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# Journal Pre-proof



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1 **Simple or complex organic substrates inhibit arsenite oxidation and *aioA* gene**  
2 **expression in two  $\beta$ -*Proteobacteria* strains**

3

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28 **Abstract**

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

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

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

60 Bacteria able to oxidize AsIII and reduce AsV have been found in many terrestrial and aquatic  
61 environments [5-10]. Some bacteria possess genes allowing oxidation as well as genes  
62 conferring the ability to reduce AsV to AsIII via the Ars resistance system. Although this  
63 AsV-reduction detoxification intracellular reaction is energy consuming, it is widely found in  
64 microbial communities because AsIII thus obtained is excreted out of the cell, or is  
65 sequestered as reduced glutathione (GSH) or other thiol conjugates, for example in yeast [11].

66 Another way in which bacteria derive energy from arsenic is via the dissimilatory reduction of  
67 AsV into AsIII by anaerobic respiration via the Arr system, using AsV as a final electron  
68 acceptor [12]. The AsIII oxidation function in bacteria is encoded by the *aio* operon [13],  
69 which comprises up to six genes (*aioB*, *aioA*, *aioS*, *aioR*, *aioX* and *aioE*). Two of them encode  
70 the two AsIII oxidase enzyme subunits: *aioB*, which encodes the small subunit containing the  
71 Rieske [2Fe-2S] cluster; and *aioA*, which encodes the large subunit containing a catalytic  
72 molybdopterin and a cluster [3Fe-4S]. These two genes were originally identified and isolated  
73 from two  $\beta$ -proteobacteria: *Alcaligenes faecalis* [14] and *Herminiimonas arsenicoxydans*

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

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

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

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

121

122

123

## 2. Materials and methods

### 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  $\beta$ -*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.

### 2.2. Culture media

"*T. arsenivorans*" was maintained in minimal CAsO1 medium [29] with 100 mg.L<sup>-1</sup> 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<sup>-1</sup> 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 KH<sub>2</sub>PO<sub>4</sub>, 0.5 g K<sub>2</sub>HPO<sub>4</sub>, 0.5 g NaCl, 0.05 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 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 CaCl<sub>2</sub> and 0.1 g MgSO<sub>4</sub>. Both solutions were autoclaved separately and mixed after cooling. 10 mL of a solution of 10 g.L<sup>-1</sup> AsIII (As<sub>2</sub>O<sub>3</sub>), filtered at 0.22  $\mu$ m,



148 were added to the culture medium. A concentrated solution of 50 g/L yeast extract was  
149 prepared, filtered at 0.22  $\mu\text{m}$  and then stored at 4°C.

### 150 2.3. *aioA* gene expression with 75 mg.L<sup>-1</sup> AsIII

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

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

168

### 169 2.4. AsIII oxidation kinetics

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

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

189 Appropriate sub-culture volumes were sampled at the end of the growth phase in order to  
190 adjust the final cell densities in the suspensions to values close to 2 x 10<sup>7</sup> cells.mL<sup>-1</sup>. After  
191 centrifugation (30 minutes at 8,500 x g), pellets were washed with 20 mL of arsenic-free  
192 CAsO1 medium. After a second centrifugation (30 minutes at 8,500 x g), pellets were re-  
193 suspended in 130 mL of arsenic-free CAsO1 medium, with or without 0.2 g.L<sup>-1</sup> of organic  
194 substrate. 20 mL of the resulting bacterial suspensions were distributed in 60 mL sterile  
195 cottoned flasks.

196 At t=0, 400 µL of 100 mg.L<sup>-1</sup> AsIII solution were added to the flasks to obtain a concentration  
197 of 2 mg.L<sup>-1</sup> AsIII, mixed immediately and sampled for bacterial cells counting and for arsenic

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

206

### 207 *2.5 Speciation and quantification of arsenic*

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

212

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

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

223 RNA was finally eluted in 60  $\mu$ L and assayed by BioPhotometer spectrophotometer  
224 (Eppendorf). The quality of extracted RNA was checked on 1% agarose gel (25 min at 100 V)  
225 and by calculation of the  $A_{260}/A_{230}$  and  $A_{260}/A_{280}$  ratios. They were stored in LoBind  
226 microtubes (Eppendorf), at  $-80^{\circ}\text{C}$  until the reverse-transcription PCR (RT-PCR) step. The  
227 reaction mixture used for the RT-PCR step consisted of 1  $\mu$ L iScript<sup>TM</sup> reverse transcriptase  
228 (BioRad), a variable volume of RNA extract (so as to obtain 50 ng per tube), 4  $\mu$ L 5X<sup>TM</sup>  
229 iScript reaction mix (BioRad), and ultrapure nuclease-free water (qsp 20  $\mu$ L). RT-PCR  
230 consisted of 5 min at  $25^{\circ}\text{C}$ , 30 min at  $46^{\circ}\text{C}$ , 5 min at  $85^{\circ}\text{C}$  and hold at  $15^{\circ}\text{C}$ .

231

### 232 2.7. *aioA* gene and transcripts quantification by qPCR

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

248 All measurements were run in duplicates. Results were expressed as ratios of *aioA* transcripts  
249 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<sup>-1</sup> AsIII

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

272 1F). Here, in the maximum *aioA* gene expression phase, expression was significantly greater  
273 in the presence of 0.2 g.L<sup>-1</sup> than 1 g.L<sup>-1</sup> of yeast extract (at t=5h, average *aioA* mRNA/DNA  
274 ratios were 1.6 x 10<sup>-3</sup> and 3.3 x 10<sup>-4</sup> in the presence of 0.2 g.L<sup>-1</sup> and 1 g.L<sup>-1</sup> of yeast extract,  
275 respectively).

276

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

283

### 284 3.2. Oxidation kinetics with 2 mg.L<sup>-1</sup> AsIII

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

297 observed for “*T. arsenivorans*” grown on succinate as a simple organic substrate (Fig. 3B). *H.*  
298 *arsenicoxydans* did not grow well in the presence of acetate, although the trend is less clear.  
299 However, for this strain, the specific AsIII oxidation rate was lower at 1 g.L<sup>-1</sup> (5.6.10<sup>-11</sup>  
300 μg/cell/min) than at 0.05 g.L<sup>-1</sup> of acetate (2.10<sup>-10</sup> μg/cell/min).

301  
302 These specific rates were compared with those obtained when AsIII-oxidation kinetics were  
303 determined in the presence of organic substrates (Fig. 4 and Table 1). A negative effect of the  
304 addition of yeast extract was observed systematically for both strains, whatever the pre-  
305 culture condition: rates decreased by 13.6% to 39.1% for “*T. arsenivorans*” and by 10% to  
306 25.9% for *H. arsenicoxydans*. In contrast, no effect on AsIII-oxidation rates was detected with  
307 simple organic substrates (acetate or succinate).

308

309

#### 310 4. Discussion

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

322 strains of *Pseudomonas* [36, 37]. In *H. arsenicoxydans*, the expression of genes in the *aio*  
323 operon is related to the presence of AsIII and to the expression of genes involved in motility  
324 [38]. Our results are in accordance with those of Nandre et al. [24], who observed a higher  
325 expression of *aioA* genes under acetate starvation condition in seven strains belonging to  
326 different genera, for one single time of incubation (8 h), in presence of 1.33 mM of As. Here,  
327 the phenomenon was observed with a complex organic substrate (yeast extract) and correlated  
328 with early growth phase.

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

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



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

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

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

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

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

395

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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399

#### 400 **Aknowledgements**

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405 *arsenicoxydans* strain.

406

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547

#### 548 **Legends to figures**

549

550 Figure 1. Expression of *aioA* gene represented as the *aioA* mRNA/DNA ratio during growth  
551 of *T. arsenivorans* (A) and *H. arsenicoxydans* (B) in the presence of  $75 \text{ mg.L}^{-1}$  AsIII. ;  
552 Evolution of cells concentrations of *T. arsenivorans* (C) and *H. arsenicoxydans* (D) during  
553 this experiment. Evolution of AsV concentration with *T. arsenivorans* (E) and *H.*  
554 *arsenicoxydans* (F) during this experiment, and in sterile culture media incubated in the same  
555 condition (G). Significance of differences between modalities  $0.2$  and  $1 \text{ g.L}^{-1}$  yeast extract  
556 was evaluated using an Anova parametric test (details in material and methods section).

557

558

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

564

565  
566 Figure 3. Specific AsIII-oxidation rates of kinetics of AsIII oxidation in minimum medium  
567 containing 2 mg/L AsIII and no organic substrate; A: *T. arsenivorans* and *H. arsenicoxydans*  
568 grown in presence of yeast extract; B: *T. arsenivorans* grown in presence of succinate.  
569 Significance of differences between modalities was evaluated using an Anova parametric test  
570 (details in material and methods section).

571  
572 Figure 4. Kinetics of AsIII oxidation in minimum medium containing 2 mg/L AsIII and 0.2  
573 g/L of organic substrate. Cells were previously grown in presence of 0.05, 0.2 or 1 g/L of  
574 organic substrate. A: “*T. arsenivorans*” on succinate; B: “*T. arsenivorans*” on yeast extract;  
575 C: *H. arsenicoxydans* on acetate; D: *H. arsenicoxydans* on yeast extract. Error bars represent  
576 the standard error of the mean (triplicates).

577

578

**Table 1.** Influence of the presence of 0.2 g.L<sup>-1</sup> organic substrate on specific AsIII oxidation rate during the kinetics: variation of AsIII oxidation rates between 0 and 0.2 g.L<sup>-1</sup> 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
<i>T. arsenivorans</i>	0.05 g/L yeast extract	-13.6
<i>T. arsenivorans</i>	0.2 g/L yeast extract	-39.1
<i>T. arsenivorans</i>	1 g/L yeast extract	-17.7
<i>T. arsenivorans</i>	0.05 g/L succinate	+7.3
<i>T. arsenivorans</i>	0.2 g/L succinate	+3.0 (NS)
<i>T. arsenivorans</i>	1 g/L succinate	+26.3 (NS)
<i>H. arsenicoxydans</i>	0.05 g/L yeast extract	-25.9
<i>H. arsenicoxydans</i>	0.2 g/L yeast extract	-17.9
<i>H. arsenicoxydans</i>	1 g/L yeast extract	-10
<i>H. arsenicoxydans</i>	0.05 g/L acetate	-11.5 (NS)
<i>H. arsenicoxydans</i>	0.2 g/L acetate	NI
<i>H. arsenicoxydans</i>	1 g/L acetate	+106.1

NI: not identified (not observable AsIII oxidation)

NS: not significant

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

**Table 2.** Comparison of the specific oxidation rates obtained in the present study with rates reported in the literature

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







