Exposure to Different Arsenic Species drives the Establishment of Iron- and Sulfur-oxidizing Bacteria on Rice Root Iron Plaques

Sarah Zecchin, Milena Colombo, Lucia Cavalca*

Dipartimento di Scienze per gli Alimenti, la Nutrizione e l'Ambiente (DeFENS), Università degli Studi di Milano, Milano, Italy

*Corresponding author.

E-mail address: lucia.cavalca@unimi.it

Postal address: via Celoria, 2, 20133, Milano (Italy)

Telephone: +39 0250319116

Fax: +39 0250319238

Abstract

Iron- and sulfur-oxidizing bacteria inhabiting rice rhizoplane play a significant role on arsenic biogeochemistry in flooded rice paddies, influencing arsenic translocation to rice grains. In the present study, the selective pressure of arsenic species on these microbial populations was evaluated. Rice roots from continuously flooded plants were incubated in iron sulfide (FeS) gradient tubes and exposed to either arsenate or arsenite. The biomass developed in the visible iron-oxidation band of the enrichments was analyzed by Scanning Electron Microscopy and Energy-Dispersive Spectroscopy (SEM-EDS) and the bacterial communities were characterized by 16S rRNA gene sequencing. Different *Proteobacteria* communities were selected depending on exposure to arsenate and arsenite. Arsenate addition favored the versatile iron-oxidizers *Dechloromonas* and *Azospira*, associated to putative iron (hydr)oxide crystals. Arsenite exposure decreased the diversity in the enrichments, with the development of the sulfur-oxidizer *Thiobacillus thioparus*, likely growing on sulfide released by FeS. Whereas sulfur-oxidizers were observed in all treatments, iron-oxidizers disappeared when exposed to arsenite.

These results reveal a strong impact of different inorganic arsenics on rhizospheric iron-oxidizers as well as a crucial role of sulfur-oxidizing bacteria in establishing rice rhizosphere communities under arsenic pressure.

Keywords: Iron-oxidizing bacteria; sulfur-oxidizing bacteria; arsenic; rice rhizosphere; rice iron plaques; gradient tubes.

Introduction

Arsenic contamination of rice in an issue of global concern, with metalloid concentrations in grains often exceeding the tolerable daily intake recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2010) as well as the limits established by the Commission regulation (EU) 2015/1006 and in China (Zhu et al. 2008). Strikingly, it has been recently demonstrated that even when plants are cultivated in European soils containing arsenic (As) concentrations below the law limits, metalloid concentrations in rice grains exceed the law limits for baby food production (Zecchin et al. 2017a).

Rice plants usually grow under complete flooding for the whole life cycle. In rice paddies, the chemistry of As is strongly influenced by its interactions with iron (Fe) and sulfur (S) minerals, as well as with the rhizosphere microbiota (Hu et al. 2007; Somenahally et al. 2011; Jia et al. 2015; Zecchin et al. 2017a, 2017b). Continuous flooding leads to a redox potential decrease in soil solution, promoting As releasing processes such as ferric iron [Fe(III)] (hydr)oxides dissolution (Kögel-Knabner 2010) and arsenate [As(V)] reduction to arsenite [As(III)] (Takahashi et al. 2004; Yamaguchi et al. 2014). Under flooded conditions in rice paddies, Fe(III)-reducing bacteria, that harvest energy by coupling organic acid oxidation to Fe(III) reduction, promote As release to pore water, thus impairing a strong impact on rice contamination by As, with an increase of metalloid accumulation in rice grains with respect to aerobic rice (Zecchin et al. 2017a; Das et al. 2016; Ma et al. 2014; Spanu et al. 2012). Sulfate-reducing bacteria (SRB) and sulfur oxidizing bacteria (SOB) similarly participate to As cycling, by promoting, respectively, the formation and the dissolution of As(III)-containing S minerals such as orpiment and realgar (Fisher et al. 2008). An opposite trend has been observed for cadmium and lead, which decrease in continuous flooding and increase in aerobic rice (Ye et al. 2018; Rizwan et al. 2016; Rinklebe et al. 2016). Probably as a consequence of the common cultivation of rice under continuous flooding, As has been

identified by the European Food Safety Authority (EFSA) as the major issue in rice consumption (EFSA 2014, 2012 and 2010).

The rhizosphere in rice paddies is a narrow redox boundary micro-environment characterized by presence of anoxic Fe(II)-rich water and a gradient of oxygen (O_2) released by the root aerenchima (Colmer 2003). In such environment, Fe(II)-oxidizing bacteria (FeOB) find their proper place in opposing gradients of electron donor [Fe(II)] and acceptor (O_2) (Dubinina and Sorokina 2014). FeOB include a wide range of species in terms of ecological niche: neutrophilic, acidophilic, aerobes and anaerobes (Hedrich et al. 2011). These organisms are widespread in Fe(II)-rich environments characterized by Fe and O_2 opposing gradients. In marine environments, FeOB are usually affiliated to the class Zetaproteobacteria, whereas freshwater populations belong to the betaproteobacterial family *Gallionellaceae* (Emerson et al. 2012). Some FeOB are characterized by extracellular deposition of Fe(III)-oxides either as stalks, like in *Gallionella ferruginea* (Emerson et al. 2010), or as sheaths that cover the entire cells like in *Acidovorax* sp. (Hohmann et al. 2010). Under continuous flooding, together with chemical Fe(II) oxidation, FeOB contribute to the formation of Fe plaques around the roots (Weiss et al. 2003; Oremland et al. 2005; Kögel-Knabner 2010). This compact thin layer enveloping the roots prevents the assimilation of As by the plant (Zhao et al. 2009; Seyfferth et al. 2010), due to high affinity of the metalloid for Fe(III) (hydr)oxides (Roberts et al. 2004). Although the documented ecological role of FeOB in rice paddies (Kögel-Knabner 2010; Zecchin et al. 2017a, 2017b), very little is known about their identity and physiology in comparison to freshwater FeOB.

Sulfur chemistry in rice paddies is more complicated, given the presence of several oxidation states of the element and of a high number of organic molecules (Hu et al. 2002). In anaerobic rice paddies, SRB actively contribute to the S cycle by respiring SO_4^{2-} to sulfide (S^{2-}) (Pester et al. 2012). S^{2-} reacts with soluble Fe(II) and As(III), leading to the formation of FeS_x and

100 As_xS_x minerals, thus decreasing As concentration in soil solution. Where O_2 leaks from the
101 roots, SOB, typically inhabiting ecosystems with steep opposing gradients of S^{2-} and O_2,
102 oxidize S^{2-} releasing As from As_xS_x minerals (Dahl et al. 2008). For this reason, SOB likely
103 have an important role in As mobilization in continuously flooded plants (Stubner et al. 1998;
104 Fisher et al. 2008; Zecchin et al. 2017a, 2017b).
105 Aerobic and anaerobic chemolithotrophic SOB use electrons derived from sulfur oxidation for
106 either respiration or autotrophic reduction of CO_2. They are mainly affiliated to the
107 Proteobacteria and can oxidize a variety of S species. These microorganisms are
108 phylogenetically and physiologically diverse, particularly in terms of pH and temperature
109 (Friedrich et al. 2005).
110 Chemolithotrophic SOB were found in a variety of ecosystems, such as anaerobic digesters,
111 microaerophilic wastewater treatment plants, bacterial sulfur mats, intertidal mud flats, soils
112 and plants rizosphere (Gosh et al. 2009). In some species, as *Thiobacillus* spp., *Beggiatoa*
113 *alba* and *Xanthomonas* spp., sulfur globules are produced as a consequence of S^{2-} oxidation,
114 either inside or outside the cells (Kleinjan et al. 2003). Given their ability to produce sulfuric
115 acid (H_2SO_4), SOB can be used to solubilize metals from minerals, a process called
116 bioleaching (Rawlings 2005).
117 The microbial enrichment cultivation based on the 'gradient tubes' was firstly described in
118 1957 by Kucera and Wolfe for the specific isolation of the FeOB *Gallionella* sp.. This
119 technique was modified to extend the specificity to other betaproteobacterial FeOB as
120 *Sideroxydans* sp. and *Leptothrix* sp. (Emerson and Moyer 1997; Weiss et al. 2007; Emerson et
121 al. 2010). These microorganisms are neutrophilic and microaerophilic and grow at the redox
122 interface where O_2 concentration is below 10 μM (Emerson et al. 2010). Despite their
123 recalcitrance in the laboratory, the employment of FeOB for As removal has been taken into
124 account in a variety of applications, such as drinking water biological filtration and

125 groundwater remediation (Pokhrel and Viraraghavan 2009; Katsoyiannis and Zouboulis 2006;
126 Mouchet 1992).
127 Genomic analyses performed on freshwater FeOB (Emerson et al. 2013) evidenced the
128 presence of gene clusters for arsenic detoxification through As(V) reduction composed by
129 *arsRCDA/ACR3* genes and *arsRC/ACR3* in *Gallionella* ES-2 and on *Syderoxidans* ES-1
130 strains, respectively. Arsenite oxidase genes were retrieved from a Fe(II)-oxidizing
131 enrichment culture obtained from As-rich groundwaters (Hassan et al. 2015), but *aioA*
132 sequence homology was related to Alphaproteobacteria and Betaproteobacteria not able to use
133 Fe(II) as energy source. Within SOB, resistance by means of As(III) extrusion with *ACR3* as
134 well as As(III) oxidation is quite common (Giloteaux et al. 2013; Cavalca et al. 2013). In
135 some species, as *Bosea* sp., heterotrophic and autotrophic growth based on the oxidation of
136 S^{2-} and As(III) can be alternated according to the environmental conditions (Walczak et al.
137 2018).
138 In order to clarify the role of rice rhizoplane-inhabiting microorganisms on As dynamics and
139 given the limited knowledge of As toxicity to FeOB and SOB, the objectives of the present
140 work were to (i) describe the rice root-associated FeOB and SOB populations and (ii) assess
141 their susceptibility to As.
142

143 # Material and Methods

144

145 ***Rice Roots Sampling***

146 Fresh roots were obtained from rice plants (*Oryza sativa* subsp. *japonica*, var. Loto)
147 cultivated in open-air macrocosms (3 replicates) under continuous flooding. The soil used for
148 the cultivation was an acidic (pH 6) sandy-loam rice field soil (Pavia, Italy), with total Fe and
149 As concentrations of 33.1 ± 1.04 g kg^{-1} and 11.4 ± 0.74 mg kg^{-1}, respectively. The plants were

sampled at flowering stage, which occurred after 100 days from germination. At this time point, the macrocosms were still flooded.

Immediately after sampling, three plants were pooled in one composite sample for each replicate, according to Somenahally et al. (2011). The rhizosphere soil was removed from roots after shaking (180 *rpm*) in tetrasodium pyrophosphate buffer (0.2%, pH 8.0) for 1 h at 30 °C. The roots were detached from the epigeal portion, washed thoroughly with sterile distilled water, ground to 2-3 cm length fragments and used for inoculation of FeS gradient tubes.

FeS Gradient Tube Enrichment Cultures

Iron-oxidizing bacteria enrichment cultures were performed with the gradient tube method according to Emerson and Moyer (1997). Briefly, sterile glass tubes (16 x 120 mm) with screw caps were filled 2/3 (v/v) with a bottom layer containing 0.75 mL of iron sulfide (FeS) and 0.75 mL of Modified Wolfe Mineral Medium (Hanert et al. 1992) added with 1% agarose, and a top layer with 4.5 mL of the same medium containing 0.15% of agarose. Before sterilization, 0.5 mM $NaHCO_3$ was added to the top layer. For the bottom layer, FeS was prepared adding 46.2 g of ferrous sulfate ($FeSO_4$, Merck KGaA, Darmstadt, Germany) and 39.6 g of sodium sulfide (Na_2S, Sigma-Aldrich, St. Louis, MO, USA) to 300 mL of distilled water at 50 °C under shaking. After 3 min of continuous stirring, the black FeS sludge was decanted into a narrow-mouthed 500 mL dark glass bottle. The bottle was filled to the top with distilled water to limit O_2 influx and capped. To wash FeS, it was allowed to settle for several hours, replacing the overlaying water at least five times. After washing, the pH of FeS solution was 7. The final concentration of FeS in the gradient tubes was 6.5-7 g L^{-1}. After hardening, 1 mL L^{-1} of Wolfe's Vitamin Solution (WVS) (Wolin et al. 1963) was added to the medium. For As amendment, 500 mM stock solutions of $Na_2HAsO_4 \cdot 7H_2O$ and

175 of $NaAsO_2$ (Sigma-Aldrich, St. Louis, MO, USA) were prepared for As(V) and As(III),
176 respectively. In the gradient tubes, As(V) and As(III) was added to a final concentration of 30
177 and 0.03 mg L^{-1}, respectively. The solutions were sterilized with 0.2 µm cellulose-acetate
178 filters (Sartorium Stedim Biotech, Germany) and included in the autoclaved top layer
179 immediately before filling the tubes.
180 In total, three gradient tubes categories were set up: i) gradient tubes not amended with As
181 (GT); ii) amended with As(V) [GT-As(V)]; iii) with As(III) [GT-As(III)]. To compare
182 biological and chemical Fe(II) oxidation, abiotic tubes were prepared for each category.
183 For each enrichment, 1 root fragment was inoculated in the top layer. The tubes were
184 incubated for 20 days at room temperature in the dark. Every 20 days, 100 mg of biomass
185 grown within the orange Fe(II) oxidation band was transferred to a fresh tube. The cultures
186 were set up in triplicate.
187
188 ***Scanning Electron Microscopy***
189 To analyze microorganisms and Fe (hydr)oxides developed in the gradient tubes, the biomass
190 grown within the orange Fe(II) oxidation band was sampled after 3 transfers and observed by
191 Scanning Electron Microscopy (SEM). From each treatment, 1 g of material was suspended in
192 5 mL of 2% glutaraldehyde (dissolved in 1x PBS) for 24 h. Suspensions were centrifuged at
193 10000 g for 5 min and subsequently washed in phosphate-buffered saline (PBS) solution (0.1
194 M, pH 7.2) for 8 h, resuspended in 1% OsO_4 (dissolved in H_2O) for 1 h at room temperature
195 and progressively dehydrated in EtOH from 2% to 100%. After final dehydration in
196 $[(CH_3)_3Si]_2NH$ (HMDS), samples were sputter-covered with gold with High Vacuum Coater
197 (Leica Mycrosystems, Wetzlar, Germany). Observations were performed with a microscope
198 Leo 1430 (Zeiss) equipped with energy-dispersive spectroscopy (EDS) with INCA probe and
199 analyzed at the microscopy facility 'NoLimits' of the University of Milano.
200

16S rRNA Gene Clone Library Preparation and Screening for As-transforming Genes

From 0.25 g of biomass collected within the orange Fe(II) oxidation band after 3 transfers, total DNA was isolated using the UltraClean® Microbial DNA Isolation kit (MO BIO, Carlsbad, USA), according to manufacturer's instructions.

To prepare 16S rRNA gene clone libraries, the target was amplified mixing 0.3 µM of primers GM-3F (5'-AGAGTTTGATCMTGGC-3') and GM-4R (5'-TACCTTGTTACGACTT-3') (Muyzer et al., 1995) with 1X Taq PCR Master Mix (QIAGEN), 20 ng of template DNA and PCR-grade water (Sigma-Aldrich) to a final volume of 25 µL. The thermal protocol was carried out on T-Gradient thermocycler (Biometra, Germany) and included 5 min of denaturation at 95°C, 35 cycles of denaturation for 1 min at 95°C, 40 sec of annealing at 55°C and 1 min and 40 sec of elongation at 72°C, and a final elongation for 10 min at 72°C. The PCR products were cloned on TOP10 chemically competent *E. coli* cells using TOPO® TA Cloning® Kit (Invitrogen) and pCR™2.1-TOPO® vector following manufacturer's instructions. From the positive clones, the plasmid was extracted with UltraClean™ 6 minutes Mini Plasmid Prep Kit (MO BIO).

To cluster the different clones in Operational Taxonomic Units based on their insert, 200 µg of the extracted plasmids were digested over night at 37 °C with 0.5 U of *Hae*III restriction enzyme and 1X REact®2 buffer (Invitrogen) in a total volume of 10 µL. Digestion products were loaded on a 3% agarose gel prepared with Tris-acetate-EDTA (TAE) 1X buffer and separated applying 50 mV for 3 h. Inserts showing the same restriction profile were clustered in unique OTUs and one representative for each OTU was sequenced.

To investigate the capacity of enriched microorganisms to transform As, an attempt to amplify arsenate reductase (*arsC*), arsenite oxidase (*aioA*) and arsenite S-methyltransferase (*arsM*) was carried out using the respective primer couples: ArsC52F (5'-AGCCAAATGGCAGAAGC-3') and ArsC323R (5'-GCTGGRTCRTCAAATCCCCA-3')

according to Bachate et al. (2009); aoxBM1-2F (5'-CCACTTCTGCATCGTGGGNTGYGGNTA-3') and aoxBM2-1R (5'-GGAGTTGTAGGCGGGCCKRTTRTGDAT-3') according to Quéméneur et al. (2008); arsMF1 (5'-TCYCTCGGCTGCGGCAAYCCVAC-3') and arsMR2 (5'-CGWCCGCCWGGCTTWAGYACCCG-5') according to Zecchin et al. (2017a).

Sequence and Community Analysis

Sequences were edited and aligned using MEGA software version 6 (Tamura et al. 2013) and compared to the GenBank database with BLASTn. Clone and reference sequences where aligned with MUSCLE (Edgar 2004) and trees were built using the maximum likelihood method based on the Tamura-Nei model (Tamura et al. 1993).

The alpha diversity within the enrichments was inferred calculating the Shannon-Wiener index and Pielou's evenness (Pielou 1966) using the R software's package Vegan (R Development Core Team 2008, Oksanen et al. 2017).

For each species retrieved in the analyzed gradient tubes, details on the morphology, physiology and As transformation were screened in the available literature and in the related genomes deposited in GenBank.

Accession Numbers

The sequences obtained in this study are deposited in GenBank with accession numbers from MH511579 to MH511605.

Results

FeS Gradient Tubes

Iron-oxidizing enrichments revealed iron oxidation after one week from inoculation, displayed as one/two orange bands formed in the top layer of the gradient tubes (Fig. 1). In the inoculated tubes, a sharp and thick orange band at 2 cm from the top surface could be observed. From this type of band after the third transplant, microbial biomass could be successfully isolated and used for further analyses. In the abiotic tubes, only one band at 4 cm from the top surface was formed, more diffused and lower with respect to that in the inoculated samples. In some inoculated tubes, a second band was present at 3 cm from the top surface. Several attempts were unsuccessfully performed to extract biomass from this band, although bacterial cells were visible under phase contrast microscope and SEM.

Scanning Electron Microscopy

To characterize microorganisms and Fe (hydr)oxides developed in the gradient tubes in the presence and in the absence of As, SEM-EDS analysis was carried out in selected samples after three transplants of enrichment cultures and abiotic controls.

In abiotic samples, amorphous structures were visualized (Fig. 2a), in which Fe was present at low concentrations (Fig. 2b). In GT enrichment cultures, cells with 2 μm diameter with a nano-globules-structured surface were observed (Fig. 3a and 3b). The shape and dimension of these structures is compatible with sulfur globules previously observed in *Thiobacillus* spp. (Kleinjan et al. 2003). The presence of Fe on these structures was confirmed by the EDS analysis (Fig. 3c and 3d, Table 1). In GT-As(V) and GT-As(III) enrichments, irregular crystalline structures enveloping putative round-shaped cells with diameter below 1 μm and nano-filaments were detected (Fig. 4a and 4b). The EDS analysis revealed the presence in the irregular crystals of Fe, C, O and As (Fig. 4c and 4d, Table 1), indicating that microorganisms were enveloped by Fe-oxides As-adsorbed minerals, embedded in exopolymeric substances.

276 ***Bacterial Community enriched in the Gradient Tubes***

277 The addition of As decreased the species diversity and evenness in enrichments GT-As(V)
278 and GT-As(III) with respect to GT, as revealed by the Shannon-Wiener indexes and Pielou's
279 evenness (Fig. 5). From all enrichments, all 16S rRNA genes sequences were classified within
280 the class *Proteobacteria* (Fig. 6). In GT enrichments, bacteria affiliated to *Alpha-*, *Beta-* and
281 *Gammaproteobacteria* were retrieved, whereas in GT-As(V) and GT-As(III) most of the
282 clones belonged to the *Betaproteobacteria*, with only one representative within the
283 *Gammaproteobacteria*. The details concerning the morphology, substrates used as either
284 electron acceptor or donor and presence of As-related genes of the retrieved species are listed
285 in Table 2.

286 In GT, the FeOB *Lysobacter pocheonensis* and *Pseudomonas* spp. and the SOB *Sulfuricella*
287 *denitrificans* were enriched. Together with these species, a number of typically rhizospheric
288 microorganisms were retrieved, some of which known to be able to perform N_2 fixation, such
289 as *Azospirillum* sp. (de Zamaroczy et al. 1989), *Rhizobium* sp. (Crook et al. 2013) and
290 *Pseudomonas* spp. (Li et al. 2017). Two dimorphic prostecate species, *Caulobacter* sp. and
291 *Asticcacaulis taihuensis*, were found, as well as *Achromobacter xylosoxidans*, *Pseudolabrys*
292 sp. and three *Kaistia* sp. strains (Fig. 6 and 7).

293 GT-As(V) was dominated by fewer species, 73% of which belonging to FeOB genera
294 *Azospira, Dechlorosoma* and *Pseudomonas* (Fig. 7). The remaining community was
295 represented by the SOB *Thiobacillus thioparus* and *Massilia timonae*. In GT-As(III)
296 enrichments, only the SOB *T. thioparus* and *M. timonae* were retrieved, none of which is
297 known to oxidize Fe(II) for metabolic purposes.

298 Although all the retrieved species, carry As-transforming or -resistance genes in their genome,
299 with the exception of *Asticcacaulis* sp. (Table 2), none of the targeted As-transforming genes
300 could be amplified from any enrichment.

Discussions

The position of the Fe oxides bands present in inoculated and non-inoculated gradient tubes was consistent with neutrophilic FeOB enrichment cultures from groundwaters performed by Hassan et al. (2015). Arsenic was detected in association with amorphous structures, in both GT-As(V) and GT-As(III) samples, evidencing the co-localization of Fe and As in Fe (hydr)oxides produced by FeOB, likely embedded in organic polymers. In previous studies, the presence of Fe encrustations on microbial cell surface has been reported as mineralized filaments, globules or periplasmic precipitates (Hohmann et al. 2010). In this study, single cells clearly enveloped by Fe encrustations could not be observed. However, within cells covered by putative S globules, Fe was measured by EDS technique. This could indicate a co-precipitation of Fe(II) and S^{2-} excreted by SOB.

The presence of a second lower band putatively indicated anaerobic oxidation of Fe(II), coupled to NO_3 reduction, possibly carried out by *Pseudomonas* sp., *Azospira* sp. and/or *Dechlorosoma* sp. strains.

In GT enrichments, not exposed to As pressure, only *Pseudomonas* spp. and *Lysobacter* sp. were related to species known to oxidize Fe(II) (Table 2), representing 15% of the total community. Together with these, the SOB *Sulfuricella denitrificans* accounted for another 15%. The genus *Pseudolabrys* could potentially contribute to sulfur oxidation, since sulfur oxidizing genes (*sox*) are present in its genome (accession numbers: KQZ00770-1, KQZ00773, KQZ02460; Bay et al. 2015). However, this metabolic capacity has never been tested *in vivo*. The absence of As pressure and CO_2 fixation carried out by FeOB and SOB likely allowed the proliferation of heterotrophic bacteria. Among these, several typically rhizospheric genera were enriched, like *Rhizobium*, *Azospirillum*, and *Kaistia* (Jin et al. 2011). *Caulobacter* and *Asticcacaulis* genera are oligotrophic dimorphic prostecate bacteria tolerant

to prolonged nutrient scarcity; their presence has been reported on Fe and manganese oxides, but whether they produce or just adhere to the minerals remains unknown (Poindexter 2006). The presence of As primed the bacterial populations in GT-As(V) and GT-As(III) gradient tubes and exerted a negative effect on the biodiversity observed in these enrichment lines. Curiously, although most species retrieved in GT were putatively As-resistant (Table 2), none of these survived in the As-added enrichments GT-As(V) and GT-As(III). For example, *P. putida* has been reported to resist to As(V) by reducing it with the arsenate reductase ArsC to As(III), which is then extruded outside the cell using the As(III)-efflux pump encoded by *arsB* gene (Achour et al. 2007, Table 2). Similarly, members of the SOB genus *Azospira* are known to be able to oxidize As(III) (Cavalca et al. 2013). However, these species did not survive in GT-As(III).

Arsenic resistance and transformation are often strain-specific characteristics. In fact, although As-transforming genes are frequently present in the genomes of several microorganisms, these capacities are not always expressed (Cavalca et al. 2013).

No FeOB resisted to As(III) pressure in the GT-As(III) enrichment, whereas *T. thioparus* was the only SOB retrieved in these conditions. This obligate chemolithoautotrophic species, typically found in Italian rice fields (Stubner et al. 1998; Wörner et al. 2016; Zecchin et al. 2017b), uses sulfide (S^{2-}) as electron donor. These microorganisms likely proliferated by the oxidation of S^{2-} present in the FeS bottom layer. The conversion of S^{2-} to SO_4^{2-} could contrast the co-precipitation of As(III) with S^{2-}, maintaining the metalloid in solution and explaining the low biodiversity in the GT-As(III) enrichments. Carbon fixed by the activity of *T. thioparus* might have supported the growth of *M. timonae*, which is an aerobic heterotroph (Table 2). The ability of *M. timonae* to resist to As(V) and to As(III) is suggested by the presence of genes homologous to *ars*C-like gene for As(V) reductase and to *arsB* for As(III) efflux pump (Table 2). Previous studies showed that S^{2-} promotes As(III) oxidation by SOB,

indicating a probable concurrence of these metabolic pathways in the same microorganism (Fisher et al. 2008). In *T. thioparus* an *aio*-like operon is not present. However, only one genome is available for this species despite the high diversity observed at strain level (Boden et al. 2012). In fact, *aioA* genes have been sequenced in other species of the genus *Thiobacillus* (NCBI Acc. no MEFJ01000004). Further investigations would clarify whether the *T. thioparus* strains retrieved in rice rhizoplane have the ability to grow chemolithoautotrophically on As(III) as electron donor, as demonstrated for the purple SOB *Ectothiorhodospira* sp. (Zargar et al. 2012).

The high selective pressure exerted by As(III) in the GT-As(III) enrichments could be explained either by the higher toxicity of As(III) with respect to As(V), but also by a different adaptation of rhizospheric FeOB populations to As species present in the original micro-habitat. In fact, in the close proximity of rice roots, O_2 leakage favors the presence of the metalloid in the form of As(V), which usually co-precipitates within the Fe plaques around the roots (Dixit et al. 2003; Yamaguchi et al. 2014).

Inorganic As species exerted different toxicity on FeOB and SOB inhabiting rice rhizoplane. While SOB were resistant to both As(V) and As(III), FeOB were highly sensitive to As(III). Due to the importance of Fe and S species on As mobility and translocation to the plant, these results contribute to understand microbially-mediated dynamics of As in rice paddies, particularly in the microhabitat surrounding rice roots.

Funding

This work was supported by Ministry of University and Research program PRIN [grant number 2010JBNLJ7-004]; Sarah Zecchin was awarded of a PhD fellowship by the University of Milan.

Declaration of interests

None.

References

Abraham W, Strömpl C, Meyer H, Lindholst S, Moore ERB, Christ R, Vancanneyt M, Tindall BJ, Bennasar A, Smit J, Tesar M. 1999. Phylogeny and polyphasic taxonomy of *Caulobacter* species. Proposal of *Maricaulis* gen. nov. with *Maricaulis maris* (Poindexter) comb. nov. as the type species, and emended description of the genera *Brevundirnonas* and *Caulobacter*. Int J Syst Bacteriol. 49:1053-1073.

Achenbach LA, Michaelidou U, Bruce RA, Fryman J, Coates JD. 2001. *Dechloromonas agitata* gen. nov., sp. nov. and *Dechlorosoma suillum* gen. nov., sp. nov., two novel environmentally dominant (per)chlorate-reducing bacteria and their phylogenetic position. Int J Syst Evol Microbiol. 51:527-533.

Achour AR, Bauda P, Billard P. 2007. Diversity of arsenite transporter genes from arsenic-resistant soil bacteria. Res Microbiol. 158:128-137.

Bachate SP, Cavalca L, Andreoni V. 2009. Arsenic-resistant bacteria isolated from agricultural soils of Bangladesh and characterization of arsenate-reducing strains. J Appl Microbiol. 107:145-156.

Bay Y, Muller DB, Srinivas G, Garrido-Oter R, Potthoff E, Rott M, Dombrowski N, Munch PC, Spaepen S, Remus-Emsermann M, Huttel B, McHardy AC, Vorholt JA, Schulze-Lefert

401 P. 2015. Functional overlap of the *Arabidopsis* leaf and root microbiota. Nature.
402 528(7582):364-369.
403
404 van Berkum P, Beyene D, Bao G, Campbell TA, Eardly BD. 1998. *Rhizobium mongolense* sp.
405 nov. is one of three rhizobial genotypes identified which nodulate and form nitrogen-fixing
406 symbioses with *Medicago ruthenica* [(L.) Ledebour]. Int J Syst Bacteriol. 48:13-22.
407
408 Cavalca L, Corsini A, Zaccheo P, Andreoni V, Muyzer G. 2013. Microbial transformations of
409 arsenic: perspectives for biological removal of arsenic from water. Future Microbiol. 86:753-
410 768.
411
412 Coenye T, Vancanneyt M, Falsen E, Swings J, Vandamme P. 2003. *Achromobacter insolitus*
413 sp. nov. and *Achromobacter spanius* sp. nov., from human clinical samples. Int J Syst Evol
414 Microbiol. 53:1819-1824.
415
416 Colmer TD. 2003. Long-distance transport of gases in plants: a perspective on internal
417 aeration and radial oxygen loss from roots. Plant Cell Environ. 26:17-36.
418
419 Commission E. 2015. Commission regulation (EC) No 2015/1006 of 25 June 2015 amending
420 Regulation (EC) No 1881/2006 as regard maximum levels of inorganic arsenic in foodstuffs.
421 OJ L 161:14–16
422
423 Crook MB, Mitra S, Ané JM, Sadowsky MJ, Gyaneshwar P. 2013. Complete genome
424 sequence of the Sesbania symbiont and rice growth-promoting endophyte *Rhizobium* sp.
425 strain IRBG74. Genome Announc. 1(6):e00934-13.

Dahl C, Friedrich C, Kletzin A. 2008. Sulfur oxidation in prokaryotes. In eLS, (Ed.).

Das S, Chou ML, Jean JS, Liu CC, Yang HJ. 2016. Water management impacts on arsenic behavior and rhizosphere bacterial communities and activities in a rice agro-ecosystem. Sci Total Environ. 542:642-652.

de Zamaroczy M, Delorme, F, Elmerich C. 1989. Regulation of transcription and promoter mapping of the structural genes for nitrogenase (*nifHDK*) of *Azospirillum brasilense* Sp7. Mol Gen Genet. 220(1):88-94.

Dixit S, Hering JG. 2003. Comparison of arsenic(V) and arsenic(III) sorption onto iron oxide minerals: implications for arsenic mobility. Environ Sci Technol. 37(2003): 4182-4189.

Dubinina GA, Sorokina, AY. 2014. Neutrophilic lithotrophic iron-oxidizing prokaryotes and their role in the biogeochemical processes of the iron cycle. Microbiology 83:1-14.

Edgar RC. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 5:113.

Emerson D, Field E, Chertkov O, Davenport K, Goodwin L, Munk C, Nolan M, Woyke T. 2013. Comparative genomics of freshwater Fe-oxidizing bacteria: implications for physiology, ecology, and systematics. Front Microbiol. 4:1-17.

450 Emerson D. 2012. Biogeochemistry and microbiology of microaerobic Fe(II) oxidation.
451 Biochem Soc Trans. 40:1211-1216.
452
453 Emerson D, Fleming EJ, McBeth JM. 2010. Iron-oxidizing bacteria: an environmental and
454 genomic perspective. Ann Rev Microbiol. 64:561-583.
455
456 Emerson D, Moyer C. 1997. Isolation and characterization of novel iron-oxidizing bacteria
457 that grow at circumneutral pH. Appl Environ Microbiol. 63(12):4784-4792.
458
459 European Food Safety Authority, 2010. Scientific opinion of lead in food. EFSA J. 8(4):1570.
460
461 European Food Safety Authority, 2012. Cadmium dietary exposure in the European
462 population. EFSA J. 10(1):2551.
463
464 European Food Safety Authority, 2014. Dietary exposure to inorganic arsenic in the European
465 population. EFSA J. 12:3597.
466
467 Fisher JC, Wallschläger D, Planer-Friedrich B, Hollibaugh JT. 2008. A new role for sulfur in
468 arsenic cycling. Environ Sci Tecnol. 42:81-85.
469
470 Friedrich CG, Bardischewsky F, Rother D, Quentimeier A, Fischer J. 2005. Prokaryotic sulfur
471 oxidation. Curr Opin Microbiol. 8:253-259.
472
473 Giloteaux L, Holmes DE, Williams KH, Wrighton KC, Wilkins MJ, Montgomery AP, Smith
474 JA, Orellana R, Thompson CA, Roper TJ, Long PE, Loveley DR. 2013. Characterization and

transcription of arsenic respiration and resistance genes during in situ uranium bioremediation. ISME J. 7:370-383.

Gosh W, Dam B. 2009. Biochemistry and molecular biology of lithotrophic sulfur oxidation by taxonomically and ecologically diverse bacteria and archaea. FEMS Microbiol Rev. 33:999-1043.

Gray JS, Birmingham JM, Fenton JI. 2010. Got swimming dots in your cell culture? Identification of *Achromobacter* as a novel cell culture contaminant. Biologicals 38(2):273-277.

Hanert, HH. 1992. The Prokaryotes: The genus *Gallionella*. New York: Springer Verlag. pp. 4082-4088.

Hassan Z, Sultana M, Westerhoff HV, Khan SI, Röling WFM. 2015. Iron cycling potentials of arsenc-contaminated groundwater in Bangladesh as revealed by enrichment cultivation. Geomicrobiol J. 33(9):779-792.

Hedrich S, Schlömann M, Johnson DB. 2011. The iron-oxidizing *Proteobacteria*. Microbiology. 157:1551-1564.

Henrici AT, Johnson DE. 1935. Studies of freshwater bacteria: II. Stalked bacteria, a new order of *Schizomycetes* 1. J Bacteriol. 30(1):61.

Hohmann C, Winkler E, Morin G, Kappler A. 2010. Anaerobic Fe(II)-oxidizing bacteria show As resistance and immobilize As during Fe(III) mineral precipitation. Environ Sci Technol. 44:94-101.

Hu Z, Yang Z, Xu C, Haneklaus S, Cao Z, Schnug E. 2002. Effect of crop growth on the distribution and mineralization of soil sulfur fractions in the rhizosphere. J Plant Nutr Soil Sci. 165:249-254.

Hu Z, Zhu Y, Li M, Zhang L, Cao Z, Smith FA. 2007. Sulfur (S)-induced enhancement of iron plaque formation in the rhizosphere reduces arsenic accumulation in rice (*Oryza sativa* L.) seedlings. Environ Pollut. 147:387-393.

Jia Y, Bao P, Zhu Y. 2015. Arsenic bioavailability to rice plant in paddy soil: influence of microbial sulfate reduction. J Soil Sediments. 15:1960-1967.

Jin L, Kim KK, Baek SH, Lee ST. 2011. *Kaistia geumhonensis* sp. nov. and *Kaistia dalseonensis* sp. nov., two members of the class *Alphaproteobacteria*. Int J Syst Evol Microbiol. 61(11):2577-2581.

Joint FAO/WHO Expert Committee on Food Additives, 2010. Joint FAO/WHO Expert Committee on Food Additives Seventy-third Meeting. World Health Organization, Geneva

Kämpfer P, Young CC, Arun AB, Shen FT, Jäckel U, Rossello-Mora R, Lai W, Rekha PD. 2006. *Pseudolabrys taiwanensis* gen. nov., sp. nov., an alphaproteobacterium isolated from soil. Int J Syst Evol Microbiol. 56(10):2469-2472.

524

525 Katsoyiannis IA, Zouboulis AI. 2006. Use of iron- and manganese-oxidizing bacteria for the
526 combined removal of iron, manganese and arsenic from contaminated groundwater. Water
527 Qual Res J Canada. 41(2):117-129.

528

529 Kelly DP, Wood AP. 2000. Confirmation of *Thiobacillus denitrificans* as a species of the
530 genus *Thiobacillus*, in the b-subclass of the *Proteobacteria*, with strain NCIMB 9548 as the
531 type strain. Int J Syst Evol Microbiol. 50:547-550.

532

533 Kleinjan WE, de Keizer A, Janssen A.J.H. 2003. Biologically produced sulfur. Top Curr
534 Chem. 230:167-188.

535

536 Kögel-Knabner I, Amelung W, Cao Z, Fiedler S, Frenzel P, Jahn R, Kalbitz K, Kölbl A,
537 Schloter M. 2010. Biogeochemistry of paddy soils. Geoderma 157:1-14.

538

539 Kojima H, Fukui M. 2010. *Sulfuricella denitrificans* gen. nov., sp. nov., a sulfur-oxidizing
540 autotroph isolated from a freshwater lake. Int J Syst Evol Microbiol. 60(12):2862-2866.

541

542 Kucera S, Wolfe RS. 1957. A selective enrichment method for *Gallionella ferruginea*. J
543 Bacteriol. 74(3):344.

544

545 La Scola B, Birtles RJ, Mallet MN, Raoult D. 1998. *Massilia timonae* gen. nov., sp. nov.,
546 isolated from blood of an immunocompromised patient with cerebellar lesions. J Clin
547 Microbiol. 36(10):2847-2852.

548

549 Li HB, Singh RK, Singh P, Song QQ, Xing YX, Yang LT, Li YR. 2017. Genetic diversity of
550 nitrogen-fixing and plant growth promoting Pseudomonas species isolated from sugarcane
551 rhizosphere. Front Microbiol. 8:1268.
552
553 Liu WJ, Zhu YG, Smith FA. 2005. Effects of iron and manganese plaques on arsenic uptake
554 by rice seedlings (*Oryza sativa* L.) grown in solution culture supplied with arsenate and
555 arsenite. Plant Soil. 277:127-138.
556
557 Ma R, Shen J, Wu J, Tang Z, Shen Q, Zhao FJ. 2014. Impact of agronomic practices on
558 arsenic accumulation and speciation in rice grain. Environ Pollut. 194:217-223.
559
560 Mouchet P. 1992. From conventional to biological removal of iron and manganese in France.
561 J Am Water Works Assoc. 84(4):158-166.
562
563 Muyzer G, Teske A, Wirsen CO, Jannasch HW. 1995. Phylogenetic relationships of
564 *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by
565 denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch Microbiol. 164(3):165-
566 172.
567
568 Neumann G, Veeranagouda Y, Karegoudar TB, Sahin Ö, Mäusezahl I, Kabelitz N,
569 Kappelmeyer U, Heipieper HJ. 2005. Cells of *Pseudomonas putida* and *Enterobacter* sp.
570 adapt to toxic organic compounds by increasing their size. Extremophiles 9(2):163-168.
571

572 Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara
573 RB, Simpson GL, Solymos P, Stevens MHH, Szoecs E, Wagner H. 2017. vegan: Community
574 Ecology Package. https://CRAN.R-project.org/package=vegan
575
576 Oremland RS, Stolz JF. 2005. Arsenic, microbes and contaminated aquifers. Trends
577 Microbiol. 13(2):45-49.
578
579 Pester M, Knorr K, Friedrich MW, Wagner M, Loy A. 2012. Sulfate-reducing
580 microorganisms in wetlands – fameless actors in carbon cycling and climate change. Front
581 Microbiol. 3:1-19.
582
583 Pielou EC. 1966. Shannon's formula as a measure of specific diversity: its use and misuse.
584 Am Nat. 100:463-465.
585
586 Poindexter JS. 1964. Biological properties and classification of the *Caulobacter* group.
587 Bacteriol Rev. 28(3):231.
588
589 Poindexter JS. 2006. The Prokaryotes: Dimorphic Prosthecate Bacteria: The Genera
590 *Caulobacter*, *Asticcacaulis*, *Hyphomicrobium*, *Pedomicrobium*, *Hyphomonas* and
591 *Thiodendron*. New York: Springer. pp. 72-90.
592
593 Pokhrel D, Viraraghavan T. 2009. Biological filtration for removal of arsenic from drinking
594 water. J Environ Manage. 90:1956-1961.
595

596 Quéméneur M, Heinrich-Salmeron A, Muller D, Lièvremont D, Jauzein M, Bertin PN,
597 Garrido F, Joulian C. 2008. Diversity surveys and evolutionary relationships of *aoxB* genes in
598 aerobic arsenite-oxidizing bacteria. Appl Environ Microbiol. 74(14):4567-4573.
599
600 Reinhold-Hurek B, Hurek T. 2000. Reassessment of the taxonomic structure of the
601 diazotrophic genus *Azoarcus* sensu lato and description of three new genera and new species,
602 *Azovibrio restrictus* gen. nov., sp. nov., *Azospira oryzae* gen. nov., sp. nov. and *Azonexus*
603 *fungiphilus* gen. nov., sp. nov. Int J Syst Evol Microbiol. 50(2):649-659.
604
605 Rinklebe J, Shaheen SM, Yu K 2016. Release of As, Ba, Cd, Cu, Pb, and Sr under pre-
606 definite redox conditions in different rice paddy soils originating from the U.S.A. and Asia.
607 Geoderma 270:21-32.
608
609 Rizwan M, Ali S, Adrees M, Rizvi H, Zia-ur-Rehman M, Hannan F, Qayyum MF, Hafeez F,
610 Ok YS 2016. Cadmium stress in rice: toxic effects, tolerance mechanisms and management: a
611 critical review. Environ Sci Pollut Res 23:17859-17879.
612
613 Roberts LC, Hug SJ, Ruettimann T, Billah MM, Khan AW, Rahman MT. 2004. Arsenic
614 removal with iron (II) and iron (III) in waters with high silicate and phosphate concentrations.
615 Environ Sci Technol. 38(1):307-315.
616
617 Seyfferth AL, Webb SM, Andrews JC, Fendorf S. 2010. Arsenic localization, speciation, and
618 co-occurrence with iron on rice (*Oryza sativa* L.) roots having variable Fe coatings. Environ
619 Sci Technol. 44(21):8108-8113.
620

621 Siddiqi MZ, Im WT. 2016. *Lysobacter pocheonensis* sp. nov., isolated from soil of a ginseng
622 field. Arch Microbiol. 198:551-557.
623
624 Somenahally AC, Hollister EB, Loeppert RH, Yan W, Gentry TJ. 2011. Microbial
625 communities in rice rhizosphere altered by intermittent and continuous flooding in fields with
626 long-term arsenic application. Soil Biol Biochem. 43:1220-1228.
627
628 Spanu A, Daga L, Orlandoni AM, Sanna G. 2012. The role of irrigation techniques in arsenic
629 bioaccumulation in rice (*Oryza sativa* L.). Environ Sci Technol. 46:8333-8340.
630
631 Stanier RY, Palleroni NJ, Doudoroff M. 1966. The aerobic pseudomonads a taxonomic study.
632 Microbiol. 43(2):159-271.
633
634 Stubner S, Wind T, Conrad R. 1998. Sulfur oxidation in rice field soil: activity, enumeration,
635 isolation and characterization of thiosulfate-oxidizing bacteria. System Appl Microbiol.
636 21:569-578.
637
638 Sullivan RF, Holtman MA, Zylstra GJ, White JF, Kobayashi DY. 2003. Taxonomic
639 positioning of two biological control agents for plant diseases as *Lysobacter enzymogenes*
640 based on phylogenetic analysis of 16S rDNA, fatty acid composition and phenotypic
641 characteristics. J Appl Microbiol. 94(6):1079-1086.
642
643 Takahashi Y, Minamikawa R, Hattori KH, Kurishima K, Kihou N, Yuita, K. 2004. Arsenic
644 behavior in paddy fields during the cycle of flooded and non-flooded periods. Environ Sci
645 Technol. 38:1038-1044.

Tamura K, Nei M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol. 10:512-526.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 30:2725-2729.

Vasilyeva LV, Omelchenko MV, Berestovskaya YY, Lysenko AM, Abraham WR, Dedysh SN, Zavarzin GA. 2006. *Asticcacaulis benevestitus* sp. nov., a psychrotolerant, dimorphic, prosthecate bacterium from tundra wetland soil. Int J Syst Evol Microbiol. 56(9):2083-2088.

Walczak AB, Yee N, Young LY. 2018. Draft genome of *Bosea* sp. WAO an arsenite and sulfide oxidizer isolated from a pyrite rock outcrop in New Jersey. Stand Genomic Sci. 13:6.

Weiss JV, Emerson D, Backer SM, Megonigal JP. 2003. Enumeration of Fe (II)-oxidizing and Fe (III)-reducing bacteria in the root zone of wetland plants: implications for a rhizosphere iron cycle. Biogeochemistry 64(1):77-96.

Weiss JV, Rentz JA, Plaia T, Neubauer SC, Merrill-Floyd M, Lilburn T, Bradburne C, Megonigal JP, Emerson D. 2007. Characterization of neutrophilic Fe(II)-oxidizing bacteria isolated from the rhizosphere of wetland plants and description of *Ferritrophicum radicicola* gen. nov. sp. nov., and *Sideroxydans paludicola* sp. nov. Geomicrobiol J. 24:559-570.

Wolin EA, Wolin M, Wolfe RS. 1963. Formation of methane by bacterial extracts. J Biol Chem. 238(8):2882-2886.

Wörner S, Zecchin S, Dan J, Hristova Todorova N, Loy A, Conrad R, Pester M. 2016. Gypsum amendment to rice paddy soil stimulated bacteria involved in sulfur cycle but largely preserved the phylogenetic composition of the total bacterial community. Environ Microbiol Rep. 8(3):413-423.

Xie CH, Yokota A. 2005. *Azospirillum oryzae* sp. nov., a nitrogen-fixing bacterium isolated from the roots of the rice plant *Oryza sativa*. Int J Syst Evolut Microbiol. 55(4):1435-1438.

Yabuuchi E, Kawamura Y, Kosako Y, Ezaki T. 1998. Emendation of genus *Achromobacter* and *Achromobacter xylosoxidans* (Yabuuchi and Yano) and proposal of *Achromobacter ruhlandii* (Packer and Vishniac) comb. nov., *Achromobacter piechaudii* (Kiredjian et al.) comb. nov., and *Achromobacter xylosoxidans* subsp. *denitrificans* (Rüger and Tan) comb. nov. Microbiol Immunol. 42(6):429-438.

Yamaguchi N, Ohkura T, Takahashi Y, Maejima Y, Arao T. 2014. Arsenic distribution and speciation near rice roots influenced by iron plaques and redox conditions of the soil matrix. Environ Sci Technol. 48:1549-1556.

Ye X, Li H, Zhang L, Chai R, Tu R, Gao H 2018. Amendment damages the function of continuous flooding in decreasing Cd and Pb uptake by rice in acid paddy soil. Ecotoxicol Environ Saf. 147:708-714.

694 Zargar K, Conrad A, Bernick DL, Lowe TM, Stolc V, Hoeft S, Oremland RS, Stolz J,
695 Saltikov CW. 2012. ArxA, a new clade of arsenite oxidase within the DMSO reductase family
696 of molybdenum oxidoreductase. Environ Microbiol. 14(7):1635-1645.
697
698 Zecchin S, Corsini A, Martin M, Romani M, Beone GM, Zanchi R, Zanzo E, Tenni D,
699 Fontanella MC, Cavalca L. 2017 a. Rhizospheric iron and arsenic bacteria affected by water
700 regime: implications for metalloid uptake by rice. Soil Biol Biochem. 106:129-137.
701
702 Zecchin S, Corsini A, Martin M, Cavalca L. 2017 b. Influence of water management on the
703 active root-associated microbiota involved in arsenic, iron, and sulfur cycles in rice paddies.
704 Appl Microbiol Biotechnol. 101:6725-6738.
705
706 Zhao FJ, Ma JF, Meharg AA, McGrath SP. 2009. Arsenic uptake and metabolism in plants.
707 New Phytol. 181:777-794.
708
709 Zhu YG, Williams PN, Meharg AA. 2008. Exposure to inorganic arsenic from rice: a global
710 health issue? Environ Pollut. 154:169-171.
711
712
713
714
715
716
717
718

Table 1 Content of C, O, Fe and As in the EDS spectra shown in Fig. 2, 3 and 4.

Element	*		1		2		3		4	
	Weight %	**Atomic %**	**Weight %**	**Atomic %**	**Weight %**	**Atomic %**	**Weight %**	**Atomic %**	**Weight %**	**Atomic %**
C	63.07	70.81	64.27	72.64	63.02	71.20	28.9	43.00	35.17	48.10
O	31.13	27.08	28.57	24.24	31.39	26.62	42.34	47.15	44.20	45.37
Fe	1.15	0.28	1.89	0.46	2.68	0.65	25.33	8.08	18.17	5.34
As	-	-	-	-	-	-	1.01	0.24	0.86	0.19

742

743

744

745

746 **Table 2** Main phenotypic and physiologic features reported in the cited literature of the taxa

747 found in the enrichment cultures.

Species	Shape	Size	E^- acceptor	E^- donor	As-related genes*	Reference
Asticcacaulis taihuensis	Rod	0.5-0.7 x 1.4-2.0 µm	O_2, $NO_3^{-\dagger}$	Sugars, starch	-	Vasilyeva et al., 2006 Liu et al., 2005
Caulobacter sp.	Vibrioid, fusiform, rod	0.4-0.5 x 1-2 µm	O_2, NO_3^-	Sugars, aminoacids	*arsC*; *arsH*; *aioA*; *aioB*	Henrici and Johnson, 1935 Poindexter, 1964 Abraham et al., 1999
Azospirillum sp.	Spiral, vibrioid	1.0 x 1.5-5 µm	O_2, NO_3^-	Sugars	*arsC*; *arsH*; *ACR3*	Xie and Yokota, 2005
Kaistia sp.	Rod, coccoid	0.6-1 µm	O_2, NO_3^-	Sugars, aminoacids, $CH_3CO_2^-$, small organic acids	*arsB*; *arsC*	Jin et al., 2011
Pseudolabrys sp.	Rod	-	O_2, NO_3^-	$CH_3CO_2^-$, aconitate, small organic acids, S(?)	*ACR3*	Kämpfer et al., 2006 Bay et al., 2015
Rhizobium sp.	Rod	1 µm	O_2	Small sugars and organic acids, $CH_3CO_2^-$	*arsB*; *arsC*; *arsH*	van Berkum et al., 1998
Achromobacter sp.	Rod, coccoid	1-5 µm	O_2, NO_3^-	Sugars, small organic acids, H_2	*arsB*; *arsC*	Yabuuchi et al., 1998 Coenye et al., 2003 Gray et al., 2010
Massilia timonae	Straight rod	1-3 µm	O_2	Sugars, aminoacids, small organic acids	*arsB*; *arsC*; *arsH*	La Scola et al., 1998
Sulfuricella	Rod	0.8-2.0 x 0.4-	O_2, NO_3^-, NO_2^-	$S_2O_3^{2-}$, S	*arsB*; *arsC; ACR3*	Kojima and Fukui,

denitrificans		0.6 µm				2010
Thiobacillus thioparus	Rod	1.0-2.0 µm	O_2	S^{2-}, SCN^-	*arsC, arsB*	Kelly and Wood, 2000
Azospira sp.	Curved rod	0.4-0.6 µm	O_2, NO_3^-, ClO_4^-	Small organic acids, Fe(II), As(III)	*arsC*	Reinhold-Hurek and Hurek, 2000 Dubinina and Sorokina, 2014
Dechlorosoma sp.	Rod	1.0 x 0.3 µm	O_2, NO_3^-, ClO_4^-	Short organic fatty acids, Fe(II)	*ACR3*	Achenbach et al., 2001 Dubinina and Sorokina, 2014
Pseudomonas sp.	Rod	0.9-1.5 µm	O_2, NO_3^-	Sugars, organic acids, aminocids, aromatic compounds, Fe(II)	*arsB*; *arsC*	Stanier et al., 1966, Neumann et al., 2005, Dubinina and Sorokina, 2014
Lysobacter sp.	Rod	0.3-0.4 x 2.5-5 µm	O_2	Sugars, organic acids, aminocids, Fe(II)	*arsC*; *arsH*	Emerson and Moyer, 1997 Sullivan et al., 2003, Siddiqi and Im, 2016

†NO_3^-=nitrate; NO_2^-=nitrite; ClO_4^-=perchlorate; $CH_3CO_2^-$=acetate; H_2= hydrogen; $S_2O_3^{2-}$=thiosulfate; S=elemental sulfur; SCN^-=thiocyanate.
*Genomes screened for the presence of As-related genes: *Achromobacter xylosoxidans* (accession number CP012046); *Pseudomonas putida* (CP024086); *Massilia timonae* (CP029343); *Thiobacillus thioparus* (SAMN02331034); *Azospira* sp. (NZ_BFBP00000000); *Dechlorosoma* sp. (CP003153); *Sulfuricella denitrificans* (AP013066); *Rhizobium* sp. (AM236080 and CP000133); *Pseudolabrys* sp. (LMFS01000000); *Caulobacter* sp. (CP002008); *Azospirillum* sp. (AP010946); *Kaistia* sp. (PSNV01000000).

765

766

767

768

Figure captions

770

771 **Figure 1** Iron oxidizing bacteria enrichment cultures. From the left: abiotic control, second
772 transfer of GT-As(III) and GT-As(V), third transfers of GT-As(III), GT-As(V) and GT, first
773 transfers of GT-As(III), GT-As(V) and GT.

774

775 **Figure 2** Scanning Electron Microscopy photograph at 1200 magnifications on a portion of
776 the orange Fe(II)-oxidation band sampled from an abiotic gradient tube (a) and EDS spectrum
777 (b) performed in the point indicated by the star.

778

779 **Figure 3** Images obtained with SEM performed on a portion of the orange Fe(II)-oxidation
780 band sampled from GT enrichment cultures at 7500 (a) and 17000 (b) magnifications, and
781 related EDS spectra (c, d).

782

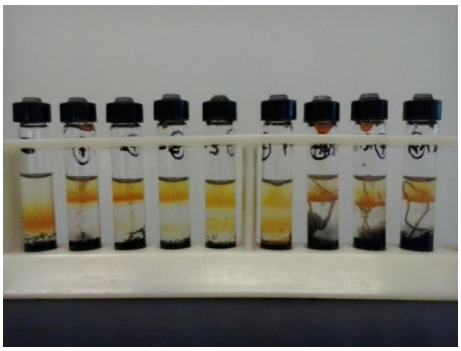
783 **Figure 4** Images obtained with SEM performed on a portion of the orange Fe(II)-oxidation
784 band sampled from GT-As(V) enrichment cultures at 5300 (a) and 10000 (b) magnifications,
785 and related EDS spectra (c, d).

786

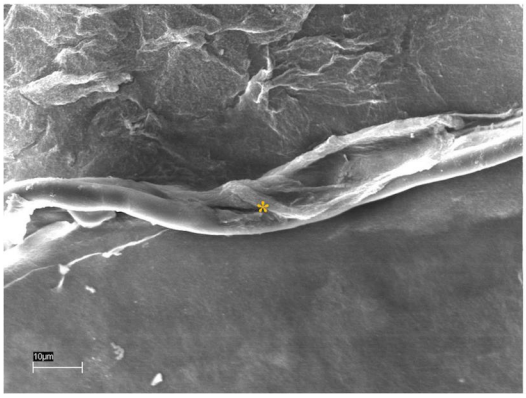
787 **Figure 5** Alpha diversity in GT, GT-As(V) and GT-As(III) enrichment cultures as a measure
788 of Shannon-Wiener indices and Pielou's evenness (Pielou 1966).

789

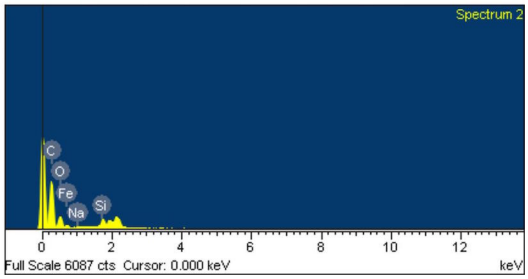
790 **Figure 6** Phylogenetic affiliation of the 16S rRNA gene clones obtained from GT, GT-As(V)
791 and GT-As(III) enrichment cultures. The evolutionary history was inferred with the
792 Maximum Likelihood method based on the Tamura-Nei model (Tamura et al., 1993). The tree
793 is drawn to scale, with branch lengths measured in the number of substitutions per site.
794 *Microcoleus vaginatus* strain CCALA 152 (KC633969), *Bacillus cellulosolyticus*
795 (AB043852) and *Chlorobium ferrooxidans* (Y18253) were used as outgroup.
796
797 **Figure 7** Relative abundance of the *Proteobacteria* species retrieved in the 16S rRNA gene
798 clone libraries obtained from GT, GT-As(V) and GT-As(III) enrichment cultures.



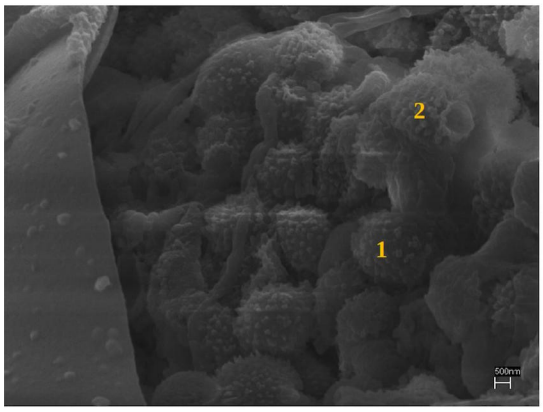
a.


10µm

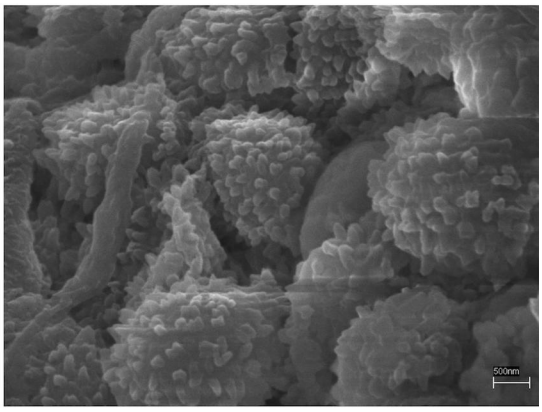
b.


Spectrum 2
C
O
Fe
Na
Si
0
2
4
6
8
10
12
Full Scale 6087 cts Cursor: 0.000 keV
keV

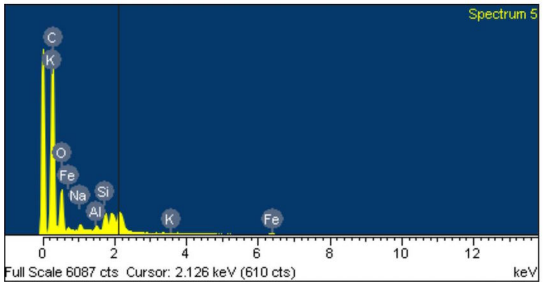
a.


2
1
500nm

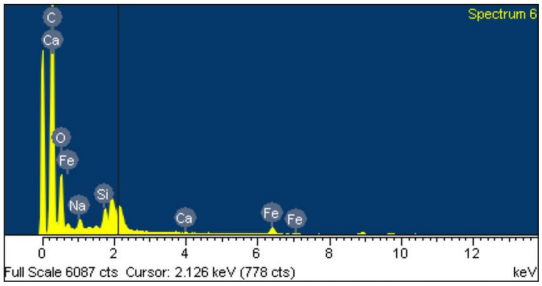
b.


500nm

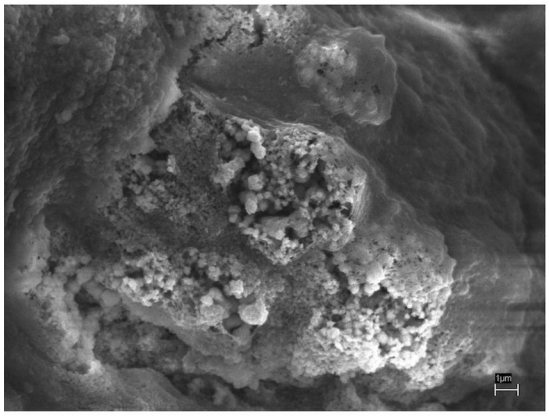
c.


Spectrum 5
C
K
O
Fe
Na
Si
Al
K
Fe
0
2
4
6
8
10
12
Full Scale 6087 cts Cursor: 2.126 keV (610 cts)
keV

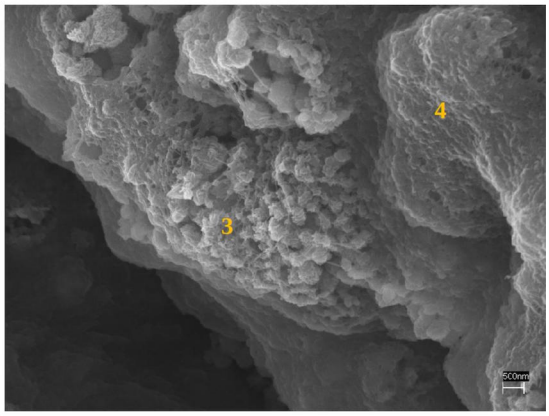
d.


Spectrum 6
C
Ca
O
Fe
Na
Si
Ca
Fe
Fe
0
2
4
6
8
10
12
Full Scale 6087 cts Cursor: 2.126 keV (778 cts)
keV

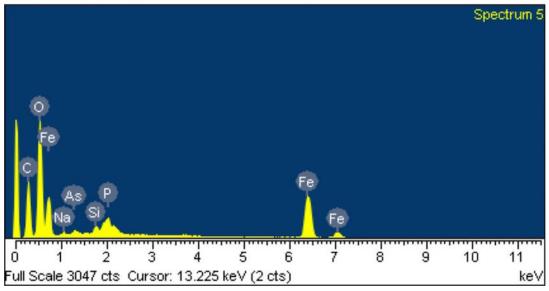
a.


1µm

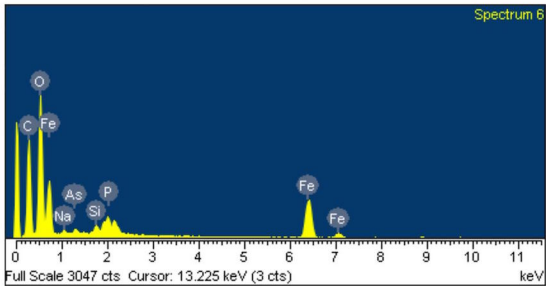
b.

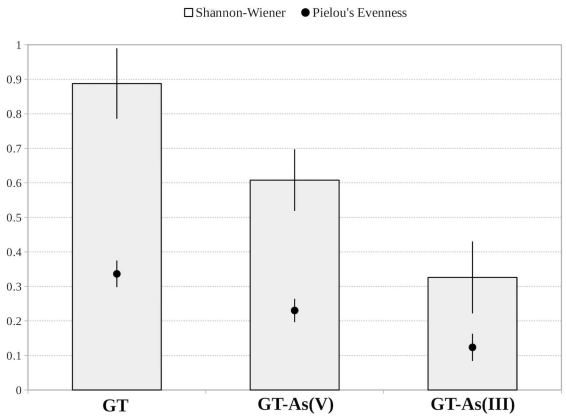

4
3
500nm

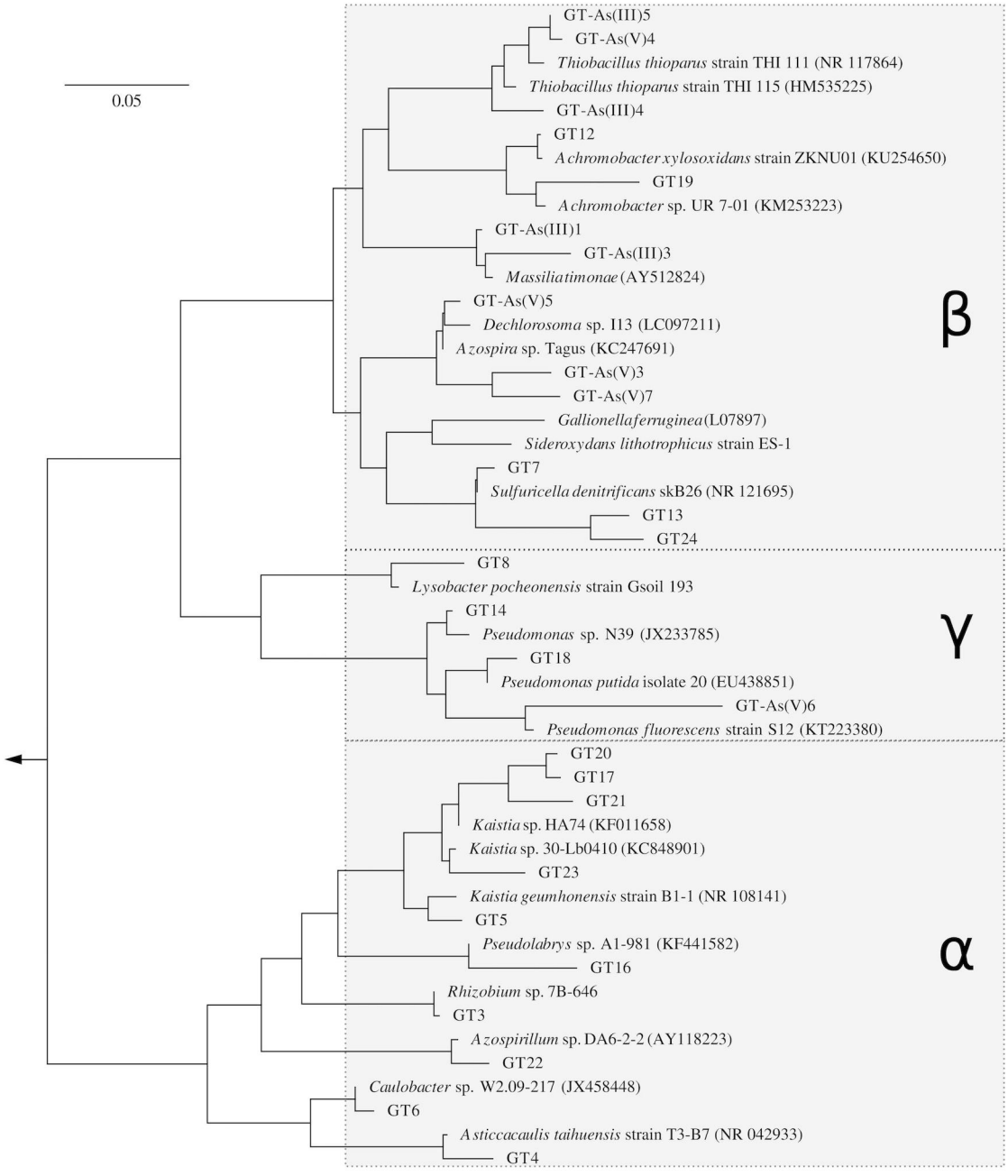
c.

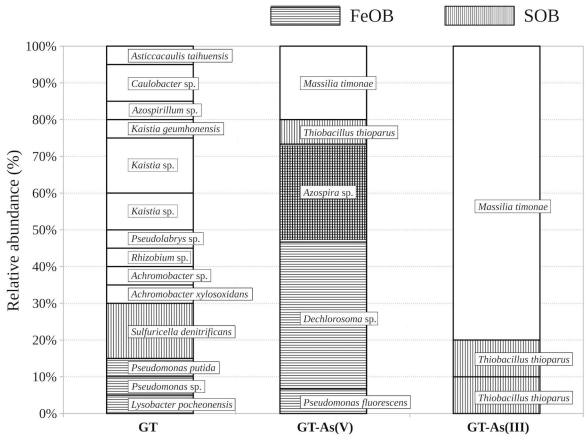

Spectrum 5
O
Fe
C
As
P
Na
Si
Fe
Fe
0 1 2 3 4 5 6 7 8 9 10 11
Full Scale 3047 cts Cursor: 13.225 keV (2 cts)
keV

d.


Spectrum 6
O
C
Fe
As
P
Na
Si
Fe
Fe
0 1 2 3 4 5 6 7 8 9 10 11
Full Scale 3047 cts Cursor: 13.225 keV (3 cts)
keV


Shannon-Wiener
Pielou's Evenness
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
GT
GT-As(V)
GT-As(III)


0.05
GT-As(III)5
GT-As(V)4
Thiobacillus thioparus strain THI 111 (NR 117864)
Thiobacillus thioparus strain THI 115 (HM535225)
GT-As(III)4
GT12
Achromobacter xylosoxidans strain ZKNU01 (KU254650)
GT19
Achromobacter sp. UR 7-01 (KM253223)
GT-As(III)1
GT-As(III)3
Massiliatimonae (AY512824)
GT-As(V)5
Dechlorosoma sp. I13 (LC097211)
Azospira sp. Tagus (KC247691)
GT-As(V)3
GT-As(V)7
Gallionella ferruginea (L07897)
Sideroxydans lithotrophicus strain ES-1
GT7
Sulfuricella denitrificans skB26 (NR 121695)
GT13
GT24
β
GT8
Lysobacter pocheonensis strain Gsoil 193
GT14
Pseudomonas sp. N39 (JX233785)
GT18
Pseudomonas putida isolate 20 (EU438851)
GT-As(V)6
Pseudomonas fluorescens strain S12 (KT223380)
γ
GT20
GT17
GT21
Kaistia sp. HA74 (KF011658)
Kaistia sp. 30-Lb0410 (KC848901)
GT23
Kaistia geumhonensis strain B1-1 (NR 108141)
GT5
Pseudolabrys sp. A1-981 (KF441582)
GT16
Rhizobium sp. 7B-646
GT3
Azospirillum sp. DA6-2-2 (AY118223)
GT22
Caulobacter sp. W2.09-217 (JX458448)
GT6
Asticcacaulis taihuensis strain T3-B7 (NR 042933)
GT4
α


FeOB
SOB
Relative abundance (%)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Asticcacaulis taihuensis
Caulobacter sp.
Azospirillum sp.
Kaistia geumhonensis
Kaistia sp.
Kaistia sp.
Pseudolabrys sp.
Rhizobium sp.
Achromobacter sp.
Achromobacter xylosoxidans
Sulfuricella denitrificans
Pseudomonas putida
Pseudomonas sp.
Lysobacter pocheonensis
Massilia timonae
Thiobacillus thioparus
Azospira sp.
Dechlorosoma sp.
Pseudomonas fluorescens
Massilia timonae
Thiobacillus thioparus
Thiobacillus thioparus
GT
GT-As(V)
GT-As(III)