2 Isolation and characterization of As-resistant bacteria*

228 By plating peptone water with suspended rock sample on solid LB medium four clearly
229 distinct colony types were obtained after one week of growth. The colonies varied in size and
230 colour, from translucent to a pale-yellow and yellow. Bigger size colonies, were covered by
231 clear sticky matrix. It is well know that bacterial communities form biofilms embedded in a
232 self-producing matrix, which help them to survive in a hostile environment (Flemming et al.,
233 2016). Extracellular matrix helps bacterial cells attachment to the mineral surface and act as
234 biosorbent of toxic metals in extreme environment (Gupta and Diwan, 2017). Thus, we
235 selected one big colony covered with matrix, one extremely small and translucent colony and
236 two colonies with similar size and differing in colour (Table 1). The bacterial isolates were
237 characterized by Gram-staining and growth capacities at different temperatures (Table 1).
238 Analysed bacterial cultures were able to grow aerobically on nutrient agar at 22 °C and at 36
239 °C, but none of them were able to grow at 42 °C (Table 1). This suggested that the recovered
240 isolates were adapted to the temperature conditions (16 °C) in the mine environment.
241

242 **Table 1.** General characteristics of isolated colonies. All isolates were non-fermentative
243 roads.

Colony morphology of Isolates	Gram stain	Growth at 22 °C	Growth at 36 °C	Growth at 42 °C
1: white - pale yellow, big size and covered by slime	-/+	+	+	-
2: pale yellow	-	+	+*	-
3: yellow	+	+	+	-
4: translucent, very small in size	+	+	+	-

244 *Production of dark brown pigment was observed when Isolate 2 was grown at 36 °C (Fig. 5).

245

246 Further, physiological resistance of isolates (1-4) exposed to elevated concentrations of
247 arsenite and arsenate was examined. All bacterial isolates were able to survive 32g/L (209
248 mM) of sodium arsenite, and 176 g/L (564 mM) of sodium arsenate (Table 2). This level of
249 resistance to arsenate is recognized as hyper-resistance (Drewniak et al., 2008). Such elevated
250 resistance was comparable to As-resistant bacteria isolated from diverse As contaminated
251 environments (Achour et al., 2007; Sunita et al., 2012). Usually bacteria exhibit lower
252 resistance to arsenite with MIC from 1-10 mM (Achour et al., 2007; Drewniak et al., 2008).
253 Only a very few reports described bacteria resistant to arsenite up to 80 mM (Achour et al.,
254 2007; Chen and Shao, 2009; Maizel et al., 2016). In this study, Isolate 2 showed exceptional
255 tolerance to arsenate and arsenite without showing any disturbance in colony formation
256 (Table 2). Its resistance (MTC) particularly to arsenite (209 mM) is 2.6 times higher than 80
257 mM so far described for an isolate closely related to *Microbacterium esteraromaticum* (Chen
258 and Shao, 2009). The metabolic capacities of the isolated strains in this study represent an
259 important asset to colonize As rich environment as previously demonstrated for
260 microorganisms colonizing mine impacted environments (Bruneel et al., 2006; Bertin et al.,
261 2011) and volcano (Medrano-Santillana et al., 2017).

262

Table 2. Determination of As-tolerance of bacterial consortium and pure cultures isolated from As-rich mineral mixture.

As-resistance	Isolate 1	Isolate 2	Isolate 3	Isolate 4
Arsenite				
MTC (g/L)	4	> 32	4	1
MIC (g/L)	8	> 32	8	2
MBC (g/L)	> 32	> 32	> 32	> 32
Arsenate				
MTC (g/L)	88	>176	88	3
MIC (g/L)	176	>176	176	6
MBC (g/L)	>176	>176	>176	>176

3.3 Identification and phylogenetic analysis

The 16S rRNA partial gene amplification (over 1260 nt), and sequencing revealed that Isolate 1 comprise mixture of various 16S rRNA genes indicating that it corresponds to a bacterial consortium. In order to identify the members of this consortium, a 16S rRNA gene library was constructed. The sequencing of 45 clones and BLAST analysis revealed that the consortium comprises three strains. Two of them were affiliated to Bacteriodetes: one bacterium representing 47% (21 clones) in the consortium shared 98.6% identity (98% coverage) with *Parapedobacter pyrenivorans* strain P-4 and the other bacterium (22%, 10 clones) shared 95% identity (94 % coverage) with *Olivibacter ginsengisoli* strain Gsoil 060 and with uncultured bacterium clone SIBG789_N12D2_16S_B (95.2% identity and 94 % coverage). The third strain was affiliated to γ-proteobacteria and representing 31% of bacteria in the consortium (14 clones) shared 99.8 % identity (89 % coverage) with *Luteimonas* sp. strain BO171. The coverage of this library was 100% (97% similarity) as shown by Good's C estimator indicating that sufficient number of clones were analyzed to reveal entire bacterial diversity (Good, 1953). The Isolates 2, 3 and 4 were obtained as pure bacterial cultures. Isolate 2 had 100% identity (100% coverage) with *Stenothrophomonas maltophilia* (γ-

proteobacteria) while Isolate 3 had 100% identity (100% coverage) with *Microbacterium murale* strain S3A-15, strain 01-Gi-001 and strain M-sp VKM Ac-2016. Isolate 4 shared 99.8% identity (99% coverage) with *Microbacterium* sp. R28 isolated from copper polluted soil and with *M. esteraromaticum* strains TRB35 and OBE2. The phylogenetic analyses based on the comparison of 16S rRNA gene sequences obtained in this study with closely related sequences deposited in database are shown in Fig. 2. The isolates from Crven Dol mine belong to bacterial phyla known for their capacity to survive in As-rich ecosystem (Liu et al., 2018). The phylogenetic tree shows that the two strains (Isolates 3 and 4) belonging to the genus *Microbacterium* were clearly separated (Fig. 2), suggesting that they correspond to two distinct species explaining their different As-survival capacities (Table 2).

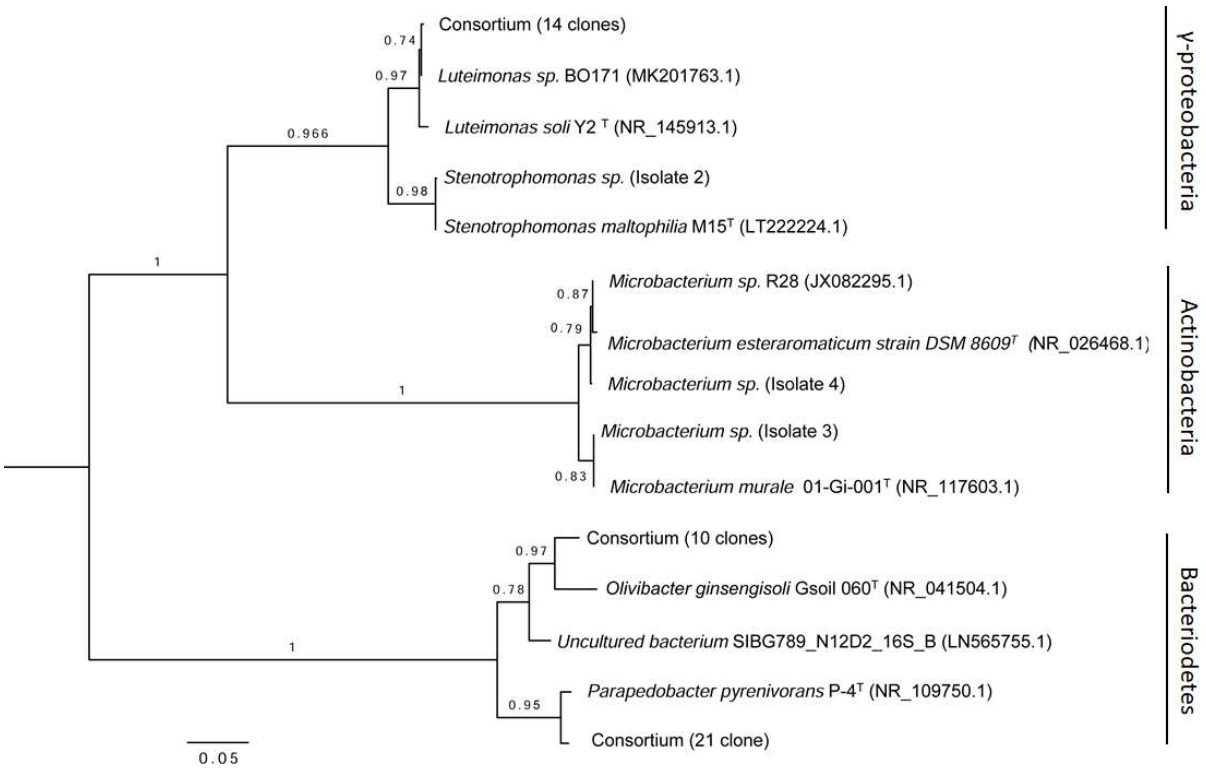


Fig. 2. Phylogenetic tree of isolated bacteria based on 16S rRNA gene sequences. aLRT values are shown for main branches.

The comprehensive overview of MALDI TOF MS-based microbial characterization at the subspecies and strain levels has been published recently (Demirev and Sandrin, 2016). The discriminating power of this method is derived from the measurement of highly abundant proteins like ribosomal proteins, which makes it almost independent from cultivation conditions. Consistently, we performed MALDI TOF MS analysis and our results showed that *Microbacterium* isolates identified by 16S rRNA approach produced clearly different protein patterns (Fig. 3). The specific protein fingerprints were obtained in mass range from 2 to 12 kDa. Thus, in line with the phylogenetic analysis MALDI-TOF MS also discriminated two isolates but at the proteome level. Noteworthy, although Biotyper database contains 37 *Microbacterium* species (including *M. murale*) MALDI TOF MS was unable to identify confidently the Isolates 3 and 4 (score was below threshold; <1.7).

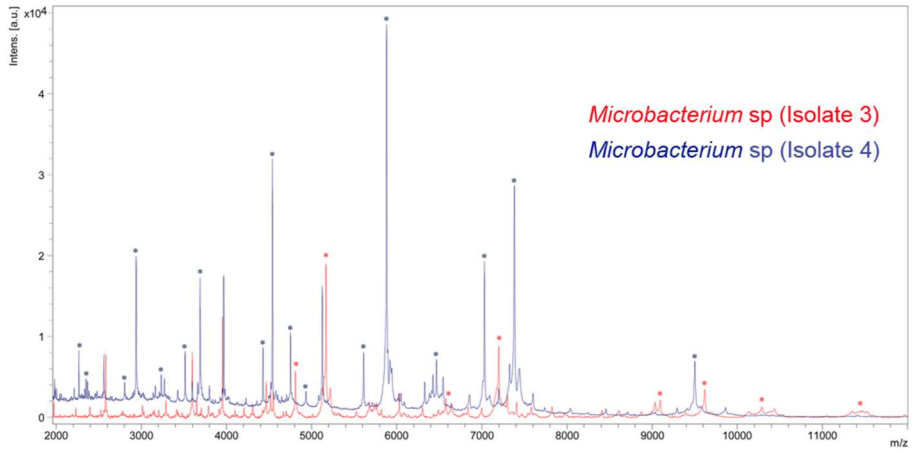


Fig 3. Comparison of MALDI TOF mass spectra obtained for *Microbacterium* species, Isolate 3 and Isolate 4. Differences in the protein profile of two species are marked with an asterisks.

MALDI TOF MS was also applied to analyse pure culture of *Stenotrophomonas* strain (Isolate 2). The score values obtained for *Stenotrophomonas* sp. varied from 1.702 -1.726. Although this scoring was low, according to the manufacturer it was sufficient to probable

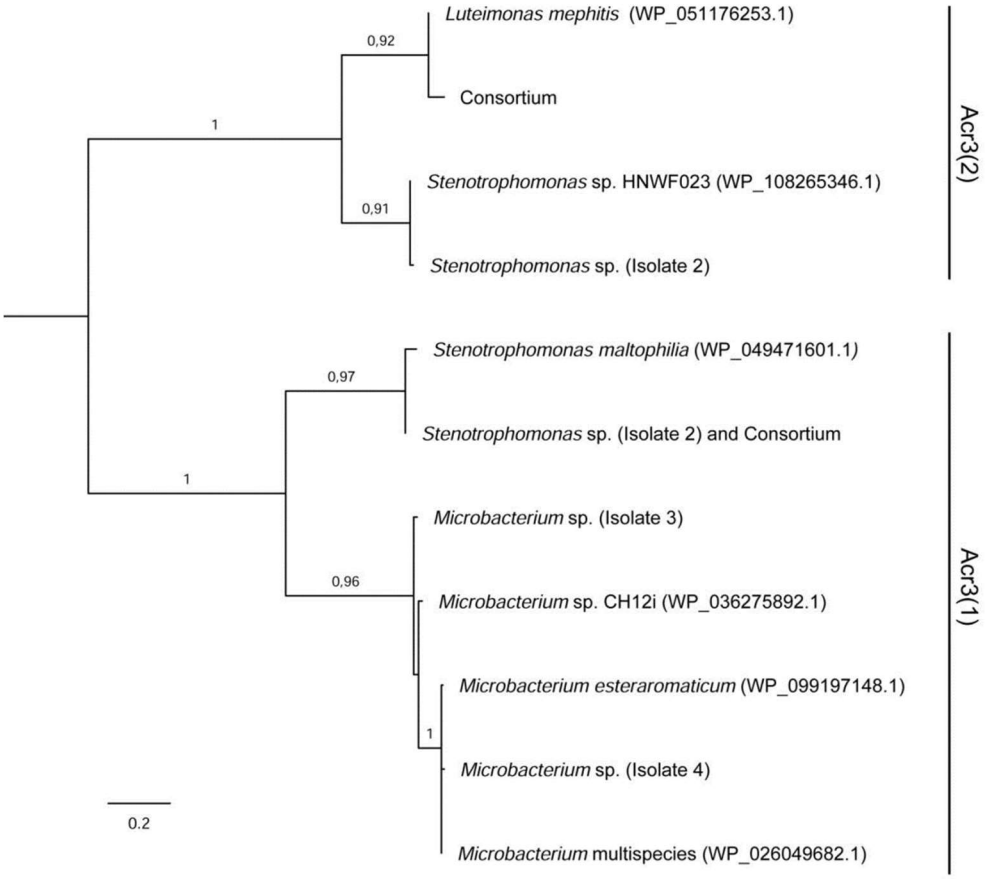
identification of isolates to the genus level (from 1.700 to 1.999). Thus, this result was in concert with 16S rRNA gene identification.

3.4. Functional analysis

The isolates were also analysed for the presence of the functional genes involved in arsenic transformations, *aioA*, and *arsC*, and for genes involved in the As-efflux system, *arsB*, and *acr3(1)* and *acr3(1).* Applying the conditions described previously (Fahy et al., 2015) we were not able to detect the presence of the genes encoding arsenite oxidase (*aioA*, large subunit) or cytoplasmic arsenate reductase (*arsC*). Either these bacteria have very divergent genes involved in As transformation, which could explain lacking of the PCR signal with the primers used in this study or other genes contribute to the hyper As-resistance mechanisms. However, PCR amplification and sequencing confirmed the presence of the genes associated with As-efflux system in all isolates (1-4). Gene encoding arsenite efflux pump (*arsB*) was detected only in bacterial consortium. Amplified nucleotide sequence exhibited 97% identity (99% coverage) with *arsB* gene encoding As-pump membrane protein from uncultured *Lysobacter* sp. clone SE1-12* and 95% identity with *Luteimonas* sp. YGD11-2. This result was not surprising because both genera belong to the same family *Xanthomonadaceae* (order Xanthomonadales γ-proteobacteria).

The *acr3(1)* and *acr3(2)* genes targeted in this study were detected in all isolates and phylogeny of their corresponding products were presented in Fig. 4. The Acr3(1) detected in Consortium exhibited 96.6% identity (99% coverage) with Acr3(1) protein from *S. maltophilia*. This is not surprising since the identical sequence of Acr3(1) protein was found in *Stenotrophomonas* strain (Isolate 2), the pure culture isolated from the Consortium. We cannot exclude the possibility of the horizontal gene transfer between the members of the same order. Namely, the *Stenotrophomonas* sp. also belongs to order Xanthomonadales as

341 *Luteimonas* sp. detected by 16S rRNA gene analysis (Fig. 2). Note that the members of this
342 order possess highly conserved Acr3(1) transporters (96.6% identity according to Blast
343 analysis). The gene products Acr3(1) from *Microbacterium* species (Isolate 3 and Isolate 4)
344 exhibited highest similarity with Acr 3(1) protein from *Microbacterium* sp. CH12i (95.8 %
345 identity and 98% coverage) and with Acr3(1) family arsenite efflux transporter
346 *Microbacterium* (identity 100% and 93 % coverage) respectively. These results further
347 confirmed the diversity of these strains.
348 The *acr3(2)* genes encoding Acr3(2) transporters were also detected in this study. In
349 consortium the Acr3(2) was most closely related to Acr3(2) from *Luteimonas mephitis* (95%
350 coverage and 95 % identity) and in *Stenotrophomonas* sp. (Isolate 2) with arsenite efflux
351 transporter from *Stenotrophomonas* sp. HMWF023 (99% identity and 96.5% coverage).
352 Consistently with previous report *acr3(2)* genes were not detected in *Microbacterium* strains
353 (Achour et al., 2007).
354 Overall, the phylogeny based on *acr3*(1) and (2) genes was congruent with 16S rRNA
355 phylogeny (Figs. 2 and 4). Since all isolates possessed As-resistance genes it is likely that
356 isolates represent primary bacterial colonizer. The hyper As-resistance of all isolates in
357 particular of *Stenotrophomonas* sp. (Isolate 2) might be the result of increased number of
358 genes involved in efflux system, as previously reported in Burkholderiales which members
359 isolated from the As-rich environments have increased number of the As-related genes on
360 their genomes (Li et al., 2014). Since *Stenotrophomonas* sp. (Isolate 2) showed exceptional
361 arsenite and arsenate resistance we further characterized the metabolic capacity of this strain.



375 F**ig. 4**. Phylogenetic tree based on the Acr3(1) and (2) As-efflux transporters. aLRT values
376 are shown for main branches.

377

378 *3.5. Characterization of* Stenotrophomonas *sp. (Isolate 2)*

379 *3.5.1. Pigment production*

380 While cultivating the isolates at 36 ºC, we noticed that *Stenotrophomonas* sp. (Isolate 2)
381 produced water-soluble brown pigment (Fig. 5). Pigment production plays a significant role in
382 protection against various environmental stresses. For example, it was reported that the
383 presence of heavy metal increase the pigmentation of various bacteria (Lima e Silva et al.,
384 2012). Melanin (brown pigment) produced by various bacteria exhibit excellent heavy metal
385 binding capacity and as such has very good potential for application in bioremediation (Thaira
386 et al., 2019). High temperature also trigger pigment production in bacteria. Similarly to our

observation, at elevated temperature *Pseudomatermonas* species SM 9913 produces pyomelanin (brown pigment) with a protective role against heat damage (Zeng et al., 2017). Thus, it is possible that *Stenotrophomonas* sp, (Isolate 2) by producing the brown pigment at elevated temperature increases its heat resistance and probably tolerance to heavy metal as demonstrated (Thaira et al., 2019). The comparison of MALDI-TOF MS pattern of *Stenotrophomonas* sp. grown at 36 ºC to that of the bacterium grown at 22 ºC revealed two peptides of approx. 2300 and 2400 Da specifically produced at 36 °C (Fig. 5). This observation suggested that these proteins might be involved in the bacterial response to exposure at higher temperature together with the production of the soluble brown-pigment. Further proteomic analyses are required to better understand the pigment production at high temperature and its role.

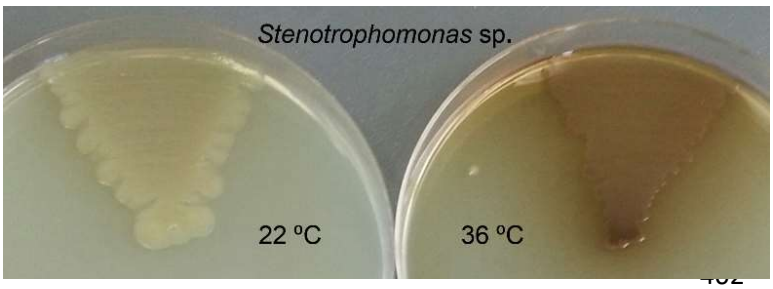
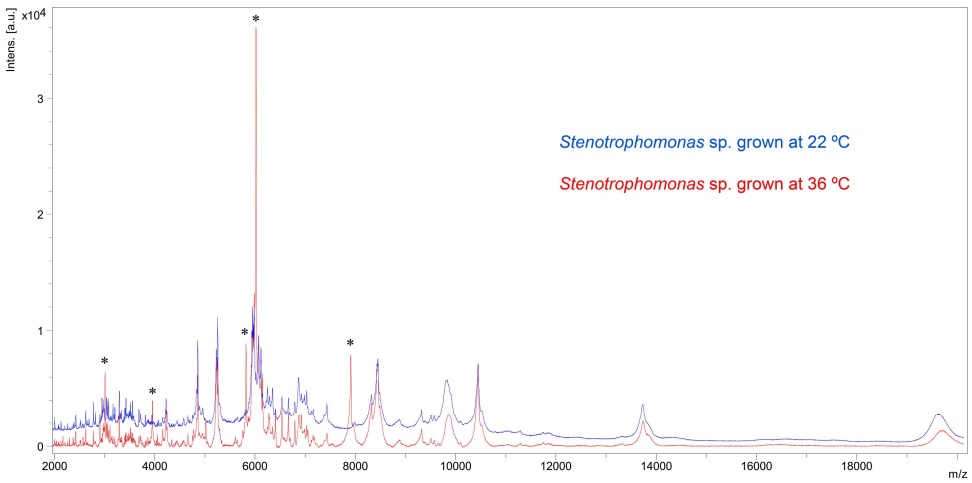
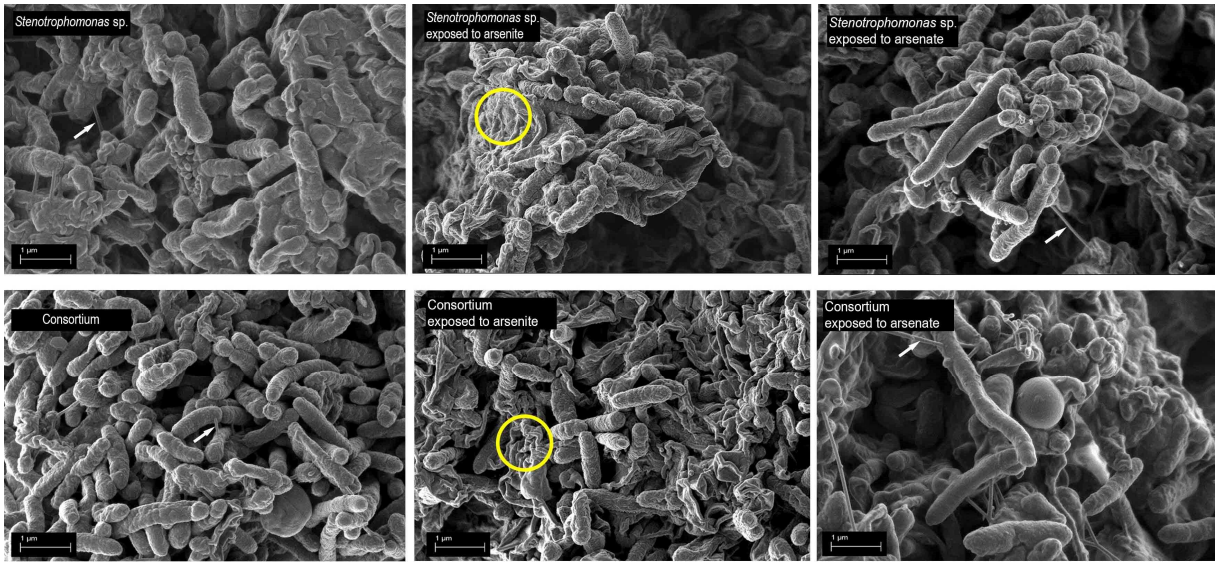


Fig. 5. Production of water-soluble brown pigment by *Stenotrophomonas* sp. at 36 ºC, but not at 22 ºC.



412 **Fig. 6.** Comparison of MALDI TOF mass spectra obtained for *Stenotrophomonas* sp. (Isolate
413 2) grown at 22 ºC (blue) and 36 ºC (red). Differences in the protein profile are marked with
414 asterisks.
415
416 *3.5.2. Antibiotic resistance*
417 *Stenotrophomonas* species range from common soil organisms to opportunistic human
418 pathogens (Hrenovic et al., 2019). The MALDI-TOF MS protein pattern of the
419 *Stenotrophomonas* sp. isolate was similar to that of *Stenotrophomonas maltophilia*, which is
420 known to be naturally resistant to a large-spectrum antibiotics (including all carbapenems) via
421 two inducible chromosomal metallo-β-lactamases (Denton and Kerr, 1998). Consistently, the
422 *Stenotrophomonas* sp. isolate was able to grow on CHROMagar revealing its carbapenem-
423 resistance capacity. Such observation is not surprising since antibiotic resistance, particularly
424 carbapenem-resistance, has been found linked with metal resistance including As (Villa et al.,
425 2013).
426
427 *3.5.3. Cell integrity and the As-sorption capacity*
428 *Stenotrophomonas* sp. was further examined by SEM/EDS in order to determine the As effect
429 on the cell integrity and the As-sorption capacity. For comparison, the Consortium was also
430 examined as a reference. In the absence of As, *Stenotrophomonas* sp. and bacterial
431 consortium showed rod-shaped cells connected together with nanowire like structures (Fig. 7).
432 Bacterial nanowires are well known to play a crucial role in transport of electrons when
433 bacteria grow on minerals (Sure et al., 2016). In the presence of arsenite at MTC, we observed
434 the production extracellular matrix (Fig. 7, middle panel). When exposed to arsenate at MTC
435 the elongation of rod cells connected with nanowires was observed for both,

436 *Stenotrophomonas* sp. and Consortium cells (Fig. 7, right panel). This observation could not
437 clarify the hyper resistance of *Stenotrophomonas* sp. (Isolate 2) to As compounds.
438



439

440 **Fig. 7.** Scanning electron micrographs of *Stenotrophomonas* sp. (Isolate 2) and Consortium.
441 Left panel is showing cells grown in the absence of As salts; middle panel cells grown in the
442 presence of arsenite at MTC; right panel cell grown in the presence of arsenate at MTC.
443 Nanowires are marked with arrows, and possible traces of the extracellular matrix with
444 circles.
445

446 To get insights on the accumulation of As compounds into the cells, EDS analysis was
447 performed. Isolates were grown at MTC as described in Materials and Methods. Note that
448 Consortium was exposed to eight times lower concentration of arsenite and two times lower
449 concentration of arsenate in comparison to *Stenotrophomonas* sp. As shown in Figure 8, the
450 *Stenotrophomonas* sp. (Isolate 2) and Consortium (Isolate 1) cells were able to sorb As-
451 compounds (0.12 – 0.34 dry wt %). In both isolates, the As content varies depending on the
452 cell exposure to arsenite or arsenate. Significantly higher difference was observed for the

Stenotrophomonas sp. (~ 3X) in contrast to Consortium cells (~ 1.3 X). Comparison of As content between isolates showed that when grown on arsenate the As-content reached its highest value and was very similar in both isolates. This might be explained by the fact that arsenate is less toxic to bacterial cells (Hughes et al., 2011) and was accumulating at the same level in the both isolates (Fig. 8). It can be also seen that Consortium accumulated more arsenic (~ 2X) than *Stenotrophomonas* sp. when exposed to arsenite (Fig. 8). Taking into account the MTC values for Consortium and Isolate 2 our results clearly show that *Stenotrophomonas* sp. has either better efflux system or transformation mechanisms to remove As-compounds from the cells when exposed to arsenite.

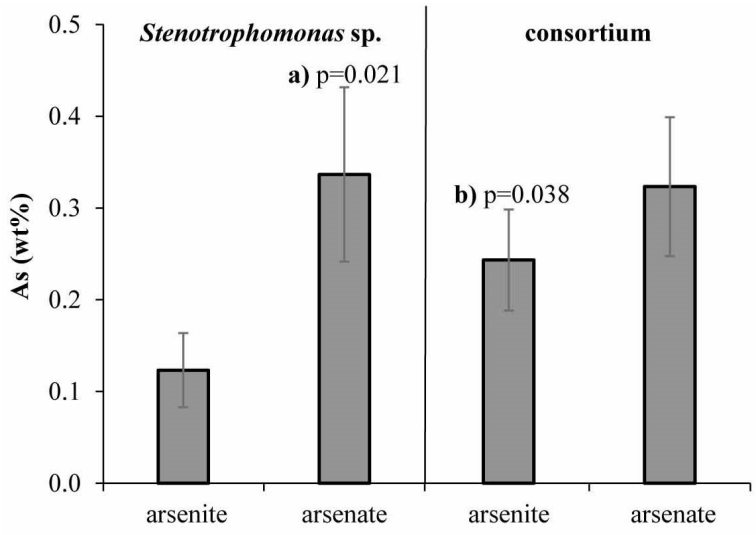


Fig. 8. The As content (wt %) in dry sample of isolate *Stenotrophomonas* sp. and Consortium determined by EDS analysis. Cells after contact with MTC values of arsenite and arsenate: a) significantly higher than cells exposed to arsenite; b) significantly higher than in cells of *Stenotrophomonas* sp. exposed to MTC of arsenite.

Based on the current knowledge, we propose that the As probably accumulate into the cell as arsenate-As(V), which is less toxic and able to interact with thiol groups present in small molecules such as glutathione (GSH) forming As-complexes retained into the cell. Because

471 the *aio*A and *ars*C genes were not detected in the isolates, it is likely that As(V) entering the
472 cell, probably via phosphate transporters (Garbinski et al., 2019), are retained inside of the
473 cell by thiol-containing molecules while arsenite-As(III) is expulsed through the ACR3
474 efflux-pumps. It should be also pointed out that only As transporters were identified in the
475 isolated bacteria. Thus, we cannot exclude the possibility that high resistance of bacterial
476 isolates is due to other As-transforming genes, such as As-reductase or oxidase, which were
477 not detected. Clearly, future research and the next generation whole genome sequencing are
478 required to gain a deeper understanding of the mechanisms that these microorganisms have
479 evolved to enhance resilience to harsh environmental conditions.
480 Finally, although arsenic efflux system are found in almost every organisms the molecular
481 mechanisms of arsenic transport and many novel genes that contribute to As resistance
482 remains to be determined (Garbinski et al., 2019). Despite this, microorganisms or plants
483 adapted to respond to environmental challenges can be very promising bioremediation agents.
484 Such as bacteria that we isolated in the Crven Dol mine or plants (Bačeva et al., 2014) with
485 capacity to accumulate heavy metals found in the surrounding environment of Allchar area.
486 Microbial-based remediation is generally considered as a promising technology for the
487 treatment of metal contaminated soils but requires optimal conditions favouring sorption
488 mechanisms while limiting desorption (Jin et al., 2018). Therefore, further studies are needed
489 to found the optimised conditions for using the Isolates obtained in this study in
490 bioremediation processes.
491

492 **4. Conclusions**

493 Bacterial consortium and three novel bacterial species belonging to the genera
494 *Stenotrophomonas* and *Microbacterium* were recovered from As-rich mineral mixture
495 collected at the Crven Dol mine, North Macedonia. These bacteria have an important role in

the transformation of primary to secondary oxidized mineral. Consortium and bacterial isolates exhibited high resistance to arsenite and arsenate in comparison to reported As-resistant bacteria. Particularly *Stenotrophomonas* sp. (Isolate 2) exhibited so far undescribed hyper-resistance to arsenite (209 mM) together with extremely high resistance to arsenate (564 mM). These results demonstrate the potential use of this isolate for bioremediation. Extreme environments, such as Crven Dol mine, are promising niches to explore for the recovery of bacterial strains having high metal tolerance capacity, which represent an important asset for the bioremediation of metal polluted areas.

Acknowledgements

This work has been supported in a part by the Croatian Science Foundation (project no. IP-2014-09-5656) and in part by the Centre of Excellence for Bioprospecting of the Adriatic Sea, Ruđer Bošković Institute, Croatia. We also acknowledge the support of the French/Croatian bilateral program PHC-Cogito 2019-20 (to DV and RD).

5. References

Achour, A.R., Bauda, P., and Billard, P. (2007). Diversity of arsenite transporter genes from arsenic-resistant soil bacteria. *Res Microbiol* 158(2)**,** 128-137. doi: 10.1016/j.resmic.2006.11.006.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25(17)**,** 3389-3402. doi: gka562 [pii].

Bačeva, K., Stafilov, T., and Matevski, V. (2014). Bioaccumulation of Heavy Metals by Endemic Viola Species from the Soil in the Vicinity of the As-Sb-Tl Mine "Allchar", Republic of Macedonia. *International Journal of Phytoremediation* 16(4)**,** 347-365. doi: 10.1080/15226514.2013.783551.

Ben Fekih, I., Zhang, C., Li, Y.P., Zhao, Y., Alwathnani, H.A., Saquib, Q., et al. (2018). Distribution of Arsenic Resistance Genes in Prokaryotes. *Front Microbiol* 9**,** 2473. doi: 10.3389/fmicb.2018.02473.

Ben Said, O., Louati, H., Soltani, A., Preud'homme, H., Cravo-Laureau, C., Got, P., et al. (2015). Changes of benthic bacteria and meiofauna assemblages during bio-treatments of anthracene-contaminated sediments from Bizerta lagoon (Tunisia). *Environ Sci Pollut Res Int* 22(20)**,** 15319-15331. doi: 10.1007/s11356-015-4105-7.

Bertin, P.N., Heinrich-Salmeron, A., Pelletier, E., Goulhen-Chollet, F., Arsene-Ploetze, F., Gallien, S., et al. (2011). Metabolic diversity among main microorganisms inside an arsenic-rich

ecosystem revealed by meta- and proteo-genomics. *ISME J* 5(11), 1735-1747. doi: 10.1038/ismej.2011.51.
Boev, B., Bermanec, V., Serafimovski, T., Lepitkova, S., Mikulčić, S., Šoufek, M., Jovanovski, G., Stafilov, T., Najdoski, M. (2002). Allchar mineral assemblage. *Geologica Macedonica* 15-16, 1-23.
Bruneel, O., Duran, R., Casiot, C., Elbaz-Poulichet, F., and Personne, J.C. (2006). Diversity of microorganisms in Fe-As-rich acid mine drainage waters of Carnoules, France. *Appl Environ Microbiol* 72(1), 551-556. doi: 10.1128/AEM.72.1.551-556.2006.
Bruneel, O., Pascault, N., Egal, M., Bancon-Montigny, C., Goni-Urriza, M.S., Elbaz-Poulichet, F., et al. (2008). Archaeal diversity in a Fe-As rich acid mine drainage at Carnoules (France). *Extremophiles* 12(4), 563-571. doi: 10.1007/s00792-008-0160-z.
Castillo, R., and Saier, M.H. (2010). Functional Promiscuity of Homologues of the Bacterial ArsA ATPases. *Int J Microbiol* 2010, 187373. doi: 10.1155/2010/187373.
Chen, S., and Shao, Z. (2009). Isolation and diversity analysis of arsenite-resistant bacteria in communities enriched from deep-sea sediments of the Southwest Indian Ocean Ridge. *Extremophiles* 13(1), 39-48. doi: 10.1007/s00792-008-0195-1.
Demirev, P., and Sandrin, T.R. (2016). *Applications of Mass Spectrometry in Microbiology.* Springer, Cham.
Denton, M., and Kerr, K.G. (1998). Microbiological and clinical aspects of infection associated with Stenotrophomonas maltophilia. *Clin Microbiol Rev* 11(1), 57-80.
Drewniak, L., and Sklodowska, A. (2013). Arsenic-transforming microbes and their role in biomining processes. *Environ Sci Pollut Res Int* 20(11), 7728-7739. doi: 10.1007/s11356-012-1449-0.
Drewniak, L., Styczek, A., Majder-Lopatka, M., and Sklodowska, A. (2008). Bacteria, hypertolerant to arsenic in the rocks of an ancient gold mine, and their potential role in dissemination of arsenic pollution. *Environ Pollut* 156(3), 1069-1074. doi: 10.1016/j.envpol.2008.04.019.
Fahy, A., Giloteaux, L., Bertin, P., Le Paslier, D., Medigue, C., Weissenbach, J., et al. (2015). 16S rRNA and As-Related Functional Diversity: Contrasting Fingerprints in Arsenic-Rich Sediments from an Acid Mine Drainage. *Microb Ecol* 70(1), 154-167. doi: 10.1007/s00248-014-0558-3.
Flemming, H.C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S.A., and Kjelleberg, S. (2016). Biofilms: an emergent form of bacterial life. *Nat Rev Microbiol* 14(9), 563-575. doi: 10.1038/nrmicro.2016.94.
Frantz, E., Palme, H., Todt, W., Elgoresy, A., and Pavicevic, M. (1994). Geochemistry of Tl-as Minerals and Host Rocks at Allchar (Fyr Macedonia). *Neues Jahrbuch Fur Mineralogie-Abhandlungen* 167(2-3), 359-399.
Garbinski, L.D., Rosen, B.P., and Chen, J. (2019). Pathways of arsenic uptake and efflux. *Environ Int* 126, 585-597. doi: 10.1016/j.envint.2019.02.058.
Ghosh, D., Bhadury, P., and Routh, J. (2018). Coping with arsenic stress: Adaptations of arsenite-oxidizing bacterial membrane lipids to increasing arsenic levels. *Microbiologyopen* 7(5), e00594. doi: 10.1002/mbo3.594.
Giloteaux, L., Goni-Urriza, M., and Duran, R. (2010). Nested PCR and New Primers for Analysis of Sulfate-Reducing Bacteria in Low-Cell-Biomass Environments. *Applied and Environmental Microbiology* 76(9), 2856-2865. doi: 10.1128/Aem.02023-09.
Good, I.J. (1953). The Population Frequencies of Species and the Estimation of Population Parameters. *Biometrika* 40(3/4), 237-264. doi: 10.2307/2333344.
Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W., and Gascuel, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol* 59(3), 307-321. doi: 10.1093/sysbio/syq010.
Gupta, P., and Diwan, B. (2017). Bacterial Exopolysaccharide mediated heavy metal removal: A Review on biosynthesis, mechanism and remediation strategies. *Biotechnol Rep (Amst)* 13, 58-71. doi: 10.1016/j.btre.2016.12.006.
Hrenovic, J., Durn, G., Kazazic, S., Dekic, S., and Music, M.S. (2019). Untreated wastewater as a source of carbapenem-resistant bacteria to the riverine ecosystem. *Water Sa* 45(1), 55-62. doi: 10.4314/wsa.v45i1.07.

584 Hughes, M.F., Beck, B.D., Chen, Y., Lewis, A.S., and Thomas, D.J. (2011). Arsenic exposure and
585 toxicology: a historical perspective. *Toxicol Sci* 123(2)**,** 305-332. doi: 10.1093/toxsci/kfr184.
586 Janković, S., Boev, B., Serafimovski, T., (1997). *Magmatism and Tertiary Mineralization of the Kozuf*
587 *Metalogenetic District, the Republic of Macedonia, With Particular Reference to the Allchar*
588 *Deposit.* Skopje: University St. Kiril and Metodij, Faculty of Mining and Geology-Stip.
589 Jin, Y., Luan, Y., Ning, Y., and Wang, L. (2018). Effects and Mechanisms of Microbial Remediation
590 of Heavy Metals in Soil: A Critical Review. *Applied Sciences* 8(8)**,** 1336.
591 Li, X., Zhang, L., and Wang, G. (2014). Genomic evidence reveals the extreme diversity and wide
592 distribution of the arsenic-related genes in Burkholderiales. *PLoS One* 9(3)**,** e92236. doi:
593 10.1371/journal.pone.0092236.
594 Lima e Silva, A.A.d., Carvalho, M.A.R.d., Souza, S.A.L.d., Dias, P.M.T., Silva Filho, R.G.d.,
595 Saramago, C.S.d.M., et al. (2012). Heavy metal tolerance (Cr, Ag and Hg) in bacteria isolated
596 from sewage. *Brazilian Journal of Microbiology* 43**,** 1620-1631.
597 Liu, J.L., Yao, J., Wang, F., Ni, W., Liu, X.Y., Sunahara, G., et al. (2018). China's most typical
598 nonferrous organic-metal facilities own specific microbial communities. *Sci Rep* 8(1)**,** 12570.
599 doi: 10.1038/s41598-018-30519-1.
600 Maizel, D., Blum, J.S., Ferrero, M.A., Utturkar, S.M., Brown, S.D., Rosen, B.P., et al. (2016).
601 Characterization of the extremely arsenic-resistant Brevibacterium linens strain AE038-8
602 isolated from contaminated groundwater in Tucuman, Argentina. *International*
603 *Biodeterioration & Biodegradation* 107**,** 147-153. doi: 10.1016/j.ibiod.2015.11.022.
604 Medrano-Santillana, M., Souza-Brito, E.M., Duran, R., Gutierrez-Corona, F., and Reyna-Lopez, G.E.
605 (2017). Bacterial diversity in fumarole environments of the Paricutin volcano, Michoacan
606 (Mexico). *Extremophiles* 21(3)**,** 499-511. doi: 10.1007/s00792-017-0920-8.
607 Oremland, R.S., and Stolz, J.F. (2003). The ecology of arsenic. *Science* 300(5621)**,** 939-944. doi:
608 10.1126/science.1081903.
609 Palinkas, S.S., Hofstra, A.H., Percival, T.J., Sostaric, S.B., Palinkas, L., Bermanec, V., et al. (2018).
610 Comparison of the Allchar Au-As-Sb-Tl Deposit, Republic of Macedonia, with Carlin-Type
611 Gold Deposits. *Diversity of Carlin-Style Gold Deposits* 20**,** 335-363. doi: 10.5382/rev.20.10.
612 Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., et al. (2011). Fast, scalable
613 generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol*
614 *Syst Biol* 7**,** 539. doi: 10.1038/msb.2011.75.
615 Suhadolnik, M.L.S., Salgado, A.P.C., Scholte, L.L.S., Bleicher, L., Costa, P.S., Reis, M.P., et al.
616 (2017). Novel arsenic-transforming bacteria and the diversity of their arsenic-related genes
617 and enzymes arising from arsenic-polluted freshwater sediment. *Sci Rep* 7(1)**,** 11231. doi:
618 10.1038/s41598-017-11548-8.
619 Sunita, M.S.L., Prashant, S., Chari, P.V.B., Rao, S.N., Balaravi, P., and Kishor, P.B.K. (2012).
620 Molecular identification of arsenic-resistant estuarine bacteria and characterization of their ars
621 genotype. *Ecotoxicology* 21(1)**,** 202-212. doi: 10.1007/s10646-011-0779-x.
622 Sure, S., Ackland, M.L., Gaur, A., Gupta, P., Adholeya, A., and Kochar, M. (2016). Probing
623 Synechocystis-Arsenic Interactions through Extracellular Nanowires. *Front Microbiol* 7. doi:
624 10.3389/Fmicb.2016.01134.
625 Thaira, H., Raval, K., Manirethan, V., and Balakrishnan, R.M. (2019). Melanin nano-pigments for
626 heavy metal remediation from water. *Separation Science and Technology* 54(2)**,** 265-274. doi:
627 10.1080/01496395.2018.1443132.
628 Villa, L., Capone, A., Fortini, D., Dolejska, M., Rodriguez, I., Taglietti, F., et al. (2013). Reversion to
629 susceptibility of a carbapenem-resistant clinical isolate of Klebsiella pneumoniae producing
630 KPC-3. *J Antimicrob Chemother* 68(11)**,** 2482-2486. doi: 10.1093/jac/dkt235.
631 Volant, A., Bruneel, O., Desoeuvre, A., Hery, M., Casiot, C., Bru, N., et al. (2014). Diversity and
632 spatiotemporal dynamics of bacterial communities: physicochemical and other drivers along
633 an acid mine drainage. *Fems Microbiology Ecology* 90(1)**,** 247-263. doi: 10.1111/1574-
634 6941.12394.
635 Yang, Y., Wu, S., Lilley, R.M., and Zhang, R. (2015). The diversity of membrane transporters
636 encoded in bacterial arsenic-resistance operons. *PeerJ* 3**,** e943. doi: 10.7717/peerj.943.

637 Zeng, Z., Cai, X., Wang, P., Guo, Y., Liu, X., Li, B., et al. (2017). Biofilm Formation and Heat Stress
638 Induce Pyomelanin Production in Deep-Sea Pseudoalteromonas sp. SM9913. *Front Microbiol*
639 8**,** 1822. doi: 10.3389/fmicb.2017.01822.

640

641 **Figure captions**

642 **Fig. 1.** A) Sampling position in Crven Dol mine and simplified geological setting of ore body.
643 GPS coordinates of the entrance to the mine are shown. B) Alteration zone around realgar
644 mineralization: the wet white mineral mixture powder (the secondary mineral) was sampled
645 for chemical and microbiological characterization in this study.

646

647 **Fig. 2.** Phylogenetic tree of isolated bacteria based on 16S rRNA gene sequences. aLRT
648 values are shown for main branches.

649

650 **Fig 3.** Comparison of MALDI TOF mass spectra obtained for *Microbacterium* species, Isolate
651 3 and Isolate 4. Differences in the protein profile of two species are marked with an asterisks.

652

653 **Fig. 4**. Phylogenetic tree based on the Acr3(1) and (2) As-efflux transporters. aLRT values
654 are shown for main branches.

655

656 **Fig. 5.** Production of water-soluble brown pigment by *Stenotrophomonas* sp. at 36 ºC, but not
657 at 22 ºC.

658

659 **Fig. 6.** Comparison of MALDI TOF mass spectra obtained for *Stenotrophomonas* sp. (Isolate
660 2) grown at 22 ºC (blue) and 36 ºC (red). Differences in the protein profile are marked with
661 asterisks.

662

663 **Fig. 7.** Scanning electron micrographs of *Stenotrophomonas* sp. (Isolate 2) and Consortium.
664 Left panel is showing cells grown in the absence of As salts; middle panel cells grown in the
665 presence of arsenite at MTC; right panel cell grown in the presence of arsenate at MTC.
666 Nanowires are marked with arrows, and possible traces of the extracellular matrix with
667 circles.

668

669 **Fig. 8.** The As content (wt %) in dry sample of isolate *Stenotrophomonas* sp. and Consortium
670 determined by EDS analysis. Cells after contact with MTC values of arsenite and arsenate: a)
671 significantly higher than cells exposed to arsenite; b) significantly higher than in cells of
672 *Stenotrophomonas* sp. exposed to MTC of arsenite.

673

674