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Flexible bacterial strains that oxidize arsenite in anoxic or aerobic conditions and utilize hydrogen or acetate as alternative electron donors

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Abstract Arsenic is a carcinogenic compound widely distributed in the groundwater around the world. The fate of arsenic in groundwater depends on the activity of microorganisms either by oxidizing arsenite (As^{III}), or by reducing arsenate (As^{V}). Because of the higher toxicity and mobility of As^{III} compared to As^{V}, microbial-catalyzed oxidation of As^{III} to As^{V} can lower the environmental impact of arsenic. Although aerobic As^{III}-oxidizing bacteria are well known, anoxic oxidation of As^{III} with nitrate as electron acceptor has also been shown to occur. In this study, three As^{III}-oxidizing bacterial strains, *Azoarcus* sp. strain EC1-pb1, *Azoarcus* sp. strain EC3-pb1 and *Diaphorobacter* sp. strain MC-pb1, have been characterized. Each strain was tested for its ability to oxidize As^{III} with four different electron acceptors, nitrate, nitrite, chlorate and oxygen. Complete As^{III} oxidation was achieved with both nitrate and oxygen, demonstrating the novel ability of these bacterial strains to oxidize As^{III} in either anoxic or aerobic conditions. Nitrate was only reduced to nitrite. Different electron donors were used to study their suitability in supporting nitrate reduction. Hydrogen and acetate were readily utilized by all the cultures. The flexibility of these As^{III}-oxidizing bacteria to use oxygen and nitrate to oxidize As^{III} as well as organic and inorganic substrates as alternative electron donors explains their presence in non-arsenic-contaminated environments. The findings suggest that at least some As^{III}-oxidizing bacteria are flexible with respect to electron-acceptors and electron-donors and that they are potentially widespread in low arsenic concentration environments.

Keywords Arsenite oxidation · Nitrate reduction · Pure culture · Metabolism · Flexibility

Introduction

Arsenic (As) is a toxic metalloid that is found in groundwater by the natural weathering of rocks (Smedley and Kinniburgh 2002). Long term exposure to As contaminated drinking water increases the risk of cancer in the skin, liver, bladder and lungs (ATSDR 2007). In response to an ever increasing awareness of the health risks associated with As, the maximum concentration level (MCL) of drinking water standard in the United States was made stricter by lowering it from 50 to 10 $\mu g\ l^{-1}$ in 2006 (USEPA 2001).

Arsenate (As^{V}, $H_2AsO_4^-$ and $HAsO_4^{2-}$) and arsenite (As^{III}, H_3AsO_3) are the predominant As

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species in circumneutral environments. As speciation is controlled mainly by redox conditions (Ascar et al. 2008; Beauchemin and Kwong 2006) with As^{V} being the dominant species in oxidizing environments while As^{III} predominates in reducing environments. As^{III} is adsorbed less than As^{V} in soil common metal oxides such as aluminum oxides (Hering 2005) and clay minerals (Lin and Puls 2000; Goldberg 2002), likewise As^{III} desorbs faster from iron (hydr)oxides than As^{V} (Tufano et al. 2008), therefore As^{III} is more mobile compared to As^{V} Thus processes governing the transformations between the different As species will have an important impact on the fate of As in the environment. Microbial activity plays a major role in the transformation between As^{V} and As^{III} (Oremland and Stolz 2003; Paez-Espino et al. 2009; Rhine et al. 2005). As^{V} can be reduced to As^{III} by dissimilatory As^{V} reducing bacteria, when an electron donor, such as organic matter or hydrogen (H_2), is present in the environment. The dissimilatory As^{V} reductase (*arrA*) are the responsible genes found in the As^{V} reducing bacteria (Saltikov and Newman 2003; Malasarn et al. 2004). On the other hand, As^{III} also can be oxidized to As^{V} by As^{III} oxidizing bacteria when an electron acceptor becomes available. A large variety of bacteria have been reported to contain the As^{III} oxidase (*aroA*) responsible for oxidation of As^{III} under aerobic (Silver and Phung 2005; Inskeep et al. 2007; Santini and vanden Hoven 2004) or anaerobic conditions (Hoeft et al. 2007; Rhine et al. 2007; Sun et al. 2010). The oxidation readily occurs under aerobic conditions. Since 1918, when bacteria capable of oxidizing As^{III} in aerobic environments were first recognized (Green 1918), numerous aerobic As^{III} oxidizing bacteria have been identified (Turner 1949; Wang and Suttigarn 2007; Krumova et al. 2008). A diversity of As metabolizing bacteria isolated from a variety of soil water systems have detectable As^{III}-oxidizing genes (Inskeep et al. 2007), indicating As^{III} oxidation plays an important role in the biogeochemical cycle of As.

Recently, As^{III} oxidation under anoxic conditions utilizing nitrate (NO_3^-) as an efficient alternative electron acceptor has been studied. Results from an urban lake have shown that nitrate (NO_3^-) levels in the anoxic zone are positively correlated with the formation of both As^{V} and As^{V}-adsorbing hydrous ferric oxides (Senn and Hemond 2002). NO_3^- injected into As-contaminated groundwater of Bangladesh was also shown to effectively lower the aqueous As concentration (Harvey et al. 2002). These results demonstrated the importance of NO_3^- as a controlling factor of As mobility in anoxic environments. Hoeft et al. (2002) reported microbial As^{III} oxidation coupled with the reduction of NO_3^- to nitrite (NO_2^-) in an arsenic-contaminated soda lake in California. Oremland et al. (2002) identified the strain *Alkalilimnicola ehrlichi* sp. MLHE-1 as a bacterial species responsible for oxidizing As^{III} when linked to NO_3^- reduction to NO_2^-. More recently, Rhine et al. (2006, 2007) identified two anaerobic As^{III} oxidizing strains from an As-contaminated soil, *Azoarcus* sp. DAO1 and *Sinorhizobium* sp. DAO10 . Based on the stoichiometry of As^{V}-formed to NO_3^--consumed as well as the presence of nitrous oxides reductase (*nosZ*) genes, these strains, DAO1 and DAO10, appear to link the As^{III} oxidation to the complete denitrification of NO_3^- to dinitrogen gas (N_2).

The three strains characterized in this paper are *Azoarcus* sp. EC1-pb1, *Azoarcus* sp. EC3-pb1 and *Diaphorobacter* sp. MC-pb1 (to be referred as EC1, EC3 and MC in the rest of the manuscript). These strains were isolated from enrichment cultures (ECs) which were originally derived from sediments or sludge from pristine environments as inoculum (Sun et al. 2009). The ECs, from which strains EC1 and EC3 were isolated, linked As^{III} oxidation with complete denitrification as evidenced from measurements of N_2 production (Sun et al. 2009). The fact that these strains originate from environments with no known As contamination suggests they must have metabolic flexibility. The scope of this research was to better understand the flexibility of EC1, EC3 and MC to utilize different electron acceptors in the oxidation of As^{III} as well as different electron donors for the reduction of nitrate.

Materials and methods

As^{III}-oxidizing pure cultures

Azoarcus sp. EC1-pb1, *Azoarcus* sp. EC3-pb1 and *Diaphorobacter* sp. MC-pb1 were isolated from enrichment cultures EC1, EC3 and MC, respectively (Sun et al. 2009). The sequences of three isolated have been deposited in the GenBank with accession numbers: HM177479, FJ514096 and FJ514095, respectively.

Medium composition

The standard basal medium (pH 7.0–7.2) was prepared using ultra pure water (Milli-Q system; Millipore). The final composition of the basal medium in the batch experiments was (mg l^{-1}) K_2HPO_4 (8.33); NH_4Cl (617.5); $MgCl_2{\cdot}6H_2O$ (173.3); $MgSO_4{\cdot}7H_2O$ (23.3); $CaCl_2$ (23.3), and trace elements in concentration (mg l^{-1}): $FeC1_3{\cdot}4H_2O$ (0.4); $CoCl_2{\cdot}6H_2O$ (0.4); $MnCl_2{\cdot}4H_2O$ (0.1); $AlCl_3{\cdot}6H_2O$ (0.018); $CuCl_2{\cdot}2H_2O$ (0.006); $ZnCl_2$ (0.01); H_3BO_3 (0.01); $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (0.01); $Na_2SeO_3{\cdot}5H_2O$ (0.032); $NiCl_2{\cdot}6H_20$ (0.01); EDTA (0.2); resazurin (0.04); HCl 36% (0.2 µl). 10 mM HCO_3^- ($NaHCO_3$) was used to buffer the pH of the medium.

Two different sets of experiments were carried out. In the first set of experiments, the electron acceptor was NO_3^- as KNO_3 (1.5 mM) and different electron donors were tested to be able to reduce NO_3^-, including $NaAsO_2$ (0.5 mM), CH_3COONa (0.11 mM), H_2 (0.4 mmol l_{liq}^{-1}), $FeCl_2{\cdot}4H_2O$ (1 mM), $Na_2S{\cdot}9H_2O$ (0.125 mM) or $MnSO_4{\cdot}H_2O$ (0.5 mM). In the second set of experiments, the electron donor was As^{III} added as $NaAsO_2$ (0.5 mM) and different electron acceptor were tested as potential As^{III} oxidants, at the concentration of KNO_3 (1.5 mM), $NaNO_2$ (0.5 mM), O_2 (0.35 mmol l_{liq}^{-1}) or $NaClO_3$ (0.25 mM), respectively. The basal medium with the electron acceptor was sterilized by autoclaving, 20 min at 121°C, while $NaHCO_3$ and the electron donor solution were sterilized using membrane filtration (0.22 µm).

Pure cultures maintenance

The three pure cultures were maintained in the absence of oxygen (O_2) in a basal medium amended with 0.5 mM As^{III} as electron donor and 5 mM NO_3^- as electron acceptor. The pure cultures were incubated in 160 ml serum bottles, with a total liquid volume of 110 ml. After the medium was autoclaved, bottles were closed using butyl rubber septa in order to ensure an anaerobic atmosphere. A flush gas mixture of N_2/CO_2 (80%:20%) was used to purge the headspace and the medium for exclusion of O_2. The gas was introduced through a 0.22 µm filter to sterilize it (needle in-needle out). As^{III} solution was added by using a 0.22 µm filter in order to sterilize the solution and then the medium was inoculated with the pure culture (6.4% vol:vol). The culture bottles were incubated in the dark on an orbital shaker (115 rpm) at 30°C. Pure cultures were transferred to fresh medium every 10–14 days once the complete oxidation of As^{III} to As^{V} was proved with 85% As^{III} converted to As^{V}.

Experimental incubations

Batch experiments were performed in 160 ml serum bottles. All assays were conducted in duplicate. In order to avoid the contamination of carry-over NO_3^- from old cultures to fresh medium during the inoculation, the cultures (10% volume of previous culture) were centrifuged in 15 ml sterilized centrifuge tubes at 1,400×*g* for 20 min. The pellets were resuspended into same volume of sterilized Milli Q water, and after two cleaning cycles they were transferred to the experiments. Several controls were run in parallel. Abiotic controls were prepared without adding pure culture to prove biological nature of the reaction. Controls lacking the electron donor or the electron acceptor but inoculated were included to demonstrate that the reaction just occurs when both reactants are added to the medium. The culture conditions were the same as those described for culture maintenance. He/CO_2 (80%:20%) was utilized as an alternative flushing gas in the experiment in which aerobic oxidation of As^{III} was studied to avoid N_2 interference when measuring O_2. In the experiments in which H_2 was added as electron donor, a H_2 stock bottle was prepared. An empty 160 ml serum bottle closed with a butyl rubber septa was flushed through a 0.22 µm filter with pure N_2 gas during 20 min and with H_2/CO_2 (80%:20%) for an extra 20 min. In a similar way, a 100% O_2 stock bottle was prepared to use in the aerobic experiments, but the serum bottle was flushed with pure O_2 for 30 min. From these bottles, the adequate volume of gas for each experiment (1.6 ml H_2/CO_2 and 1 ml O_2) was taken with a syringe and injected in the corresponding assays.

Analytical methods

Aliquot samples were taken from sealed anaerobic serum flasks by piercing the stoppers using sterile

syringes with 16-gauge needles. All samples were centrifuged (10 min, 14,000×*g*) immediately after sampling and stored in polypropylene vials. As^V, sulfate (SO_4^{2-}), chlorate (ClO_3^-), NO_3^- and NO_2^- were analyzed by suppressed conductivity ion chromatography using a Dionex IC-3000 system (Sunnyvale, CA, USA) fitted with a Dionex IonPac AS11 analytical column (4 × 250 mm) and AG16 guard column (4 mm × 40 mm). During each injection the eluent (KOH) used was 30 mM for 10 min. Mn^{2+} was measured by using an inductively coupled plasma-optical emission spectrometry (ICP-OES) system model Optima 2100 DV from Perkin-Elmer TM (Shelton, CT, USA). Fe^{2+} was quantified by the 5-ortho-phenanthroline colorimetric method, using an UV–visible spectrophotometer (Agilent 8453, Palo Alto, CA, USA). Total Fe was obtained by reducing Fe^{3+} with a hydroquinone solution in pH 4.5 (0.05 M acetate buffer) (Fortune and Mellon 1938).

Headspace samples were taken with a pressure lock gas tight syringe (1710RN, 100 µl (22 s/2″/2), Hamilton Company). N_2, O_2 and nitrous oxide (N_2O) were analyzed using a Hewlett Packard 5890 Series II gas chromatograph fitted with a Carboxen™ 1010 Plot column (30 m × 0.32 mm) and a thermal conductivity detector. Helium (He) was used as the carrier gas. H_2 and acetic acid were detected in an Agilent Technologies 7890A gas chromatography system. A Carboxen™ 1010 Plot Fused Capillary Column (30 m × 0.53 mm) with a thermal conductivity detector was used to analyze H_2 with N_2 used as the carrier gas. A Restek Stabilwax®-DA Column (30 m × 0.35 mm, ID 0.25 µm) with flame ionization detector, and He used as a carried gas, was used to detect acetic acid.

DNA concentration was measured with a TBS-360 Mini-Fluorometer (Turner Biosystems, Sunnyvale, CA, USA) for DNA quantification using PicoGreen dsDNA Quantitation Reagent (Molecular Probes, Inc., Eugene, OR, USA) (Ahn et al. 1996).

Metabolizing genes including *nosZ*, RuBisCO genes, *aroA* and *arrA* genes were PCR amplified with specifically designed primer sets. Successfully purified PCR products were cloned to identify the sequences. The methodology and conditions for these techniques are described in the Electronic Supplementary Material (ESM).

Results

Anoxic As^{III} oxidation linked to nitrate reduction

Figure 1A shows that As^{III} was oxidized to As^V in the full treatments amended with 0.5 mM As^{III} as electron donor and 1.5 mM NO_3^- as electron acceptor in the presence of inoculum. As^V formation began after a lag phase of 1.5 days, and complete oxidation was achieved after 3 days of incubation by observing the formation of 0.49 ± 0.01 mM of As^V as an average for the three pure cultures. No conversion of As^{III} to As^V was observed in control treatments run in parallel.

The end product of the NO_3^- reduction was evaluated. NO_3^- and NO_2^- concentrations were measured throughout the experiment (shown in Fig. 1B, C, respectively). NO_3^- concentration decreased from 1.7 ± 0.2 to 1.1 ± 0.1 mM, which occurred concomitantly with NO_2^- production of 0.49 ± 0.01 mM in the full treatments, whereas no appreciable NO_2^- formation was observed in control assays. These results imply that for each mole of NO_3^- reduced, approximately one mole of As^{III} is oxidized and one mole of NO_2^- is formed (Table 1A) in accordance with the reaction shown in Eq. 1:

$$H_3AsO_3 + NO_3^- \rightarrow HAsO_4^{2-} + NO_2^- + 2H^+ \quad (1)$$

Aerobic As^{III} oxidation

The three pure cultures were tested for their capacity to oxidize As^{III} in aerobic conditions. As^V formation was observed when 0.35 ± 0.06 mmol O_2 l_{liq}^{-1} was added as electron acceptor as shown in Fig. 2. In Fig. 2A, the first evidence of As^V formation was observed after only 1 day of incubation and the complete oxidation was achieved after 2.5 days in all the cultures. No As^{III} conversion was observed in the control assays (lacking either inocula or O_2). The O_2 concentration was also monitored (Fig. 2B). As shown in Table 1B, O_2 concentration was 0.32 ± 0.03 mmol l_{liq}^{-1} in the controls after 3.5 days while the concentration in the full treatments was 0.112 ± 0.01 mmol l_{liq}^{-1}, confirming the fact that biological As^{III} oxidation is linked to the O_2 consumption. The measured molar ratios (As^{III}:O_2) of the reaction were 2.25–2.4, depending on the strain,

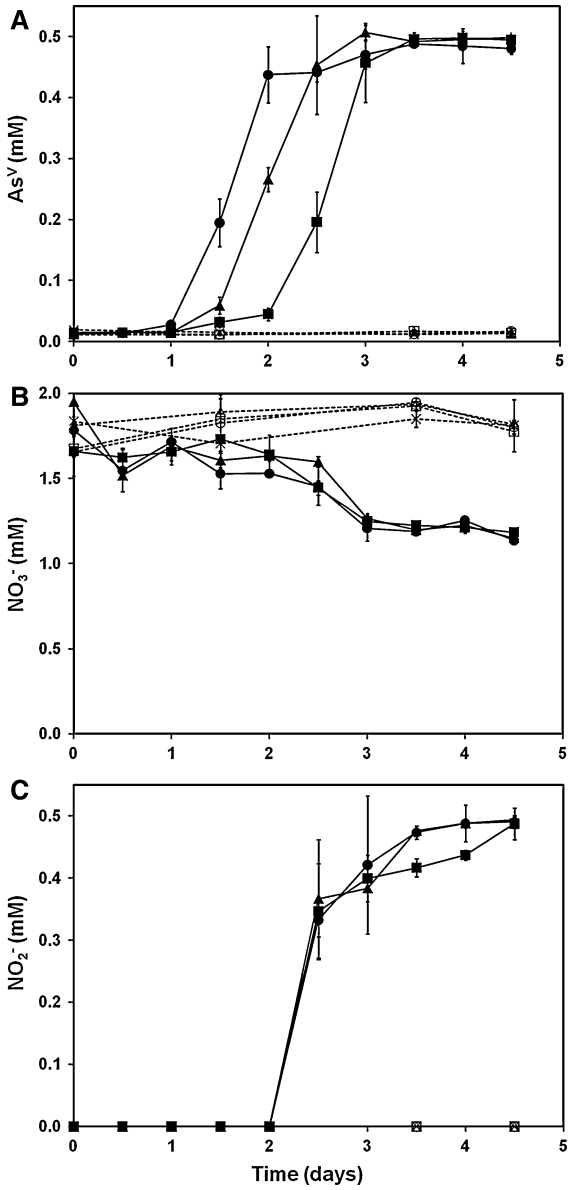

Fig. 1 As^V formation by pure cultures EC1, EC3 and MC from oxidation of of As^{III} (0.5 mM) linked to NO_3^- (1.5 mM) reduction. Legends: **A** As^V formation, complete treatments containing As^{III} and NO_3^- and inoculated with EC1 (*filled square*), EC3 (*filled circle*) and MC (*filled triangle*); biological controls (*dashed lines*) containing As^{III} but lacking NO_3^-: EC1 (*open square*), EC3 (*open circle*) and MC (*open triangle*); non inoculated control (*times symbol*); **B** NO_3^- consumption; **C** NO_2^- formation. Biological treatments supplied with NO_3^- and As^{III}, EC1 (*filled square*), EC3 (*filled circle*) and MC (*filled triangle*); biological controls (*dashed lines*) supplied with As^{III} but no NO_3, EC1 (*open square*), EC3 (*open circle*) and MC (*open triangle*); non inoculated control (*times symbol*)

approximately corresponding to the reaction shown in Eq. 2:

$$2H_3AsO_3 + O_2 \rightarrow 2HAsO_4^{2-} + 4H^+ \quad (2)$$

Oxidation of As^{III} by alternative electron acceptors

Aside from NO_3^- and O_2, NO_2^- and ClO_3^- were also tested as potential electron acceptors. The summary of the results obtained from testing electron acceptors is shown in Table 2. Strains MC and EC3 were able to link a partial oxidation of As^{III} when NO_2^- was present in the active treatments as electron acceptor. An As^V concentration of 0.24 ± 0.04 mM was reached in less than 18 days, and no further increase was observed after an additional 7 days of incubation. As^V formation was not detected when strain EC1 was used as the inoculum. No changes in As speciation occurred in the control bottles (lacking either NO_2^- or inocula) with an As^V concentration of 0.068 ± 0.01 mM at the end of the experiment. ClO_3^- was not utilized as an electron acceptor by any of the pure cultures even after 24 days of incubation for strains MC and EC3. A small As^{III} oxidation was observed by strain EC1, but it accounted for less than a 10% in 54 days of incubation; the concentration of As^V in the full treatment was 0.121 ± 0.051 mM, while in the control bottles was constant at 0.070 ± 0.02 mM throughout the entire experiment.

Use of alternative electron donors under nitrate-reducing and aerobic conditions

In order to test the ability of the three pure cultures to reduce NO_3^- with different substrates under anaerobic conditions, four inorganic electron donors and an organic electron donor were evaluated. The three strains were able to reduce NO_3^- when H_2 or acetate was amended to the treatment, but the other electron donors tested, ferrous iron (Fe^{2+}), sulfide (S^{2-}) and manganese (Mn^{2+}), were not utilized by the cultures to reduce NO_3^-.

Figure 3A shows the consumption of H_2 linked to the NO_3^- consumption represented in Fig. 3B. H_2 and NO_3^- removal were first noticeable after 1 day, and the reaction was completed by day 1.5. H_2 concentration decreased from 0.356 ± 0.014 to

Table 1 Stoichiometric calculations of the biological catalyzed reactions mediated by three pure culture strains EC1, MC and EC3

A	$\Delta(NO_3^-)$	$\Delta(NO_2^-)$	$\Delta(As^V)$	$\frac{\Delta(As^V)}{\Delta(NO_3^-)}$
	mM	mM	mM	
EC1	0.480	0.487	0.481	1.00
MC	0.531	0.494	0.483	0.91
EC3	0.583	0.490	0.468	0.80
B	$\Delta(O_2)$	$\Delta(As^V)$		$\frac{\Delta(As^V)}{\Delta(O_2)}$
	mmol l_{liq}^{-1}	mM		
EC1	0.217	0.488		2.25
MC	0.2436	0.556		2.28
EC3	0.233	0.561		2.41
C	$\Delta(H_2)$	$\Delta(NO_2^-)$	$\Delta(NO_3^-)$	$\frac{\Delta(H_2)}{\Delta(NO_3^-)}$
	mmol l_{liq}^{-1}	mM	mM	
EC1	0.355	0.353	0.387	0.92
MC	0.352	0.364	0.328	1.07
EC3	0.352	0.359	0.336	1.05

The stoichiometry has been calculated as the moles of electron donor consumed (moles of As^V produced are equivalent to the moles of As^{III} oxidized) per mole of electron acceptor utilized. Molar relationship of the As^{III} oxidation under denitrifying conditions (A), and aerobic conditions (B). Molar relationship of the H_2 oxidation under denitrifying conditions (C)

0.0036 ± 0.0001 mmol H_2 l_{liq}^{-1} in the complete treatments, while it was not oxidized in the controls treatments (lacking either NO_3^- or inocula). In parallel, NO_3^- concentration decreased an average of 0.350 ± 0.024 mM in the course of the experiment in the complete treatments and no NO_3^- removal was observed in the control treatments (lacking either H_2 or inocula). NO_3^- was reduced reduction to NO_2^- which accumulated to a concentration of 0.359 ± 0.004 mM at the end of the experiment (Fig. 3C). The experimental results indicated a molar ratio of H_2 consumed and NO_3^- reduced of 1.12 (Table 1C). This was close to the stoichiometric molar ratio of 1 shown in Eq. 3:

$$H_2 + NO_3^- \rightarrow NO_2^- + H_2O \quad (3)$$

H_2 was also tested as a possible electron donor under aerobic conditions. To pursue this experiment, the three strains were incubated with an atmosphere consisting of 0.503 mmol H_2 l_{liq}^{-1} and 0.251 mmol O_2 l_{liq}^{-1} and the H_2 consumption was monitored. The initial H_2 concentration was completely consumed in less than 20 h in bottles containing O_2 and inoculated with pure cultures. No H_2 consumption was observed in the controls (lacking either inoculum or O_2), confirming a biological mediated oxidation of H_2 by O_2.

As^{III} oxidation after pre-incubation of cultures with alternative electron donors and acceptors

The ability of the pure cultures to oxidize As^{III} with NO_3^- was evaluated after being previously incubated with H_2/NO_3^- or H_2/O_2 (Fig. 4A, B, respectively). In both cases, As^{III} oxidation linked to NO_3^- reduction was confirmed following the pre-incubation. Complete As^{III} oxidation took place in less than 1 day in both experiments. NO_3^- consumption was also confirmed in the full treatments with inoculum and As^{III}. These results indicate that the use of alternative electron acceptors and donors did not suppress the capacity of the bacteria to oxidize As^{III}. The pre-incubation with H_2/NO_3^- or H_2/O_2 likely increased the cell concentration in the assays, which may have been the reason for a faster oxidation of As^{III} compared to earlier experiments in which As^{III} grown cells were used as inoculum.

The difference in cell concentration when the cultures are incubated either with NO_3^-/As^{III} or H_2/O_2 was estimated by quantifying the DNA at the beginning

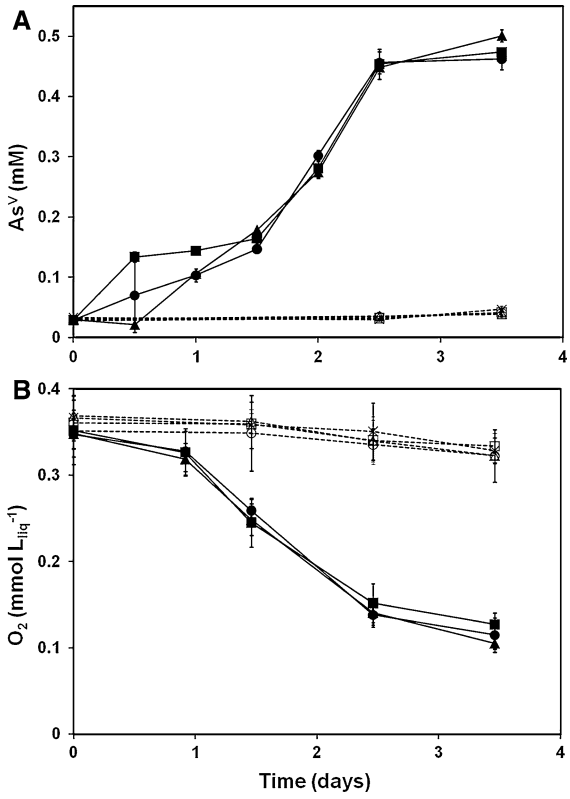

Fig. 2 As^V production by pure cultures EC1, EC3 and MC with O_2 as electron acceptor. Oxidation of 0.5 mM of As^{III} with 0.35 mmol O_2 l_{liq}^{-1}. **A** As^V formation. Biological treatments supplied with O_2 and As^{III}, EC1 (*filled square*), EC3 (*filled circle*) and MC (*filled triangle*); controls (*dashed lines*) supplied with As^{III} but no O_2, EC1 (*open square*), EC3 (*open circle*) and MC (*open triangle*); non inoculated control (*times symbol*); **B** O_2 consumption. Full treatments supplied with O_2 and As^{III}, EC1 (*filled square*), EC3 (*filled circle*) and MC (*filled triangle*); controls (*dashed lines*) supplied with O_2 but no As^{III}, EC1 (*open square*), EC3 (*open circle*) and MC (*open triangle*); non inoculated control (*times symbol*)

and at the end of the experiments. These two experiments were the two extreme scenarios, the lowest cell yield (NO_3^-/As^{III}) and the highest cell yield (H_2/O_2). In both cases, DNA production was shown to be clearly linked to the use of As(III) or H_2 as a growth substrate (Fig S1 and Fig S2 in ESM). There was no increase in DNA concentration in non-inoculated controls, nor in controls lacking either the electron acceptor or electron donor. The concentration of DNA was approximately five times greater at the end of the experiment when the pure cultures were incubated with H_2/O_2 compared with NO_3^-/As^{III}. This outcome also confirms the hypothesis that the cell yield was significantly increased with pre-incubation with the better growth substrate, H_2.

Functional gene PCR and cloning

Functional gene PCR targeting *aroA* and *arrA* genes, *nosZ* and RuBisCO genes were performed on all three As^{III}-oxidizing, NO_3^--reducing pure cultures. Various primer sets were used to detect the existence of *aroA* or *arrA* genes. No PCR products were observed for any of the primer sets for *aroA*, *nosZ* which are genes responsible for As^{III} oxidation (Inskeep et al. 2007) and nitrous oxide reductase (Scala and Kerkhof 1998), respectively. PCR products were found for primer set #1 of *arrA*, a gene encoding for dissmilatory As^V reductase (Saltikov and Newman 2003) but is related in structure to As^{III} oxidizing enzymes believed to catalyze the reverse reaction of the dissimilatory reductase (Richey et al. 2009). However, the sequence of the amplicons did not correspond to *arrA*. PCR products were obtained from a primer set targeting the large unit, *cbbL*, of ribulose-1,5-biphosphate carboxylase oxygenase genes (RuBisCO genes, an indicator of autotrophy) in all the three pure cultures (Fig. S3 in ESM). The amplicons were 800 bp in agreement with the expected size (Elsaied and Naganuma 2001). The amplicons of two of the strains, EC1 and EC3, were also checked by cloning and sequencing. The sequences were deposited in GenBank with accession numbers JN008173 and JN008174, respectively. The EC1 and EC3 derived sequences corresponded 95 and 80% similarity to the RuBisCO large unit cbbL genes of *Nitrosomonas* sp. ENI-11 [GenBank accession number AB061373] (Hirota et al. 2002) and an uncultured organism [GenBank accession number AB505078] (Kojima et al. 2009), respectively.

Discussion

The three As^{III}-oxidizing bacteria presented in this study, EC1, EC3 and MC, were found to be flexible in their ability to use different electron acceptors and electron donors. The ability to oxidize As^{III} in anoxic nitrate-reducing as well as aerobic conditions is unique and has not been observed before by a single strain. The strains therefore have the ability to adapt to changing redox conditions in the environment. Functional gene PCR was used to determine which genes might be involved in arsenic metabolism. As^{III}

Table 2 Summary of As^{III} oxidation with various electron acceptors tested

Pure cultures	Electron acceptors[a]							
	KNO_3		$NaNO_2$		O_2		$NaClO_3$	
	As^V formation (mM)	Time to completion (days)	As^V formation (mM)	Time to completion (days)	As^V formation (mM)	Time to completion (days)	As^V formation (mM)	Time to completion (days)
EC1	**0.496 ± 0.011**	**4**	0.053 ± 0.052	54	**0.473 ± 0.002**	**2.5**	0.121 ± 0.051	54
MC	**0.496 ± 0.004**	**3**	**0.240 ± 0.008**	**24**	**0.500 ± 0.010**	**2.5**	0.072 ± 0.002	24
EC3	**0.485 ± 0.028**	**3**	**0.284 ± 0.116**	**24**	**0.472 ± 0.018**	**2.5**	0.063 ± 0.021	24

As^{III} concentration was 0.5 mM

[a] Concentrations of the different electron acceptor tested were 1.5 mM KNO_3, 0.5 mM $NaNO_2$, 0.35 mmol O_2 l_{liq}^{-1} and 0.25 mM $NaClO_3$. Electron acceptors that have been positively utilized by the cultures to oxidize As^{III} are shown in bold text

oxidase (Aro) and As^V respiratory reductase (Arr) are two As metabolizing enzymes within the dimethyl sulfoxide (DMSO) reductase family (Silver and Phung 2005) that are involved in growth linked metabolism of As^{III} and As^V, respectively. However this approach provided no definitive evidence for the presence of *aroA* nor *arrA* genes in any of the three pure cultures.

As^{III} was not the only substrate that can be used as electron donor by the isolated strains. H_2 and acetate were readily oxidized under NO_3^--reducing conditions. Also H_2 was shown to be utilized as electron donor with O_2. The flexibility to utilize different substrates in order to support growth seems to be a common characteristic of many As^{III} oxidizing isolates. The facultative, As^{III} oxidizing chemoautotroph *Alkalimnicola ehrlichii* strain MLHE-1, belonging to the *γ-Proteobacteria* group, was able to grow with As^{III}, H_2, sulfide, thiosulfate and acetate while reducing NO_3^- to NO_2^-. Aerobic oxidation was also possible with H_2 and acetate (Hoeft et al. 2007). Two autotrophic As^{III} oxidizing denitrifying bacteria, *Azoarcus* strain DAO1 and *Sinorhizobium* strain DAO10 were also capable of using H_2, acetate, glucose, lactate and other organic substrates as electron donors (Rhine et al. 2006). Three newly isolated aerobic As^{III} oxidizing strains *Ancylobacter* strain OL-1, *Thiobacillus* strain S-1 and *Hydrogenophaga* strain CL-3 can also oxidize different sulfur species (thiosulfate, elemental sulfur and sulfide) to sulfate (Garcia-Dominguez et al. 2008). Ammonium was also found to be an electron donor for strains S-1 and CL-3 and the latter can also oxidize NO_2^- to NO_3^- under aerobic conditions.

None of the three bacterial strains characterized in this study were able to completely denitrify NO_3^- to N_2. Instead, NO_3^- was partially reduced to NO_2^-, which accumulated as a product at the end of the experiments. The enrichment cultures, EC1 and EC3, from which *Azoarcus* sp. strains EC1 and EC3 were isolated, respectively, were shown to produce N_2 gas as the final product of denitrification linked to As^{III} oxidation (Sun et al. 2009). Also, strain DAO1 isolated by Rhine et al. (2006), closely related to *Azoarcus* (96% 16S rRNA gene sequence similarity to strains EC1 and EC3) was able to perform complete denitrification. The dilution to extinction technique used to isolate the pure cultures may have favored subpopulations lacking the full set of denitrifying genes, in particular the *nosZ* gene. The functional PCR assay confirmed the absence of *nosZ* genes which corresponds to their inability to completely denitrify of NO_3^- to N_2.

The presence of RuBisCO genes, a key enzyme of CO_2 fixation for cell growth (Miziorko and Lorimer 1983) in all of the strains studied here, provides DNA-based evidence of autotrophic nature of pure cultures. Two amplicon sequences were confirmed to be related to RuBisCO large unit *cbbL* genes. There are several examples of autotrophic anoxic As^{III}-oxidizing bacteria reported before. *Azoarcus* sp. DAO1, *Sinorhizobium* sp. DAO10 and *Alkalimnicola ehrlichii* sp. MLHE1 were shown to contain RuBisCO genes (Hoeft et al. 2007; Rhine et al. 2006). Likewise, many aerobic As^{III}-oxidizing bacteria also contain RuBisCO genes such as *Ancylobacter* strain OL-1, *Thiobacillus* strain S-1 and *Hydrogenophaga* strain CL-3 (Garcia-Dominguez et al. 2008).

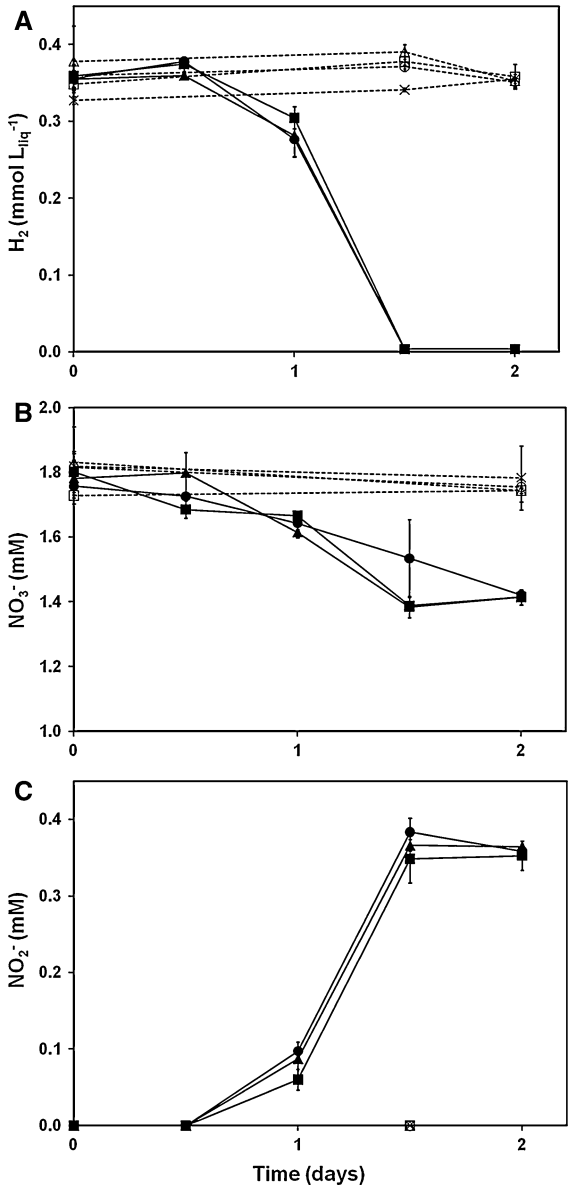

Fig. 3 H_2 oxidation linked to NO_3^- reduction. Treatments with 0.35 mmol H_2 l_{liq}^{-1} and 1.5 mM of NO_3^-. **A** H_2 oxidation biological treatments supplied with NO_3^- and H_2, EC1 (*filled square*), EC3 (*filled circle*) and MC (*filled triangle*); controls (*dashed lines*) supplied with H_2 but no NO_3^-, EC1 (*open square*), EC3 (*open circle*) and MC (*open triangle*); non inoculated control (*times symbol*); **B** decrease of NO_3^- concentration; **C** NO_2^- formation. Full treatments supplied with NO_3^- and H_2 and inoculated with EC1 (*filled square*), EC3 (*filled circle*) and MC (*filled triangle*); controls (*dashed lines*) with only H_2 and no NO_3^-: EC1 (*open square*), EC3 (*open circle*) and MC (*open triangle*); non inoculated control (*times symbol*)

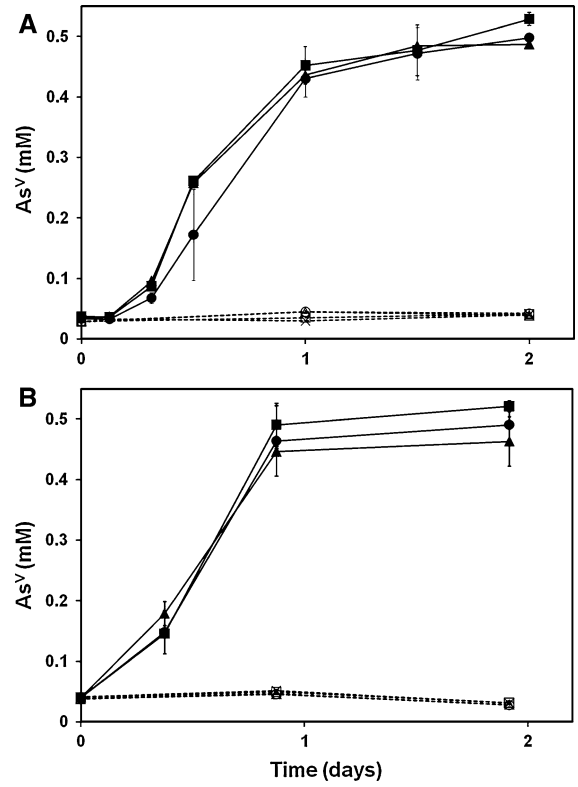

Fig. 4 Ability of the pure cultures to oxidize As^{III} under denitrifying conditions after being incubated with **A** 0.3 mM NO_3^- and 0.4 mmol l_{liq}^{-1} H_2 or **B** 0.4 mmol H_2 l_{liq}^{-1} and 0.35 mmol O_2 l_{liq}^{-1}. Treatment with 0.5 mM of As^{III} and 0.3 mM of NO_3^-: EC1 (*filled square*), EC3 (*filled circle*) and MC (*filled triangle*). Controls (*dashed lines*) with only 0.5 mM of As^{III} and no NO_3^-: EC1 (*open square*), EC3 (*open circle*) and MC (*open triangle*). Non inoculated controls (*times symbol*)

The most well studied aerobic As^{III}-oxidizing strains have been isolated from the As bearing sediments of a gold mine in Australia (Santini et al. 2000; Santini et al. 2002). Earlier anoxic As^{III}-oxidizing isolates have either been isolated from an alkaline lake, with high levels of As, in California (Oremland et al. 2002; Oremland et al. 2004; Oremland et al. 2005) or were isolated from As contaminated soil and sediments (Rhine et al. 2006). The three As^{III}-oxidizing bacteria presented in this study (EC1, EC3 and MC) were isolated from anoxic As^{III}-oxidizing EC and a mixed culture originating from pristine, non-contaminated environments (Sun et al. 2009).

The ability of As^{III}-oxidizing strains to survive in environments in which As is present in negligible

concentrations is most likely related to their flexibility in using different electron donors. The isolates are able to survive with environmentally relevant electron donors such as H_2 and acetate, which can be expected in anaerobic conditions where organic matter is decomposing. Therefore, anoxic As^{III} oxidizing bacteria like those described in this study are probably more widespread in the environment than previously thought due to the high level of flexibility in the use of substrates.

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References

Ahn SJ, Costa J, Emanuel JR (1996) PicoGreen quantitation of DNA: effective evaluation of samples pre- or post-PCR. Nucleic Acids Res 24(16):2623–2625

Ascar L, Ahumada I, Richter P (2008) Influence of redox potential (Eh) on the availability of arsenic species in soils and soils amended with biosolid. Chemosphere 72(10): 1548–1552

Beauchemin S, Kwong YTJ (2006) Impact of redox conditions on arsenic mobilization from tailings in a wetland with neutral drainage. Environ Sci Technol 40(20):6297–6303

DR ATS (2007) Toxicological profile for arsenic. US Department of Health and Human Services, Public Health Service, Atlanta

Elsaied H, Naganuma T (2001) Phylogenetic diversity of ribulose-1, 5-bisphosphate carboxylase/oxygenase large-subunit genes from deep-sea microorganisms. Appl Environ Microbiol 67(4):1751–1765

Fortune WB, Mellon MG (1938) Determination of iron with o-phenanthroline—a spectrophotometric study. Ind Eng Chem 10:0060–0064

Garcia-Dominguez E, Mumford A, Rhine ED, Paschal A, Young LY (2008) Novel autotrophic arsenite-oxidizing bacteria isolated from soil and sediments. FEMS Microbiol Ecol 66(2):401–410

Goldberg S (2002) Competitive adsorption of arsenate and arsenite on oxides and clay minerals. Soil Sci Soc Am J 66(2):413–421

Green HH (1918) Description of a bacterium which oxidizes arsenite to arsenate, and of one which reduces arsenate to arsenite, isolated from a cattle-dipping tank. S Afr J Sci 14:465–467

Harvey CF, Swartz CH, Badruzzaman ABM, Keon-Blute N, Yu W, Ali MA, Jay J, Beckie R, Niedan V, Brabander D, Oates PM, Ashfaque KN, Islam S, Hemond HF, Ahmed MF (2002) Arsenic mobility and groundwater extraction in Bangladesh. Science 298:1602–1606

Hering JG (2005) Contrasting sorption behavior of arsenic(III) and arsenic(V) in suspensions of iron and aluminum oxyhydroxides. Paper presented at the advances in arsenic research, Washington DC, pp 8–24

Hirota R, Kato J, Morita H, Kuroda A, Ikeda T, Takiguchi N, Ohtake H (2002) Isolation and characterization of cbbL and cbbS genes encoding form I ribulose-1, 5-bisphosphate carboxylase/oxygenase large and small subunits in Nitrosomonas sp strain ENI-11. Biosci Biotechnol Biochem 66(3):632–635

Hoeft SE, Lucas F, Hollibaugh JT, Oremland RS (2002) Characterization of microbial arsenate reduction in the anoxic bottom waters of Mono Lake, California. Geomicrobiol J 19(1):23–40

Hoeft SE, Blum JS, Stolz JF, Tabita FR, Witte B, King GM, Santini JM, Oremland RS (2007) *Alkalilimnicola ehrlichii* sp nov., a novel, arsenite-oxidizing haloalkaliphilic gammaproteobacterium capable of chemoautotrophic or heterotrophic growth with nitrate or oxygen as the electron acceptor. Int J Syst Evol Microbiol 57:504–512

Inskeep WP, Macur RE, Hamamura N, Warelow TP, Ward SA, Santini JM (2007) Detection, diversity and expression of aerobic bacterial arsenite oxidase genes. Environ Microbiol 9(4):934–943

Kojima H, Fukuhara H, Fukui M (2009) Community structure of microorganisms associated with reddish-brown iron-rich snow. Syst Appl Microbiol 32(6):429–437

Krumova K, Nikolovska M, Groudeva V (2008) Characterization of arsenic-transforming bacteria from arsenic contaminated sites in Bulgaria. Biotechnol Biotechnol Eq 22(2):729–735

Lin Z, Puls RW (2000) Adsorption, desorption and oxidation of arsenic affected by clay minerals and aging process. Environ Geol 39(7):753–759

Malasarn D, Saltikov W, Campbell KM, Santini JM, Hering JG, Newman DK (2004) *ArrA* is a reliable marker for As(V) respiration. Science 306(5695):455

Miziorko HM, Lorimer GH (1983) Ribulose-1, 5-bisphosphate carboxylase-oxygenase. Annu Rev Biochem 52:507–535

Oremland RS, Stolz JF (2003) The ecology of arsenic. Science 300(5621):939–944

Oremland RS, Hoeft SE, Santini JA, Bano N, Hollibaugh RA, Hollibaugh JT (2002) Anaerobic oxidation of arsenite in Mono Lake water and by facultative, arsenite-oxidizing chemoautotroph, strain MLHE-1. Appl Environ Microbiol 68(10):4795–4802

Oremland RS, Stolz JF, Hollibaugh JT (2004) The microbial arsenic cycle in Mono Lake, California. FEMS Microbiol Ecol 48(1):15–27

Oremland RS, Kulp TR, Blum JS, Hoeft SE, Baesman S, Miller LG, Stolz JF (2005) A microbial arsenic cycle in a salt-saturated, extreme environment. Science 308(5726): 1305–1308

Paez-Espino D, Tamames J, de Lorenzo V, Canovas D (2009) Microbial responses to environmental arsenic. Biometals 22(1):117–130

Rhine ED, Garcia-Dominguez E, Phelps CD, Young LY (2005) Environmental microbes can speciate and cycle arsenic. Environ Sci Technol 39(24):9569–9573

Rhine ED, Phelps CD, Young LY (2006) Anaerobic arsenite oxidation by novel denitrifying isolates. Environ Microbiol 8(5):899–908

Rhine ED, Ni Chadhain SM, Zylstra GJ, Young LY (2007) The arsenite oxidase genes (*aroAB*) in novel chemoautotrophic arsenite oxidizers. Biochem Biophys Res Commun 354(3): 662–667

Richey C, Chovanec P, Hoeft SE, Oremland RS, Basu P, Stolz JF (2009) Respiratory arsenate reductase as a bidirectional enzyme. Biochem Biophys Res Commun 382(2):298–302

Saltikov CW, Newman DK (2003) Genetic identification of a respiratory arsenate reductase. Proc Natl Acad Sci USA 100(19):10983–10988

Santini JM, vanden Hoven RN (2004) Molybdenum-containing arsenite oxidase of the chemolithoautotrophic arsenite oxidizer NT-26. J Bacteriol 186(6):1614–1619

Santini JM, Sly LI, Schnagl RD, Macy JM (2000) A new chemolithoautotrophic arsenite-oxidizing bacterium isolated from a gold mine: phylogenetic, physiological, and preliminary biochemical studies. Appl Environ Microbiol 66(1):92–97

Santini JM, Sly LI, Wen AM, Comrie D, De Wulf-Durand P, Macy JM (2002) New arsenite-oxidizing bacteria isolated from Australian gold mining environments - Phylogenetic relationships. Geomicrobiol J 19(1):67–76

Scala DJ, Kerkhof LJ (1998) Nitrous oxide reductase (*nosZ*) gene-specific PCR primers for detection of denitrifiers and three *nosZ* genes from marine sediments. FEMS Microbiol Lett 162(1):61–68

Senn DB, Hemond HF (2002) Nitrate controls on iron and arsenic in an urban lake. Science 296(5577):2373–2376

Silver S, Phung LT (2005) Genes and enzymes involved in bacterial oxidation and reduction of inorganic arsenic. Appl Environ Microbiol 71(2):599–608

Smedley PL, Kinniburgh DG (2002) A review of the source, behaviour and distribution of arsenic in natural waters. Appl Geochem 17(5):517–568

Sun WJ, Sierra-Alvarez R, Fernandez N, Sanz JL, Amils R, Legatzki A, Maier RM, Field JA (2009) Molecular characterization and in situ quantification of anoxic arsenite-oxidizing denitrifying enrichment cultures. FEMS Microbiol Ecol 68(1):72–85

Sun WJ, Sierra-Alvarez R, Milner L, Field JA (2010) Anoxic oxidation of arsenite linked to chlorate reduction. Appl Environ Microbiol 76:6804–6811

Tufano KJ, Reyes C, Saltikov CW, Fendorf S (2008) Reductive processes controlling arsenic retention: revealing the relative importance of iron and arsenic reduction. Environ Sci Technol 42(22):8283–8289

Turner AW (1949) Bacterial oxidation of arsenite. Nature 164(4158):76–77

USEPA (2001) National primary drinking water regulations; arsenic and clarifications to compliance and new source contaminants monitoring. Fed Regist 66(14):6975–7066

Wang YT, Suttigarn A (2007) Arsenite oxidation by *Alcaligenes faecalis* strain O1201 in a continuous-flow bioreactor. J Environ Eng 133(5):471–476