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Simple or complex organic substrates inhibit arsenite oxidation and *aioA* gene expression in two β -*Proteobacteria* strains

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1	Simple or complex organic substrates inhibit arsenite oxidation and aioA gene
2	expression in two β- <i>Proteobacteria</i> strains
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4	Tiffanie Lescure ^{a,b} , Catherine Joulian ^a , Clément Charles ^a , Taoikal Ben Ali Saanda ^a , Mickael
5	Charron ^a , Dominique Breeze ^a , Pascale Bauda ^c , Fabienne Battaglia-Brunet ^{a,b*}
6	
7	^a BRGM, 3 avenue Claude Guillemin, 45060 Orléans, France
8	^b BRGM, ISTO, UMR 7327, BP 36009, 45060 Orléans, France
9	^c LIEC UMR 7360 CNRS-Université de Lorraine, Campus Bridoux, rue du Général
10	Delestraint, 57070 Metz, France
11	*Corresponding author: f.battaglia@brgm.fr, (+33)2.38.64.39.30
12	
13	E-mail addresses: tiffanielescure@gmail.com (T. Lescure), c.joulian@brgm.fr (C. Joulian),
14	clement_cm45@yahoo.fr (Charles C.), taoikalali@hotmail.fr (Ben Ali Saanda T.),
15	m.charron@brgm.fr (Charron M.), d.breeze@brgm.fr (Breeze D.), pascale.bauda@univ-
16	lorraine.fr (Bauda P.), <u>f.battaglia@brgm.fr</u> (Battaglia-Brunet F., *correspondence and
17	reprints)
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28 Abstract

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Microbial transformation of arsenic species and their interaction with the carbon cycle play a 29 major role in the mobility of this toxic metalloid in the environment. The influence of simple 30 or complex organic substrates on arsenic bio-oxidation was studied using two bacterial 31 strains: one - the arsenivorans strain of Thiomonas delicata - is able to use AsIII as sole 32 energy source; the other, Herminiimonas arsenicoxydans, is not. Experiments were performed 33 at two AsIII concentrations (75 and 2 mg/L). At 75 mg/L As, for both strains, expression of 34 aioA gene decreased when yeast extract concentration was raised from 0.2 to 1 g/L. At 2 35 mg/L As, the presence of either yeast extract or simple (succinate or acetate) organic 36 substrates in the medium during bacterial growth decreased the AsIII-oxidation rate by both 37 38 strains. When added specifically during oxidation test, yeast extract but not simple organic substrates seems to have a negative effect on AsIII oxidation. Taken together, results confirm 39 the negative influence of simple or complex organic substrates on the kinetics of microbial 40 AsIII oxidation and suggest that this effect results from different mechanisms depending on 41 the type of organic substrate. Further, for the first time, the influence of a complex organic 42 43 substrate, yeast extract, on *aioA* gene expression has been evidenced.

44

45 Keywords: *Betaproteobacteria* strains; organic substrates; arsenite oxidation; *aioA* gene
46 expression

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1. Introduction

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High concentrations of the toxic metalloid arsenic (As) in soils may be the result of pollution from mining, industrial activities or agricultural practices, or they may be due to the geochemical background. In the case of mining and metallurgical activities, As is released as a by-product in the waste (tailings) or from drainage galleries [1].

Biological arsenite (AsIII) oxidation processes, coupled to iron oxidation and precipitation, can be used advantageously for effective bioremediation of mine water [1,2] and, where soils are concerned, to attenuate the environment toxicity, since arsenate (AsV) is less toxic than AsIII. This oxidizing reaction is of significant ecological importance as it leads to the stabilization of arsenic in the environment since AsV is more readily adsorbed than AsIII by carrier phases such as iron or manganese oxides [3,4].

Bacteria able to oxidize AsIII and reduce AsV have been found in many terrestrial and aquatic 60 environments [5-10]. Some bacteria possess genes allowing oxidation as well as genes 61 conferring the ability to reduce AsV to AsIII via the Ars resistance system. Although this 62 AsV-reduction detoxification intracellular reaction is energy consuming, it is widely found in 63 microbial communities because AsIII thus obtained is excreted out of the cell, or is 64 sequestered as reduced glutathione (GSH) or other thiol conjugates, for example in yeast [11]. 65 Another way in which bacteria derive energy from arsenic is via the dissimilatory reduction of 66 AsV into AsIII by anaerobic respiration via the Arr system, using AsV as a final electron 67 acceptor [12]. The AsIII oxidation function in bacteria is encoded by the *aio* operon [13], 68 which comprises up to six genes (aioB, aioA, aioS, aioR, aioX and aioE). Two of them encode 69 the two AsIII oxidase enzyme subunits: aioB, which encodes the small subunit containing the 70 Rieske [2Fe-2S] cluster; and *aioA*, which encodes the large subunit containing a catalytic 71 molybdopterin and a cluster [3Fe-4S]. These two genes were originally identified and isolated 72 from two β -proteobacteria: Alcaligenes faecalis [14] and Herminiimonas arsenicoxydans 73

[15]. The other four *aio* genes correspond to the following functions: *aioS* encodes a histidine 74 kinase sensor, *aioR* a transcriptional regulator, *aioX* an oxyanions-ligand protein, and *aioE* an 75 AsIII oxidation electron transporter [16]. Santini and van den Hoven [17] studied a 76 chemolithoautotrophic bacterium, strain NT26 that derives energy from AsIII oxidation but is 77 not able to grow on minimal medium in the presence of arsenic when its *aioA* gene is 78 inactivated. This experiment demonstrated the use of arsenic as an energy source by some 79 bacteria. Many other bacteria have been identified as carrying genes encoding AsIII oxidase 80 This is the case for the "Thiomonas (T.) arsenivorans" strain, a [18. 19]. 81 chemolithoautotrophic, acid-tolerant and mobile Gram-negative rod [5,20]. It has been proven 82 elsewhere that this bacterium, by oxidizing AsIII into AsV, can both create energy and 83 detoxify the environment [5]. Some publications suggest a very significant effect of organic 84 substrates on bacterial AsIII oxidation in the presence of oxygen. Challan-Belval et al. [21] 85 86 have shown that addition of yeast extract substantially reduces the rate of AsIII oxidation by bacteria organized in biofilms. Bachate et al. [22] observed a decrease in enzymatic AsIII-87 oxidase activity of two pure strains as the total concentration of organic substrates (acetate 88 and yeast extract) in the growth medium increased. Lescure et al. [23] observed a negative 89 effect of organic substrates input, with concentrations from 0.08 to 0.4 g of carbon/L, 90 corresponding to 0.2 to 1 g/L yeast extract, on the AsIII-oxidation rate constant of soil 91 microbial communities. It therefore appears that organic substrates slow down the 92 transformation of AsIII into AsV by bacteria in aerated conditions, and may decrease the 93 retention of As in the environment. Nandre et al. [24] showed that the presence of acetate in 94 the growth medium induced a lower *aio*A gene expression in diverse bacterial strains isolated 95 from tannery effluent and soils. Organic substrates may inhibit the aio encoded oxidation 96 processes, because bacteria would preferentially metabolize simple or complex organic 97 substrates providing more energy than AsIII. Organic substrates may also induce a decrease 98

in AsIII bioavailability by complexation reactions [25]. Because of these complexation
reactions, if AsIII oxidation was mainly or exclusively a detoxification mechanism, the
presence of a high concentration of organic substrate may have a protective effect against
AsIII, inducing an attenuation of the detoxification process.

However, to date, the influence of the concentration and nature of different organic substrateson biological AsIII oxidation has not been studied systematically.

The objective of this work was to study the influence of two types of organic substrates, 105 complex or simple organic substrates, on the ability to oxidize AsIII of two β -proteobacteria 106 strains presenting different metabolisms. One of these, a strain of Thiomonas delicata referred 107 AsIII arsenivorans" uses as energetic 108 to as *"T*. _ substrate: the other. Herminiimonas (H.) arsenicoxydans, does not. The hypothesis to be tested trough this 109 experiment was that bacterial AsIII oxidation could be inhibited differently by organic 110 111 substrates when AsIII is used or not as energy source. A common complex organic substrate was chosen: yeast extract, a reference complex organic substrate in laboratory studies that can 112 promote the growth of both strains. In parallel, because the strains do not use any common 113 simple organic substrate, two simple organic substrates were selected; acetate for H. 114 arsenicoxydans and succinate for "T. arsenivorans". Two sets of experiments were carried 115 out. First, AsIII oxidation and *aioA* gene expression were studied in batch growth experiments 116 with a relatively high concentration of arsenic allowing strain growth with AsIII as sole 117 energy source: 75 mg.L⁻¹ (or 1 mM) AsIII. Then, AsIII oxidation kinetics were determined at 118 an initial AsIII concentration more compatible with the concentrations commonly found in the 119 pore water of polluted soils $(0.5-2 \text{ mg.L}^{-1} \text{ AsIII}, [26])$. 120

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124 **2. Materials and methods**

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126 2.1.Bacterial strains

Two AsIII-oxidizing microorganisms carrying an *aioA* gene were selected as
models; "*Thiomonas arsenivorans*" (DSM 16361), a mixotrophic and optional autotrophic
strain able to grow on AsIII and using only the AsIII oxidation reaction as an energy source; *Herminiimonas arsenicoxydans* (DSM 17148), a heterotrophic strain perfectly characterized
[27].

These two strains belong to the subdivision of β -*Proteobacteria*. "*T. arsenivorans*" was isolated by BRGM [5]. *H. arsenicoxydans* [28] was kindly provided for this study by the laboratory of Molecular Genetics, Genomics, Microbiology (GMGM) of Louis Pasteur University (Strasbourg, France), where the strain was isolated.

136

137 2.2. Culture media

"T. arsenivorans" was maintained in minimal CAsO1 medium [29] with 100 mg.L⁻¹ AsIII,
and *H. arsenicoxydans*, which is a heterotrophic bacterium, in the Luria-Bertani (LB)
medium, then sub-cultured in CAsO1 medium amended with 1 g.L⁻¹ yeast extract before each
experiment.

The CAsO1 liquid medium is a mixture of 500 mL of each of two solutions. Solution A contains 0.5 g KH₂PO₄, 0.5 g K₂HPO₄, 0.5 g NaCl, 0.05 g (NH₄)₂SO₄ and 1 mL of trace elements in solution [29]. The pH of this solution was adjusted to 6.0 before autoclaving for subsequent growth of "*T. arsenivorans*", and to 7.2 for growth of *H. arsenicoxydans*. Solution B (500 mL) contains 0.1 g CaCl₂ and 0.1 g MgSO₄. Both solutions were autoclaved separately and mixed after cooling. 10 mL of a solution of 10 g.L⁻¹ AsIII (As₂O₃), filtered at 0.22 μ m, were added to the culture medium. A concentrated solution of 50 g/L yeast extract was prepared, filtered at 0.22 μ m and then stored at 4°C.

150 2.3. aioA gene expression with 75 mg. L^{-1} AsIII

The aim of the first experiment was to determine the influence of a complex organic substrate, yeast extract, on the expression of the *aioA* gene during the growth of the bacteria, in the presence of 1 mM AsIII, a concentration that provides enough energy for the growth of *"Thiomonas arsenivorans"* in batch conditions [5].

"T. arsenivorans" inoculum was cultivated in 20 mL of CAsO1 medium (pH 6.0) containing 155 75 mg.L⁻¹ AsIII without organic substrate, in a cottoned 40 mL glass bottle. The inoculum of 156 H. arsenicoxydans was prepared in CAsO1 medium (pH 7.2) containing 75 mg.L⁻¹ AsIII and 157 supplemented with 1 g.L⁻¹ yeast extract. Two CAsO1 culture media containing, respectively, 158 0.2 and 1 g.L⁻¹ yeast extract were inoculated, at pH 7.2 for *H. arsenicoxydans* and pH 6.0 for 159 "T. arsenivorans". All flasks were incubated aerobically at 25°C under stirring. At t=0 and 160 then twice per day, bacterial growth was monitored by enumerating bacterial cells with a 161 162 Thoma cell under an optical microscope and by absorbance measurements at 620 nm. 1.5 mL of each culture were sampled in a 2 mL sterile microtube and centrifuged for 10 min at 163 5,000 x g. Pellets were frozen in liquid nitrogen and stored at -80°C until RNA/DNA co-164 extraction. In parallel, 2 mL samples were filtered at 0.22 microns in sterile flasks and kept at 165 4°C until further AsV analysis. Abiotic controls prepared in the same conditions showed that 166 AsIII was not oxidized abiotically [23]. 167

168

169 2.4. AsIII oxidation kinetics

The aim of the second experiment was to quantify the influence of simple and complex organic substrates on the AsIII-oxidation rate when concentrations of AsIII are so low that the kinetics must be carried out over a short time, i.e. less than one hour. For this purpose, a

standardized cell concentration was used, allowing easier comparison of the results obtained 173 in the different conditions. Acetate was used for *H. arsenicoxydans* as it is the only simple 174 organic substrate used as the sole source of carbon and energy by this strain [27]. For "T. 175 arsenivorans", which does not use acetate, succinate was chosen, since it is a simple organic 176 substrate used efficiently by this strain [5]. Yeast extract was chosen as a complex organic 177 substrate used by both strains, allowing comparison of results with a common substrate. Each 178 strain was sub-cultured (1% volume) into 100 mL of CAsO1 medium containing 2 mg.L⁻¹ of 179 AsIII at the appropriate pH (pH 6.0 for "T. arsenivorans", pH 7.2 for H. arsenicoxydans) and 180 different concentrations of organic substrate: 0.05, 0.2 or 1 g.L⁻¹ yeast extract for both strains; 181 0.05, 0.2 or 1 g.L^{-1} of succinate for "T. arsenivorans" or acetate for H. arsenicoxydans. 182 Incubations were carried out aerobically for 3 days at 25°C under static conditions, to avoid 183 the formation of cell aggregates (oxygen was not limiting). For all of the sub-culture 184 conditions (0.05, 0.2 and 1 g.L^{-1} of organic substrates), two conditions were compared in 185 triplicate to determine AsIII oxidation kinetics: the liquid phase of the bacterial suspension 186 that oxidized 2 mg/L AsIII was either deprived of organic substrate, or contained 0.2 g.L⁻¹ of 187 organic substrate. 188

Appropriate sub-culture volumes were sampled at the end of the growth phase in order to adjust the final cell densities in the suspensions to values close to 2×10^7 cells.mL⁻¹. After centrifugation (30 minutes at 8,500 x g), pellets were washed with 20 mL of arsenic-free CAsO1 medium. After a second centrifugation (30 minutes at 8,500 x g), pellets were resuspended in 130 mL of arsenic-free CAsO1 medium, with or without 0.2 g.L⁻¹ of organic substrate. 20 mL of the resulting bacterial suspensions were distributed in 60 mL sterile cottoned flasks.

At t=0, 400 μ L of 100 mg.L⁻¹ AsIII solution were added to the flasks to obtain a concentration of 2 mg.L⁻¹ AsIII, mixed immediately and sampled for bacterial cells counting and for arsenic

speciation. The flasks were incubated at 25°C, under static conditions, and AsIII oxidation 198 was monitored over 45 minutes, with 2.5 mL-sampling every 15 minutes. The samples were 199 filtered at 0.2 µm in sterile microtubes immediately after sampling and were promptly diluted 200 tenfold in sterile ultrapure water and stored at 4°C for subsequent As speciation. Blank 201 experiments were prepared in the same conditions without cells. The specific AsIII-oxidation 202 rate was calculated as the ratio of linear AsIII oxidation over 45 minutes and the cell 203 concentration measured at the start of the experiment (this concentration did not significantly 204 vary during the 45 minutes of the experiment). 205

206

207 2.5 Speciation and quantification of arsenic

AsIII and AsV were separated using resins (AG® 1-X8 Resin, Cat. # 140-1431, Biorad) as described in [2], and a fraction of the sample remaining in the tube was used for total As quantification. Quantification of As in tubes containing AsV, AsIII and total As was carried out by electrothermal atomic absorption spectrophotometry (Varian SpectrAA 220Z).

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213 2.6. RNA and DNA co-extraction and cDNA synthesis

RNA and DNA co-extractions were performed on cell pellets stored at -80°C, using the 214 NucleoSpin® RNA II kit (Macherey-Nagel) in accordance with the manufacturer's 215 recommendations, including a separate DNA elution step with the NucleoSpin® DNA/RNA 216 Buffer Set (Macherey-Nagel). The column was then placed on a new 1.5 mL collection tube 217 and the manufacturer's protocol was continued with the digestion of DNA followed by RNA 218 elution. An additional digestion step was needed to remove residual DNA from the RNA 219 extract. To that end, extracted RNA was mixed with 7 μ L of buffer and 1 μ L of Turbo DNase 220 (2 U; Ambion, ThermoScientific) and then incubated at 37°C for 10 min. Finally, a new RNA 221 purification step was performed with the NucleoSpin® RNA II kit (Macherey-Nagel). The 222

RNA was finally eluted in 60 µL and assayed by BioPhotometer spectrophotometer 223 (Eppendorf). The quality of extracted RNA was checked on 1% agarose gel (25 min at 100 V) 224 and by calculation of the A260/A230 and A260/A280 ratios. They were stored in LoBind 225 microtubes (Eppendorf), at -80°C until the reverse-transcription PCR (RT-PCR) step. The 226 reaction mixture used for the RT-PCR step consisted of 1 µL iScriptTM reverse transcriptase 227 (BioRad), a variable volume of RNA extract (so as to obtain 50 ng per tube), 4 µL 5X TM 228 iScript reaction mix (BioRad), and ultrapure nuclease-free water (qsp 20 µL). RT-PCR 229 consisted of 5 min at 25°C, 30 min at 46°C, 5 min at 85°C and hold at 15°C. 230

231

232 2.7. aioA gene and transcripts quantification by qPCR

The aioA gene involved in AsIII oxidation and its transcripts were quantified by quantitative 233 real-time PCR (qPCR), respectively from DNAs and cDNAs obtained from cultures of "T. 234 arsenivorans" and H. arsenicoxydans. qPCR was carried out using m4-1F forward 235 (GCCGGCGGGGGGNTWYGARRAYA) m2-1R reverse 236 and (GGAGTTGTAGGCGGGCCKRTTRTGDAT) primers, as designed in [30] and applied in 237 [31]. The expected product size was 110 bp. Each primer was used at a concentration of 238 0.3 µM, with 100 ng of T4GP32 (MP Biomedicals), in 1X IQ SYBR Green Supermix 239 (BioRad) and a final volume of 20 µL. The program was run on a CFX Connect (BioRad) and 240 consisted of an initial denaturation at 95°C for 3 min, followed by 50 cycles of 95°C for 10 s. 241 54°C for 20 s, 72°C for 10 s, and a data acquisition step at 80°C for 30 s. At the end, a 242 melting curve analysis was performed by measurement of the SYBR Green I signal intensities 243 during a 0.5°C temperature increment every 10 s, from 65°C to 95°C. Negative template 244controls confirmed the absence of contaminant DNA. Six-point serial decimal dilution of a 245 linearized plasmid carrying the H. arsenicoxidans aioA gene was used to generate a linear 246 calibration curve of threshold cycle versus a number of gene copies ranging from 10^1 to 10^6 . 247

All measurements were run in duplicates. Results were expressed as ratios of *aioA* transcripts relative to the *aioA* gene (*aioA* mRNA/DNA ratio).

250

251 2.8. Statistical analysis

252 Statistical analysis were performed with XLSTAT 2019: normality of the data was first 253 verified using the tests of Shapiro-Wilk, Anderson-Darling, Lilliefors and Jarque-Bera. Then, 254 significance of differences between results obtained at different substrate concentrations was 255 evaluated using an Anova parametric test. Differences between modalities were analysed with 256 a confidence interval of 95% using the methods of Tukey and Dunnett.

257

258 **3. Results**

259 3.1. Expression of aioA gene during bacterial growth in the presence of 75 mg. L^{-1} AsIII

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Arsenite oxidation activity in batch conditions was monitored together with the detection and 260 quantification of *aioA* gene expression and growth of the pure strains. Fig. 1A. shows that 261 expression of the *aioA* gene by "*T. arsenivorans*" was mainly detectable during early growth 262 (Fig. 1C). Maximum aioA gene expression was measured at 24 h, corresponding to about 263 60 mg.L^{-1} , i.e. 80% of oxidized AsIII (Fig. 1E), and tended to be higher in the presence of 264 0.2 g.L⁻¹ than 1 g.L⁻¹ of yeast extract (average *aioA* mRNA/DNA ratios were, respectively, 265 0.032 and 0.0015 in the presence of 0.2 g.L⁻¹ and 1 g.L⁻¹ yeast extract). However, due to a 266 large variability between replicates, the difference observed between ratios obtained at 0.2 267 and 1 g/L yeast extract was not statistically significant. Expression of the *aioA* gene for 268 H. arsenicoxydans was detectable throughout the duration of the experiment (Fig. 1B). As for 269 "T. arsenivorans", maximum expression was detected in the early growth (Fig. 1D) and AsIII 270 oxidation phase (t=5 h), and corresponds to 20-30 mg.L⁻¹ (26-40%) of oxidized AsIII (Fig. 271

1F). Here, in the maximum *aioA* gene expression phase, expression was significantly greater in the presence of 0.2 g.L⁻¹ than 1 g.L⁻¹ of yeast extract (at t=5h, average *aioA* mRNA/DNA ratios were 1.6 x 10^{-3} and 3.3 x 10^{-4} in the presence of 0.2 g.L⁻¹ and 1 g.L⁻¹ of yeast extract, respectively).

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For *T. arsenivorans*, AsV concentrations were always similar whatever the concentrations of yeast extract (Fig 1E), whereas for *H. arsenicoxydans*, AsV concentration was slightly higher with 1 g.L⁻¹ yeast extract at t=5h (Fig. 1F). No significant AsV oxidation was observed in sterile media (Fig. 1G). For both strains, bacterial growth was higher at 1 g.L⁻¹ than at 0.2 g.L⁻¹ ¹ of yeast extract (Fig. 1C and 1D). Consequently, the lower the yeast extract concentration, the higher the specific AsIII oxidation.

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284 3.2. Oxidation kinetics with 2 mg.L^{-1} AsIII

Cells at the end of the growth phase, that grew at different concentrations of a simple organic 285 substrate or yeast extract oxidized AsIII in a minimum medium containing 2 mg.L⁻¹ of AsIII 286 and no organic substrate (Fig. 2) (cell growth was negligible throughout the 45 min duration 287 of the AsIII-oxidation kinetics). In the blank experiments without cells, AsV concentration 288 remained lower than the quantification limit. Examination of the kinetics shows that oxidation 289 of AsIII was more efficient with cells that grew on yeast extract (Fig. 2B and 2D) than on 290 simple substrates (Fig. 2A and 2C), and more efficient with "T. arsenivorans" (Fig. 2A and 291 2B) than with H. arsenicoxydans (Fig. 2C and 2D). H. arsenicoxydans did not grow well on 292 acetate, thus the rates obtained with this strain and a simple substrate were very low (Fig. 2C). 293 The results expressed as specific oxidation rates (Fig. 3) confirm these observations: for both 294 strains, a significant decrease of specific AsIII oxidation rates was observed when yeast 295 extract concentration in the growth medium increased (Fig. 3A). The same trend was 296

observed for "*T. arsenivorans*" grown on succinate as a simple organic substrate (Fig. 3B). *H. arsenicoxydans* did not grow well in the presence of acetate, although the trend is less clear. However, for this strain, the specific AsIII oxidation rate was lower at 1 g.L^{-1} (5.6.10⁻¹¹ μ g/cell/min) than at 0.05 g.L⁻¹ of acetate (2.10⁻¹⁰ μ g/cell/min).

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These specific rates were compared with those obtained when AsIII-oxidation kinetics were determined in the presence of organic substrates (Fig. 4 and Table 1). A negative effect of the addition of yeast extract was observed systematically for both strains, whatever the preculture condition: rates decreased by 13.6% to 39.1% for "*T. arsenivorans*" and by 10% to 25.9% for *H. arsenicoxydans*. In contrast, no effect on AsIII-oxidation rates was detected with simple organic substrates (acetate or succinate).

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310 **4. Discussion**

A negative effect of organic substrates on bacterial AsIII-oxidation activity was observed with 311 two pure strains presenting contrasting metabolisms, one able to use AsIII as an energy source 312 ("T. arsenivorans"), the other not (H. arsenicoxidans). This study has shown, for the first 313 time, the negative effect of a complex organic substrate, yeast extract, on the expression of the 314 aioA gene encoding the catalytic subunit of the AsIII-oxidase in the Aio system. So far, the 315 main factor studied influencing the expression of the aioA gene was the presence and 316 concentration of arsenic III or V. Expression of the *aioA* gene is induced by the presence of 317 As in most AsIII-oxidizing bacteria [32] including several strains belonging to the *Thiomonas* 318 genus [33]. However, in some strains, *aio*A gene expression is also observed in the absence of 319 arsenic: Thiomonas sp. 3AS [7], Ralstonia sp. 22 [34] and Agrobacterium tumefaciens 5A 320 strain, for which AsIII oxidation seems regulated by quorum-sensing [35], and for some 321

14

strains of *Pseudomonas* [36, 37]. In *H. arsenicoxydans*, the expression of genes in the *aio* operon is related to the presence of AsIII and to the expression of genes involved in motility [38]. Our results are in accordance with those of Nandre et al. [24], who observed a higher expression of *aio*A genes under acetate starvation condition in seven strains belonging to different genera, for one single time of incubation (8 h), in presence of 1.33 mM of As. Here, the phenomenon was observed with a complex organic substrate (yeast extract) and correlated with early growth phase.

The specific AsIII-oxidation rates obtained as part of this study are of the same order of magnitude (Table 2) as the V_{max} of AsIII oxidation determined for *Variovorax* sp. 24 [39] and *Agrobacterium albertimagni* AOL15 [40].

The specific oxidation rate was lower when cells had been grown with a high substrate 332 concentration. Several authors have observed that simple or complex organic substrates 333 influence the activity of AsIII-oxidizing bacteria. Santini et al. [41] reported that the AsIII-334 oxidizing strain NT-26, able to use AsIII for energy, showed strong AsIII-oxidase activity 335 when it was grown without any organic substrate, the addition of yeast extract resulting in a 336 decrease in AsIII-oxidizing activity. Challan-Belval et al. [21] observed that AsIII oxidation 337 by a bacterial consortium containing "T. arsenivorans", grown as biofilm in microplate 338 experiments with pozzolana, was drastically decreased in the presence of yeast extract, 339 dropping from 100% to 34%. This result was explained by the supply of nutrients present in 340 yeast extract, including electron donors other than AsIII. Bachate et al. [22] studied the 341 influence of various parameters (temperature, pH and growth medium) on AsIII-oxidation 342 rate by bacterial cells washed and re-suspended in 75 mg. L^{-1} AsIII-containing medium. They 343 followed AsIII oxidation by these cells for 1 hour. The strains were heterotrophic bacteria, 344 phylogenetically related to the genera Bordetella and Achromobacter. These authors observed 345 that the growth conditions have an effect on AsIII-oxidation rate: the latter was maximal 346

when the strains were cultured with only 0.4 g.L⁻¹ yeast extract, decreased by 25 to 50% with 347 1 g.L⁻¹ yeast extract, and by 85% with 0.4 g.L⁻¹ of yeast extract and 20 mM (1.18 g.L⁻¹) of 348 acetate. They also observed a decrease in the enzymatic AsIII-oxidase activity of both strains 349 with the increase of total organic substrate concentration in the growth medium. The present 350 results are consistent with those of Bachate et al. [22], although the studied strains belong to 351 different genera and come from very different environments: "T. arsenivorans" and H. 352 arsenicoxydans were isolated from a mining site and a wastewater treatment plant 353 respectively, whereas the origin of strains of Bachate and al. [22] was a garden soil. 354

Here, bacterial specific AsIII-oxidation rate was adversely affected for both strains by the 355 presence of simple or complex organic substrates in growth medium. This phenomenon was 356 observed both at relatively high AsIII concentrations (75 mg.L⁻¹) and at the lower 357 concentrations of arsenic (2 mg.L⁻¹) commonly detected in groundwater or soil pore water on 358 polluted sites. This negative effect on AsIII oxidation was observed with a complex organic 359 substrate (yeast extract), as well as with at less one simple organic substrate that do not 360 provide nitrogen, i.e. succinate, suggesting that the observed phenomenon is not linked to 361 nitrogen metabolism but rather to energy or carbon source. Thus for both "T. arsenivorans", a 362 mixotrophic strain using AsIII as energy source, and *H. arsenicoxydans*, a heterotrophic 363 strain, arsenic oxidation and metabolism of organic substrates seem to be linked. 364

Several hypotheses may explain the negative effect of organic substrates on *aio*A gene expression and the bacterial specific AsIII-oxidation rate. Nandre et al. [24] hypothesized cross protection in the carbon starvation stress wherein nutrient deprived cells show higher resistance against other stresses. However, this phenomenon could hardly explain results obtained with bacteria such as "*T. arsenivorans*" usually growing in mineral or oligotrophic media. In bacteria using AsIII as an energy source, such as NT-26 or "*T. arsenivorans*", the phenomenon could be related to competition between AsIII and organic substrates as an

energy source: in the presence of high energy-providing organic molecules, cells do not have to acquire energy from the AsIII oxidation reaction, and therefore express the *aio*A gene more weakly. In bacteria unable to gain energy from AsIII oxidation (like *H. arsenicoxydans*) and in which the ability to oxidize AsIII can be a detoxification mechanism [41], the presence of a high concentration of organic substrate may have a protective effect against AsIII.

Another hypothesis would be linked to complexation reactions that may occur between As 377 and some compounds present in complex organic substrates, and thus reduce AsIII toxicity 378 and/or bioavailability. Here, the results of experiments at 2 mg. L^{-1} As suggest that the 379 complex organic substrate (i.e. yeast extract) has a negative effect on AsIII oxidation, 380 considering the comparison of kinetics with cells in suspension in solution with or without 381 yeast extract. This phenomenon may be related to the complexation of AsIII with certain 382 components of yeast extract such as thiol groups of amino acids that would make As less 383 384 bioavailable. These complexation reactions would not occur with simple organic substrates. Direct binding of AsIII to cysteine residues of proteins has been observed [43]. Another 385 possible explanation of the negative effect of simple or complex organic substrates on AsIII 386 oxidation may be that the availability of energy sources would favor the AsV-reducing Ars 387 arsenic resistance system [12] because this resistance process consumes energy. Regarding 388 the Ars resistance system, most studies have focused on arsB and ACR3 genes carriers 389 [35,44,45] whose expression was shown to be controlled by the concentration of arsenic and 390 phosphate. Further studies should focus on determination of the effect of organic substrates on 391 AsIII complexation, and on the expression of the *arsC* gene encoding an AsV reductase in the 392 Ars resistance system, which, in parallel with the Aio system, could influence the balance of 393 speciation of inorganic arsenic in aerated or microaerophilic environments. 394

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396 **Conflict of interest**

397 The authors declare that there is no conflict of interest regarding the publication of this article.

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548 Legends to figures
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Figure 1. Expression of *aio*A gene represented as the *aio*A mRNA/DNA ratio during growth of *T. arsenivorans* (A) and *H. arsenicoxydans* (B) in the presence of 75 mg.L⁻¹ AsIII. ; Evolution of cells concentrations of *T. arsenivorans* (C) and *H. arsenicoxydans* (D) during this experiment. Evolution of AsV concentration with *T. arsenivorans* (E) and *H. arsenicoxydans* (F) during this experiment, and in sterile culture media incubated in the same condition (G). Significance of differences between modalities 0.2 and 1 g.L⁻¹ yeast extract was evaluated using an Anova parametric test (details in material and methods section).

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Figure 2. Kinetics of AsIII oxidation in minimum medium containing 2 mg/L AsIII and no organic substrate. Cells were previously grown in presence of 0.05, 0.2 or 1 g/L of organic substrate. A: *"T. arsenivorans"* on succinate; B: *"T. arsenivorans"* on yeast extract; C: H. arsenicoxydans on acetate; D: H. arsenicoxydans on yeast extract. Error bars represent the standard error of the mean (triplicates).

Figure 3. Specific AsIII-oxidation rates of kinetics of AsIII oxidation in minimum medium containing 2 mg/L AsIII and no organic substrate; A: *T. arsenivorans* and *H. arsenicoxydans* grown in presence of yeast extract; B: *T. arsenivorans* grown in presence of succinate. Significance of differences between modalities was evaluated using an Anova parametric test (details in material and methods section).

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Figure 4. Kinetics of AsIII oxidation in minimum medium containing 2 mg/L AsIII and 0.2
g/L of organic substrate. Cells were previously grown in presence of 0.05, 0.2 or 1 g/L of
organic substrate. A: *"T. arsenivorans"* on succinate; B: *"T. arsenivorans"* on yeast extract;
C: *H. arsenicoxydans* on acetate; D: *H. arsenicoxydans* on yeast extract. Error bars represent
the standard error of the mean (triplicates).

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Table 1. Influence of the presence of 0.2 g.L^{-1} organic substrate on specific AsIII oxidation rate during the kinetics: variation of AsIII oxidation rates between 0 and 0.2 g.L⁻¹ organic substrates conditions, with pre-cultures grown under different conditions. Significance of variation was tested by ANOVA parametric test.

Strain	Pre-culture conditions	Variation (%) of AsIII oxidation rates between 0 and 0.2 g/L organic substrates during the kinetics					
T. arsenivorans	0.05 g/L yeast extract	-13.6					
T. arsenivorans	0.2 g/L yeast extract	-39.1					
T. arsenivorans	1 g/L yeast extract	-17.7					
T. arsenivorans	0.05 g/L succinate	+7.3					
T. arsenivorans	0.2 g/L succinate	+3.0 (NS)					
T. arsenivorans	1 g/L succinate	+26.3 (NS)					
H. arsenicoxydans	0.05 g/L yeast extract	-25.9					
H. arsenicoxydans	0.2 g/L yeast extract	-17.9					
H. arsenicoxydans	1 g/L y east extract	-10					
H. arsenicoxydans	0.05 g/L acetate	-11.5 (NS)					
H. arsenicoxydans	0.2 g/L acetate	NI					
H. arsenicoxydans	1 g/L acetate	+106.1					
NI: not identified (not observable AsIII oxidation)							
NS: not significant							

Negative	Organic substrate (0.2 g/L) within the kinetics medium has a negative effect on AsIII
effect	oxidation rate
Positive	Organic substrate (0.2 g/L) within the kinetics medium has a positive effect on AsIII
effect	oxidation rate

Table 2.	Comparison	of the	specific	oxidation	rates	obtained	in	the	present	study	with	rates
reported	in the literatu	re										

Bacterial strains	Specific rate (µg/L/min per cell)	References			
Comamonas sp. ASR11	$3.6 \ge 10^{-11}$	[46]			
Pseudomonas sp. ASR1	7.35 x 10 ⁻¹¹	[46]			
Variovorax sp. 34	V max 8.85 x 10 ⁻¹¹	[38]			
H. arsenicoxydans	0.5 to 9.5 x 10^{-10}	Present study			
Agrobacterium albertimagni AOL15	V max $1.36 \ge 10^{-10}$	[39]			
T. arsenivorans	1 to 3 x 10^{-9}	Present study			
Bordetella sp. SPB-24	V max 1.46 x 10 ⁻⁶	[22]			
Variovorax sp. MM-1	V max 9.23 x 10 ⁻⁶	[47]			







