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

Tiffanie Lescure, Catherine Joulian, Clément Charles, Taoikal Ben Ali Saanda, Mickael Charron, Dominique Breeze, Pascale Bauda, Fabienne Battaglia-Brunet

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

Tiffanie Lescure, Catherine Joulian, Clément Charles, Taoikal Ben Ali Saanda, Mickael Charron, Dominique Breeze, Pascale Bauda, Fabienne Battaglia-Brunet

PII: S0923-2508(19)30100-7

DOI: https://doi.org/10.1016/j.resmic.2019.09.006

Reference: RESMIC 3741

To appear in: *Research in Microbiology*

Received Date: 21 June 2019

Revised Date: 4 September 2019

Accepted Date: 6 September 2019

Please cite this article as: T. Lescure, C. Joulian, C. Charles, T. Ben Ali Saanda, M. Charron, D. Breeze, P. Bauda, F. Battaglia-Brunet, Simple or complex organic substrates inhibit arsenite oxidation and *aioA* gene expression in two β-*Proteobacteria* strains, *Research in Microbiologoy*, https://doi.org/10.1016/j.resmic.2019.09.006.

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

Tiffanie Lescure[a,b], Catherine Joulian[a], Clément Charles[a], Taoikal Ben Ali Saanda[a], Mickael Charron[a], Dominique Breeze[a], Pascale Bauda[c], Fabienne Battaglia-Brunet[a,b*]

[a]BRGM, 3 avenue Claude Guillemin, 45060 Orléans, France

[b]BRGM, ISTO, UMR 7327, BP 36009, 45060 Orléans, France

[c]LIEC UMR 7360 CNRS-Université de Lorraine, Campus Bridoux, rue du Général Delestraint, 57070 Metz, France

*Corresponding author: f.battaglia@brgm.fr, (+33)2.38.64.39.30

E-mail addresses: tiffanielescure@gmail.com (T. Lescure), c.joulian@brgm.fr (C. Joulian), clement_cm45@yahoo.fr (Charles C.), taoikalali@hotmail.fr (Ben Ali Saanda T.), m.charron@brgm.fr (Charron M.), d.breeze@brgm.fr (Breeze D.), pascale.bauda@univ-lorraine.fr (Bauda P.), f.battaglia@brgm.fr (Battaglia-Brunet F., *correspondence and reprints)

Abstract

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

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

1. Introduction

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

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 substrates on biological AsIII oxidation has not been studied systematically.

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

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 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.

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 KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g NaCl, 0.05 g $(NH_4)_2SO_4$ 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 $CaCl_2$ and 0.1 g $MgSO_4$. Both solutions were autoclaved separately and mixed after cooling. 10 mL of a solution of 10 $g.L^{-1}$ AsIII (As_2O_3), filtered at 0.22 µm,

were added to the culture medium. A concentrated solution of 50 g/L yeast extract was prepared, filtered at 0.22 µm and then stored at 4°C.

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 75 mg.L^{-1} AsIII without organic substrate, in a cottoned 40 mL glass bottle. The inoculum of *H. arsenicoxydans* was prepared in CAsO1 medium (pH 7.2) containing 75 mg.L^{-1} AsIII and supplemented with 1 g.L^{-1} yeast extract. Two CAsO1 culture media containing, respectively, 0.2 and 1 g.L^{-1} yeast extract were inoculated, at pH 7.2 for *H. arsenicoxydans* and pH 6.0 for "*T. arsenivorans"*. All flasks were incubated aerobically at 25°C under stirring. At t=0 and then twice per day, bacterial growth was monitored by enumerating bacterial cells with a 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 5,000 x g. Pellets were frozen in liquid nitrogen and stored at -80°C until RNA/DNA co-extraction. In parallel, 2 mL samples were filtered at 0.22 microns in sterile flasks and kept at 4°C until further AsV analysis. Abiotic controls prepared in the same conditions showed that AsIII was not oxidized abiotically [23].

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

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

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).

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

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

2.7. aioA gene and transcripts quantification by qPCR

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

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

2.8. Statistical analysis

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

3. Results

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

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

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).

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 $g.L^{-1}$ of yeast extract (Fig. 1C and 1D). Consequently, the lower the yeast extract concentration, the higher the specific AsIII oxidation.

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

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 $g.L^{-1}$ ($5.6.10^{-11}$ µg/cell/min) than at 0.05 $g.L^{-1}$ of acetate (2.10^{-10} µg/cell/min).

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 pre-culture 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).

4. Discussion

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

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

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

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

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 and some compounds present in complex organic substrates, and thus reduce AsIII toxicity and/or bioavailability. Here, the results of experiments at 2 $mg.L^{-1}$ As suggest that the complex organic substrate (i.e. yeast extract) has a negative effect on AsIII oxidation, considering the comparison of kinetics with cells in suspension in solution with or without yeast extract. This phenomenon may be related to the complexation of AsIII with certain components of yeast extract such as thiol groups of amino acids that would make As less 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 possible explanation of the negative effect of simple or complex organic substrates on AsIII oxidation may be that the availability of energy sources would favor the AsV-reducing Ars arsenic resistance system [12] because this resistance process consumes energy. Regarding the Ars resistance system, most studies have focused on *ars*B and *ACR*3 genes carriers [35,44,45] whose expression was shown to be controlled by the concentration of arsenic and phosphate. Further studies should focus on determination of the effect of organic substrates on AsIII complexation, and on the expression of the *arsC* gene encoding an AsV reductase in the Ars resistance system, which, in parallel with the Aio system, could influence the balance of speciation of inorganic arsenic in aerated or microaerophilic environments.

Conflict of interest

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

Aknowledgements

This work was supported by the French Environment and Energy Management Agency (ADEME), BRGM (convention TEZ 11-16), and the Labex Voltaire (ANR-10-LABX-100-01). We are grateful to the laboratory of Molecular Genetics, Genomics, Microbiology (GMGM) of Louis Pasteur University (Strasbourg, France) for providing the *H. arsenicoxydans* strain.

References

[1] Casiot C, Morin G, Juillot F, Bruneel O, Personné J-C, Leblanc M, Duquesne K, Bonnefoy V, Elbaz-Poulichet F. Bacterial Immobilization and Oxidation of Arsenic in Acid Mine Drainage (Carnoulès Creek, France). Wat Res 2003;37: 2929–36.

[2] Battaglia-Brunet F, Itard Y, Garrido F, Delorme F, Crouzet C, Greffié C, Joulian C. A simple biogeochemical process removing arsenic from a mine drainage water. Geomicrobiol 2006;23: 201-211.

[3] Fuller CC, Davis JA, Waychunas GA. Surface chemistry of ferrihydrite: Part 2. Kinetics of arsenate adsorption and coprecipitation. Geochim Cosmochim Acta 1993;199357: 227 l-2282.

[4] Smedley PL, Kinniburgh DG. A review of the source, behaviour and distribution of arsenic in natural waters. Appl Geochem 2002;17: 517-568.

[5] Battaglia-Brunet F, Joulian C, Garrido F, Dictor MC, Morin D, Coupland K, Johnson BD, Hallberg KB, Baranger P. Oxidation of arsenite by *Thiomonas* strains and characterization of *Thiomonas arsenivorans sp. nov*.. Antonie Van Leeuwenhoek 2006;89:99-108.

[6] Campos VL, Valenzuela C, Yarza P, Kampfer P, Vidal R, Zaror C, Mondaca MA, Lopez-Lopez A, Rossello-Mora R. *Pseudomonas arsenicoxydans* sp nov., an arsenite-oxidizing strain isolated from the Atacama desert. Syst Appl Microbiol 2010;33:193-197.

[7] Duquesne K, Lieutaud A, Ratouchniak J, Muller D, Lett MC, Bonnefoy V. Arsenite oxidation by a chemoautotrophic moderately acidophilic *Thiomonas* sp. : from the strain isolation to the gene study. Env Microbiol 2008;10: 228-237.

[8] Hoeft SE, Kulp TR, Stolz JF, Hollibaugh JT, Oremland RS. Dissimilatory Arsenate Reduction with Sulfide as Electron Donor: Experiments with Mono Lake Water and Isolation of Strain MLMS-1, a Chemoautotrophic Arsenate Respirer. Appl Environ Microbiol 2004;70: 2741-2747.

[9] Quéméneur M, Cébron A, Billard P, Battaglia-Brunet F, Garrido F, Leyval C, Joulian C. Population structure and abundance of arsenite-oxidizing bacteria along an arsenic pollution gradient in waters of the upper isle River Basin, France. Appl Environ Microbiol 2010;76: 4566-4570.

[10] Cordi A, Pagnout C, Devin S, Poirel J, Billard P, Dollard MA, Bauda P. Determination of physiological, taxonomic, and molecular characteristics of a cultivable arsenic-resistant bacterial community. Environ Sci Poll Res 2015;22:13753–13763.

[11] Rosen BP. Biochemistry of As detoxification. FEBS Lett 2002;529:86-92.

[12] Silver S, Phung LT. Genes and enzymes involved in bacterial oxidation and reduction of inorganic arsenic. Appl Environ Microbiol 2005;71:599-608.

[13] Lett MC, Muller D, Lievremont D, Silver S, Santini J. Unified nomenclature for genes involved in prokaryotic aerobic arsenite oxidation. J Bacteriol 2012;194:207-208.

444 [14] Anderson LC, Bruland KW. Biogeochemistry of Arsenic in Natural Waters: The
445 Importance of Methylated Species. Environ Sci Technol 1991;25:420-427.
446 [15] Muller D, Lievremont D, Simeonova DD, Hubert JC, Lett MC. Arsenite Oxidase *aox*
447 Genes from a Metal-Resistant-Proteobacterium. J Bacteriol 2003;185:135-141.
448 [16] Wang Q, Han Y, Shi K, et al. An Oxidoreductase AioE is Responsible for Bacterial
449 Arsenite Oxidation and Resistance. Scientific Reports 2017;7:41536.
450 [17] Santini JM, van den Hoven RN Molybdenum-Containing Arsenite Oxidase of the
451 Chemolithoautotrophic Arsenite Oxidizer NT-26. J Bacteriol 2004;186:1614-1619.
452
453 [18] Oremland RS, Hoeft SE, Santini JM, Bano N, Hollibaugh RA, Hollibaugh JT. Anaerobic
454 Oxidation of Arsenite in Mono Lake Water and by a Facultative, Arsenite-Oxidizing
455 Chemoautotroph, Strain MLHE-1. Appl Environ Microbiol 2002;68:4795-4802.
456 [19] Santini JM, Sly LI, Wen A, Comrie D, Wulf-Durand PD, Macy JM. New Arsenite-
457 Oxidizing Bacteria Isolated from Australian Gold Mining Environments-Phylogenetic
458 Relationships. Geomicrobiol J 2002;19:67-76.
459 [20] Battaglia-Brunet F, El Achbouni H, Quemeneur M, Hallberg KB, Kelly DP, Joulian C.
460 Proposal that the arsenite-oxidizing organisms, *Thiomonas cuprina* and '*Thiomonas*
461 *arsenivorans*' be reclassified as strains of *Thiomonas delicata*, and emended description of
462 *Thiomonas delicata*. Intl J Syst Environ Microbiol 2011;61:2816-2821.
463 [21] Challan-Belval S, Garnier F, Michel C, Chautard S, Breeze D, Garrido F. Enhancing
464 pozzolana colonization by As(III)-oxidizing bacteria for bioremediation purposes. Appl
465 Microbiol Biotechnol 2009;84: 565-573.
466 [22] Bachate SP, Khapare RM, Kodam KM (2012) Oxidation of arsenite by two beta-
467 proteobacteria isolated from soil. Applied Microbiology and Biotechnology 93: 2135-2145

[23] Lescure T, Moreau J, Charles C, Ben Ali Saanda T, Thouin H, Pillas N, Bauda P, Lamy I, Battaglia-Brunet F. Influence of organic matters on AsIII oxidation by the microflora of polluted soils. Env Geochem Health. 2016;38:911–925.

[24] Nandre VS, Bachate SP, Salunkhe RC et al. Enhanced Detoxification of Arsenic Under Carbon Starvation: A New Insight into Microbial Arsenic Physiology. Curr Microbiol 2017;74:614.

[25] Spuches AM, Kruszyna HG, Rich AM, Wilcox DE. Thermodynamics of the AsIII-thiol interaction: arsenite and monomethylarsenite complexes with glutathione, dihydroglucoic acid and other thiol ligants. Inorg Chem 2005;44:2964-2972.

[26] Battaglia-Brunet F, Joulian C, Breeze D, Poirier L, Coulon S, Guérin V (2010) Monitoring of arsenic bio-oxidation and bio-redution activities in highly As-polluted soils during a phytostabilization field experiment (Phytoperf project). Consoil 2010, 11th International UFZ- Deltares/TNO Conference on Management of Soil, Groundwater and Sediment, Salzburg.

[27] Muller D, Simeonova DD, Riegel P, Mangenot S, Koechler S, Lièvremont D, Bertin PN, Lett MC. *Herminiimonas arsenicoxydans* sp. nov., a metalloresistant bacterium. Int J Syst Evol Microbiol 2003; 56:1765–1769.

[28] Weeger W, Lièvremont D, Perret M, Lagarde F, Hubert JC, Leroy M, Lett MC. Oxidation of arsenite to arsenate by a bacterium isolated from an aquatic environment. Biometals 1999; 12: 141-149.

[29] Battaglia-Brunet F, Dictor MC, Garrido F, Crouzet C, Morin D, Dekeyser K, Clarens M, Baranger P. An arsenic(III)-oxidizing bacterial population: selection, characterisation, and performance in reactors. J Appl Microbiol 2002;93:656-667.

[30] Quéméneur M. Les processus biogéochimiques impliqués dans la mobilité de l'arsenic : recherche de bioindicateurs. Thèse de doctorat d'université. Thesis, Laboratoire des

493 Interactions Microorganismes-Minéraux-Matière Organique dans les Sols (LIMOS), UMR
494 7137 CNRS, Université Henri Poincaré de Nancy. 2008.
495 [31] Fernandez-Rojo L, Hery M, Le Pape P, Braungardt C, Desoeuvre A, Torres E, Tardy V,
496 Resongles E, Laroche E, Delpoux S, Joulian C, Battaglia-Brunet F, Boisson J, Grapin G,
497 Morin G, Casiot C. Biological attenuation of arsenic and iron in a continuous flow
498 bioreactor treating acid mine drainage (AMD). Water Research 2017):123:594-606.
499 [32] Slyemi D, Bonnefoy V. How prokaryotes deal with arsenic. Env Microbiol Reports
500 2012; 4: 571-586.
501 [33] Hovasse A, Bruneel O, Casiot C, Desoeuvre A, Farasin J, Hery M, Van Dorsselaer A,
502 Carapito C, Arsène-Ploetze F. Spatio-Temporal Detection of the *Thiomonas* Population and
503 the *Thiomonas* Arsenite Oxidase Involved in Natural Arsenite Attenuation Processes in the
504 Carnoulès Acid Mine Drainage. Front Cell Dev Biol 2016;4:1-14.
505 [34] Lieutaud A, van Lis R, Duval S, Capowiez L, Muller D, Lebrun R, Lignon S, Fardeau
506 ML, Lett MC, Nitschke W, Shoepp-Cothenet B. Arsenite oxidase from *Ralstonia* sp. 22:
507 characterization of the enzyme and its interaction with soluble cytochromes. Journal of
508 Biological Chemistry 2010;285:2433-2441.
509 [35] Kashyap DR, Botero LM, Franck WL, Hassett DJ, McDermott TR. Complex regulation
510 of arsenite oxidation in *Agrobacterium tumefaciens*. J Bacteriol 2006;188:1081-1088.
511 [36] Poirel J, Joulian C, Leyval C, Billard P. Arsenite-induced changes in abundance and
512 expression of arsenite transporter and arsenite oxidase genes of a soil microbial community.
513 Res Microbiol 2013;164:457-465.
514 [37] Koechler S, Arsène-Ploetze F, Brochier-Armanet C, Goulhen-Chollet F, Heinrich-
515 Salmeron A, Jost B, Lièvremont D, Philipps M, Plewniak F, Bertin PN, Lett M-C.
516 Constitutive arsenite oxidase expression detected in arsenic-hypertolerant *Pseudomonas*
517 *xanthomarina* S11. Res Microbiol 2015;166:205-214.

518 [38] Koechler S, Cleiss-Arnold J, Proux C, Sismeiro O, Dillies MA, Goulhen-Chollet F,
519 Hommais F, Lievremont D, Arsene-Ploetze F, Coppee JY, Bertin PN. Multiple controls affect
520 arsenite oxidase gene expression in *Herminiimonas arsenicoxydans*. BMC Microbiol
521 2010;10: 53.
522 [39] Janda JM, Abbott SL. 16S rRNA gene sequencing for bacterial identification in the
523 diagnostic laboratory: pluses, perils, and pitfalls. J Clin Microbiol 2007;45: 2761-2764.
524 [40] Salmassi TM, Venkateswaran K, Satomi M, Nealson K, Newman DK, Hering JG.
525 Oxidation of arsenite by *Agrobacterium albertimagni* AOL15, sp. Nov., isolated from hot
526 creek California. Geomicrobiol J 2002;19:53-66.

527 [41] Santini JM, Sly LI, Schnagl RD, Macy JM. A new chemolithoautotrophic arsenite-
528 oxidizing bacterium isolated from a gold mine: phylogenetic, physiological, preliminary
529 biochemical studies. Appl Environ Microbiol 2000;66:92-97.

530 [42] Ghosh P, Rathinasabapathi B, Teplitski M, Ma LQ. Bacterial ability in AsIII oxidation
531 and AsV reduction: Relation to arsenic tolerance, P uptake, and siderophore production.
532 Chemosphere 2015: 138;995-1000.

533 [43] Kitchin KT, Wallace K. The role of protein binding of trivalent arsenicals in arsenic
534 carcinogenesis and toxicity. J Inorg Biochem 2008;102:532–539.
535 [44] Achour AR, Bauda P, Billard P Diversity of arsenite transporter genes from arsenic-
536 resistant soil bacteria. Res Microbiol 2007;158: 128-137.
537 [45] Achour-Rokbani A, Cordi A, Poupin P, Bauda P, Billar P. Characterization of the *ars*
538 gene cluster from extremely arsenic-resistant *Microbacterium* sp. strain A33. Appl Environ
539 Microbiol 2010;76:948-955.

[46] Das S, Jean JS, Kar S, Chou ML, Chen CY. Screening of plant growth-promoting traits in arsenic-resistant bacteria isolated from agricultural soil and their potential implication for arsenic bioremediation. J Haz Mat 2014;272:112-120.

[47] Bahar MM, Megharaj M, Naidu R. Kinetics of arsenite oxidation by *Variovorax* sp. MM-1 isolated from a soil and identification of arsenite oxidase gene. Journal of Hazardous Materials 2013;262:997-1003.

Legends to figures

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).

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).

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).

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.

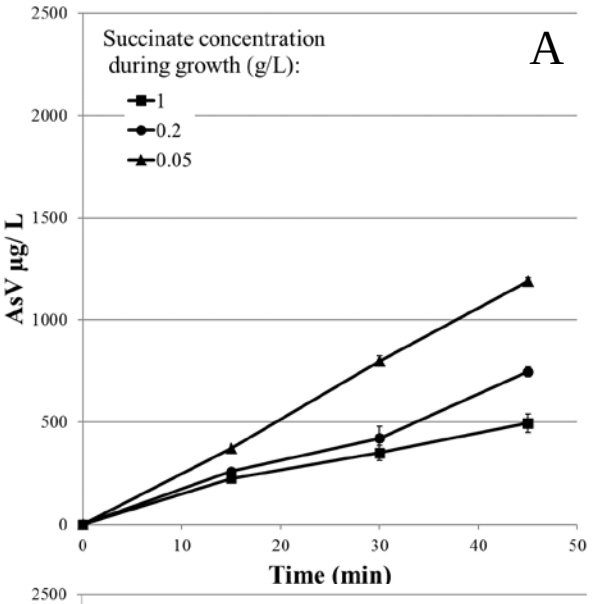
Strain	Pre-culture conditions	Variation (%) of AsIII oxidation rates between 0 and 0.2 g/L organic substrates during the kinetics
T. arsenivorans	0.05 g/L yeast extract	-13.6
T. arsenivorans	0.2 g/L yeast extract	-39.1
T. arsenivorans	1 g/L yeast extract	-17.7
T. arsenivorans	0.05 g/L succinate	+7.3
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

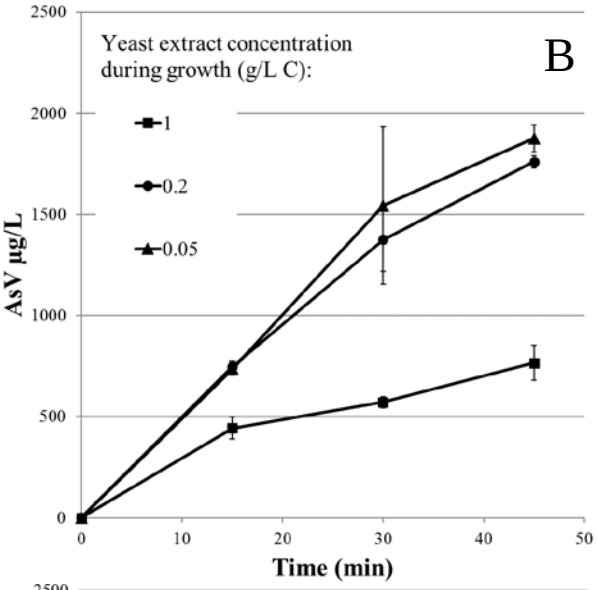
NI: not identified (not observable AsIII oxidation)
NS: not significant

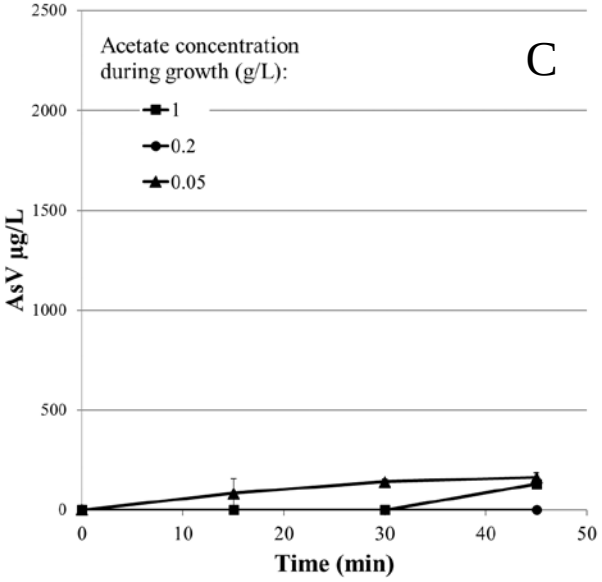
Negative effect	Organic substrate (0.2 g/L) within the kinetics medium has a negative effect on AsIII oxidation rate
Positive effect	Organic substrate (0.2 g/L) within the kinetics medium has a positive effect on AsIII oxidation rate

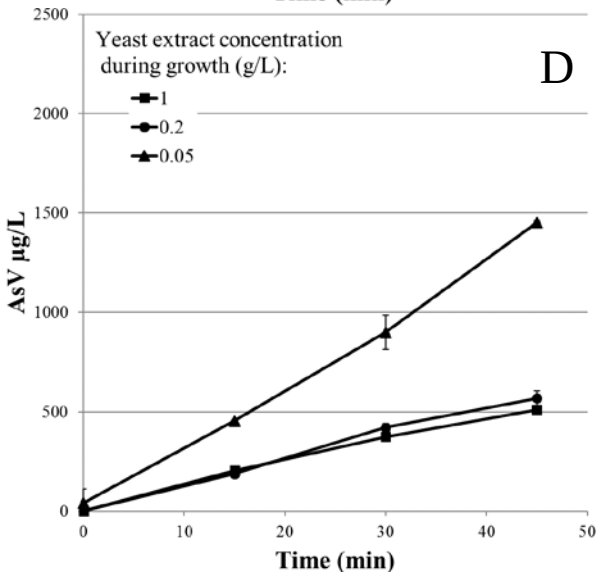
Table 2. Comparison of the specific oxidation rates obtained in the present study with rates reported in the literature

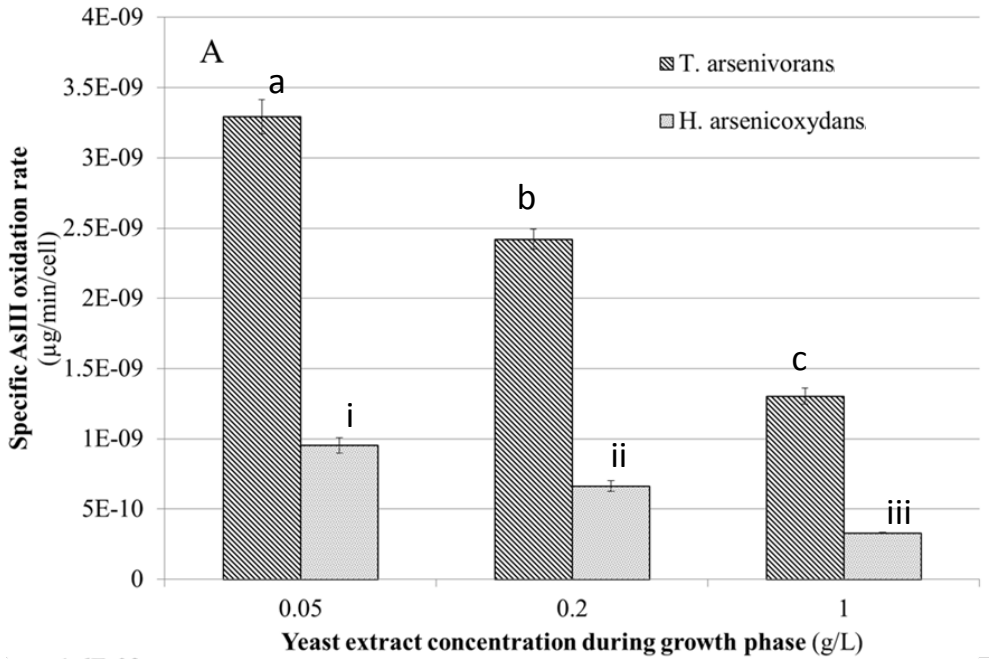
Bacterial strains	Specific rate (µg/L/min per cell)	References
Comamonas sp. ASR11	3.6×10^{-11}	[46]
Pseudomonas sp. ASR1	7.35×10^{-11}	[46]
Variovorax sp. 34	V max 8.85×10^{-11}	[38]
H. arsenicoxydans	0.5 to 9.5×10^{-10}	Present study
Agrobacterium albertimagni AOL15	V max 1.36×10^{-10}	[39]
T. arsenivorans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

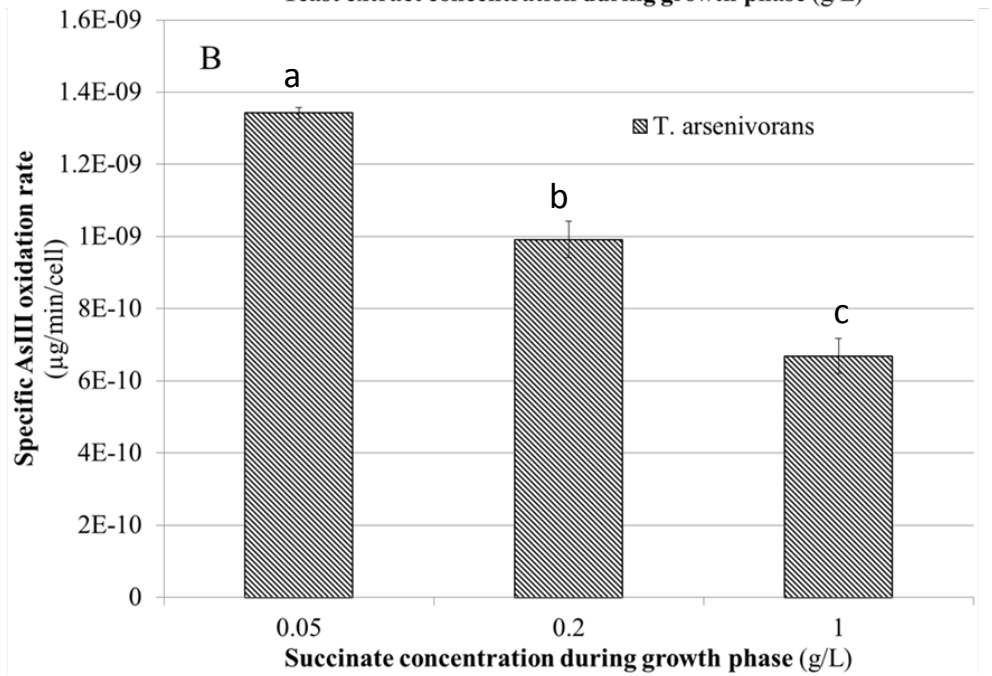

Succinate concentration during growth (g/L):
A
1
0.2
0.05
AsV µg/ L
Time (min)


Yeast extract concentration during growth (g/L C):
B
1
0.2
0.05
AsV µg/L
Time (min)


Acetate concentration during growth (g/L):
C
1
0.2
0.05
AsV µg/L
Time (min)


Yeast extract concentration during growth (g/L):
D
1
0.2
0.05
AsV µg/L
Time (min)


A
T. arsenivorans
H. arsenicoxydans
Specific AsIII oxidation rate (µg/min/cell)
4E-09
3.5E-09
3E-09
2.5E-09
2E-09
1.5E-09
1E-09
5E-10
0
a
b
c
i
ii
iii
0.05
0.2
1
Yeast extract concentration during growth phase (g/L)


B
T. arsenivorans
Specific AsIII oxidation rate (µg/min/cell)
1.6E-09
1.4E-09
1.2E-09
1E-09
8E-10
6E-10
4E-10
2E-10
0
a
b
c
0.05
0.2
1
Succinate concentration during growth phase (g/L)