Published in final edited form as:

Biodegradation. 2012 February ; 23(1): 133–143. doi:10.1007/s10532-011-9493-x.

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

Lucía Rodríguez-Freire^{1,*}, Wenjie Sun¹, Reyes Sierra-Alvarez¹, and Jim A. Field¹

¹ Department of Chemical and Environmental Engineering, University of Arizona, Tucson, AZ, USA

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, *Azoarcus* sp. strain EC3 and *Diaphorobacter* sp. strain MC, 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 $\mu\text{g/L}$ in 2006 (EPA 2006).

*Department of Chemical and Environmental Engineering, University of Arizona, P.O. Box 210011, Tucson, Arizona Tel. 520-621-2591 Fax. 520-621-6048 luciar@email.arizona.edu.

Arsenate (As^{V} , H_2AsO_4^- and HAsO_4^{2-}) and arsenite (As^{III} , H_3AsO_3) are the predominant As 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 was first recognized (Green 1918), numerous aerobic As^{III} oxidizing bacteria have been identified (Turner 1949; Wang and Suttigarn 2007; Krumova et al. 2008). A biodiversity 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. In 2001, Hoeft *et al.* reported microbial As^{III} oxidation coupled with the reduction of NO_3^- to nitrite (NO_2^-) in an arsenic-contaminated soda lake in California (Hoeft et al. 2002). Oremland *et al.* identified the strain *Alkalilimnicola ehrlichii* sp. MLHE-1 as a bacterial species responsible for oxidizing As^{III} when linked to NO_3^- reduction to NO_2^- (Oremland et al. 2002). More recently, in 2006, Rhine *et al.*, identified two anaerobic As^{III} oxidizing strains from an As-contaminated soil, *Azoarcus* sp. DAO1 and *Sinorhizobium* sp. DAO10 (Rhine et al. 2006; Rhine et al. 2007). 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); $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (173.3); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (23.3); CaCl_2 (23.3), and trace elements in concentration (mg L^{-1}): $\text{FeCl}_3 \cdot 4\text{H}_2\text{O}$ (0.4); $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.4); $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (0.1); $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ (0.018); $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ (0.006); ZnCl_2 (0.01); H_3BO_3 (0.01); $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (0.01); $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$ (0.032); $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ (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 $\text{L}_{\text{liq}}^{-1}$), $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (1 mM), $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (0.125 mM) or $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (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 (0.4 mmol $\text{L}_{\text{liq}}^{-1}$) 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 d 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 MiliQ 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/CO₂ (80%:20%) was utilized as an alternative flushing gas in the experiment in which aerobic oxidation of As^{III} was studied to avoid N₂ interference when measuring O₂. In the experiments in which H₂ was added as electron donor, a H₂ 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 N₂ gas during 20 min and with H₂/CO₂ (80%:20%) for an extra 20 min. In a similar way, a 100% O₂ stock bottle was prepared to use in the aerobic experiments, but the serum bottle was flushed with pure O₂ for 30 min. From these bottles, the adequate volume of gas for each experiment (1.6 mL H₂/CO₂ and 1 mL O₂) 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 (SO₄²⁻), chlorate (ClO₃⁻), NO₃⁻ and NO₂⁻ 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. Mn²⁺ 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). Fe²⁺ 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 Fe³⁺ 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 (22s/2"/2), Hamilton Company). N₂, O₂ and nitrous oxide (N₂O) were analyzed using a Hewlett Packard 5890 Series II gas chromatograph fitted with a CarboxenTM 1010 Plot column (30 m × 0.32 mm) and a thermal conductivity detector. Helium (He) was used as the carrier gas. H₂ and acetic acid were detected in an Agilent Technologies 7890A gas chromatography system. A CarboxenTM 1010 Plot Fused Capillary Column (30 m × 0.53 mm) with a thermal conductivity detector was used to analyze H₂ with N₂ 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

Fig. 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 NO₃⁻ as electron acceptor in the presence of inoculum.

As^V formation began after a lag phase of 1.5 d, and complete oxidation was achieved after 3 d 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 NO₃⁻ reduction was evaluated. NO₃⁻ and NO₂⁻ concentrations were measured throughout the experiment (shown in Fig. 1B and 1C, respectively). NO₃⁻ concentration decreased from 1.7 ± 0.2 mM to 1.1 ± 0.1 mM, which occurred concomitantly with NO₂⁻ production of 0.49 ± 0.01 mM in the full treatments, whereas no appreciable NO₂⁻ formation was observed in control assays. These results imply that for each mole of NO₃⁻ reduced, approximately one mole of As^{III} is oxidized and one mole of NO₂⁻ is formed (Table 1A) in accordance with the reaction shown in equation 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 O₂ 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 d of incubation and the complete oxidation was achieved after 2.5 d in all the cultures. No As^{III} conversion was observed in the control assays (lacking either inocula or O₂). The O₂ concentration was also monitored (Fig. 2B). As shown in Table 1B, O₂ concentration was 0.32 ± 0.03 mmol L_{liq}⁻¹ in the controls after 3.5 d 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 O₂ consumption. The measured molar ratios (As^{III}:O₂) of the reaction were 2.25 to 2.4, depending on the strain, approximately corresponding to the reaction shown in equation 2:

Oxidation of As^{III} by alternative electron acceptors

Aside from NO₃⁻ and O₂, NO₂⁻ and ClO₃⁻ 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 NO₂⁻ 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 d, and no further increase was observed after an additional 7 d 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 NO₂⁻ or inocula) with an As^V concentration of 0.068 ± 0.01 mM at the end of the experiment. ClO₃⁻ was not utilized as an electron acceptor by any of the pure cultures even after 24 d 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 NO₃⁻ with different substrates under anaerobic conditions, four inorganic electron donors and an organic electron donor were evaluated. The three strains were able to reduce NO₃⁻ when H₂ 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^- .

Fig. 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 d, and the reaction was completed by day 1.5. H_2 concentration decreased from $0.356 \pm 0.014 \text{ mmol H}_2 \text{ L}_{\text{liq}}^{-1}$ to $0.0036 \pm 0.0001 \text{ mmol H}_2 \text{ L}_{\text{liq}}^{-1}$ 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 $0.350 \pm 0.024 \text{ mM}$ 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 to NO_2^- which accumulated to a concentration of $0.359 \pm 0.004 \text{ mM}$ 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 equation 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 $0.503 \text{ mmol H}_2 \text{ L}_{\text{liq}}^{-1}$ and $0.251 \text{ mmol O}_2 \text{ L}_{\text{liq}}^{-1}$ and the H_2 consumption was monitored. The initial H_2 concentration was completely consumed in less than 20 hours 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 and 4B, 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 $\text{NO}_3^-/\text{As}^{\text{III}}$ or H_2/O_2 was estimated by quantifying the DNA at the beginning and at the end of the experiments. These two experiments were the two extreme scenarios, the lowest cell yield ($\text{NO}_3^-/\text{As}^{\text{III}}$) 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 $\text{NO}_3^-/\text{As}^{\text{III}}$. 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 dissimilatory 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} 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. H₂ and acetate were readily oxidized under NO₃⁻-reducing conditions. Also H₂ was shown to be utilized as electron-donor with O₂. 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}, H₂, sulfide, thiosulfate and acetate while reducing NO₃⁻ to NO₂⁻. Aerobic oxidation was also possible with H₂ 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 H₂, 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 NO₂⁻ to NO₃⁻ under aerobic conditions.

None of the three bacterial strains characterized in this study were able to completely denitrify NO₃⁻ to N₂. Instead, NO₃⁻ was partially reduced to NO₂⁻, 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 N₂ gas as the final product of denitrification linked to As^{III} oxidation (Sun et al. 2009). Also, strain DAO1 isolated by Rhine *et al.* (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).

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The work presented here was funded by a U.S. Geological Survey, National Institute for Water Resources 104G grant (2005AZ114G), and by a grant of the National Institute of Environment and Health Sciences-supported Superfund Basic Research Program (NIH ES-04940). The use of trade, product, or firm names in this report is for descriptive purposes only and does not constitute endorsement by the U.S. Geological Survey.

References

- Ahn SJ, Costa J, Emanuel JR. PicoGreen quantitation of DNA: Effective evaluation of samples pre- or post-PCR. *Nucleic Acids Res.* 1996; 24(16):2623–2625. [PubMed: 8692708]
- Ascar L, Ahumada I, Richter P. Influence of redox potential (Eh) on the availability of arsenic species in soils and soils amended with biosolid. *Chemosphere.* 2008; 72(10):1548–1552. [PubMed: 18550147]
- ATSDR. Toxicological profile for Arsenic. U.S. Department of Health and Human Services, Public Health Service; Atlanta, GA: 2007.

- Beauchemin S, Kwong YTJ. Impact of redox conditions on arsenic mobilization from tailings in a wetland with neutral drainage. *Environ Sci Technol*. 2006; 40(20):6297–6303. [PubMed: 17120556]
- Elsaied H, Naganuma T. Phylogenetic diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes from deep-sea microorganisms. *Appl Environ Microbiol*. 2001; 67(4):1751–1765. [PubMed: 11282630]
- Fortune WB, Mellon MG. Determination of iron with o-phenanthroline - A spectrophotometric study. *Ind Eng Chem-Anal Ed*. 1938; 10:0060–0064.
- Garcia-Dominguez E, Mumford A, Rhine ED, Paschal A, Young LY. Novel autotrophic arsenite-oxidizing bacteria isolated from soil and sediments. *FEMS Microbiol Ecol*. 2008; 66(2):401–410. [PubMed: 18717738]
- Goldberg S. Competitive adsorption of arsenate and arsenite on oxides and clay minerals. *Soil Sci Soc Am J*. 2002; 66(2):413–421.
- Green HH. 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*. 1918; 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, Ashfaq KN, Islam S, Hemond HF, Ahmed MF. Arsenic mobility and groundwater extraction in Bangladesh. *Sci*. 2002; 298:1602–1606.
- Hering, JG. Paper presented at the Advances in Arsenic Research. Washington DC: 2005. Contrasting sorption behavior of arsenic(III) and arsenic(V) in suspensions of iron and aluminum oxyhydroxides.; p. 8-24.
- Hirota R, Kato J, Morita H, Kuroda A, Ikeda T, Takiguchi N, Ohtake H. 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 Biotech Bioch*. 2002; 66(3):632–635.
- Hoeft SE, Blum JS, Stolz JF, Tabita FR, Witte B, King GM, Santini JM, Oremland RS. *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*. 2007; 57:504–512. [PubMed: 17329775]
- Hoeft SE, Lucas F, Hollibaugh JT, Oremland RS. Characterization of microbial arsenate reduction in the anoxic bottom waters of Mono Lake, California. *Geomicrobiol J*. 2002; 19(1):23–40.
- Inskeep WP, Macur RE, Hamamura N, Warelou TP, Ward SA, Santini JM. Detection, diversity and expression of aerobic bacterial arsenite oxidase genes. *Environ Microbiol*. 2007; 9(4):934–943. [PubMed: 17359265]
- Kojima H, Fukuhara H, Fukui M. Community structure of microorganisms associated with reddish-brown iron-rich snow. *Syst Appl Microbiol*. 2009; 32(6):429–437. [PubMed: 19560891]
- Krumova K, Nikolovska M, Groudeva V. Characterization of arsenic-transforming bacteria from arsenic contaminated sites in Bulgaria. *Biotechnol Biotec Eq*. 2008; 22(2):729–735.
- Lin Z, Puls RW. Adsorption, desorption and oxidation of arsenic affected by clay minerals and aging process. *Environ Geol*. 2000; 39(7):753–759.
- Malasarn D, Saltikov W, Campbell KM, Santini JM, Hering JG, Newman DK. *ArrA* is a reliable marker for As(V) respiration. *Sci*. 2004; 306(5695):455–455.
- Miziorko HM, Lorimer GH. Ribulose-1,5-Bisphosphate Carboxylase-Oxygenase. *Annu Rev Biochem*. 1983; 52:507–535. [PubMed: 6351728]
- Oremland RS, Hoeft SE, Santini JA, Bano N, Hollibaugh RA, Hollibaugh JT. Anaerobic oxidation of arsenite in Mono Lake water and by facultative, arsenite-oxidizing chemoautotroph, strain MLHE-1. *Appl and Environ Microbiol*. 2002; 68(10):4795–4802. [PubMed: 12324322]
- Oremland RS, Kulp TR, Blum JS, Hoeft SE, Baesman S, Miller LG, Stolz JF. A microbial arsenic cycle in a salt-saturated, extreme environment. *Sci*. 2005; 308(5726):1305–1308.
- Oremland RS, Stolz JF. The ecology of arsenic. *Sci*. 2003; 300(5621):939–944.
- Oremland RS, Stolz JF, Hollibaugh JT. The microbial arsenic cycle in Mono Lake, California. *FEMS Microbiol Ecol*. 2004; 48(1):15–27. [PubMed: 19712427]
- Paez-Espino D, Tamames J, de Lorenzo V, Canovas D. Microbial responses to environmental arsenic. *Biometals*. 2009; 22(1):117–130. [PubMed: 19130261]

- Rhine ED, Garcia-Dominguez E, Phelps CD, Young LY. Environmental microbes can speciate and cycle arsenic. *Environ Sci Technol*. 2005; 39(24):9569–9573. [PubMed: 16475337]
- Rhine ED, Ni Chadhain SM, Zylstra GJ, Young LY. The arsenite oxidase genes (*aroAB*) in novel chemoautotrophic arsenite oxidizers. *Biochem Biophys Res Commun*. 2007; 354(3):662–667. [PubMed: 17257587]
- Rhine ED, Phelps CD, Young LY. Anaerobic arsenite oxidation by novel denitrifying isolates. *Environ Microbiol*. 2006; 8(5):899–908. [PubMed: 16623746]
- Richey C, Chovanec P, Hoeft SE, Oremland RS, Basu P, Stolz JF. Respiratory arsenate reductase as a bidirectional enzyme. *Biochem Biophys Res Commun*. 2009; 382(2):298–302. [PubMed: 19285953]
- Saltikov CW, Newman DK. Genetic identification of a respiratory arsenate reductase. *Proc Natl Acad Sci U S A*. 2003; 100(19):10983–10988. [PubMed: 12939408]
- Santini JM, Sly LI, Schnagl RD, Macy JM. A new chemolithoautotrophic arsenite-oxidizing bacterium isolated from a gold mine: phylogenetic, physiological, and preliminary biochemical studies. *Appl Environ Microbiol*. 2000; 66(1):92–97. [PubMed: 10618208]
- Santini JM, Sly LI, Wen AM, Comrie D, De Wulf-Durand P, Macy JM. New arsenite-oxidizing bacteria isolated from Australian gold mining environments - Phylogenetic relationships. *Geomicrobiol J*. 2002; 19(1):67–76.
- Santini JM, vanden Hoven RN. Molybdenum-containing arsenite oxidase of the chemolithoautotrophic arsenite oxidizer NT-26. *J Bacteriol*. 2004; 186(6):1614–1619. [PubMed: 14996791]
- Scala DJ, Kerkhof LJ. Nitrous oxide reductase (*nosZ*) gene-specific PCR primers for detection of denitrifiers and three *nosZ* genes from marine sediments. *FEMS Microbiol Lett*. 1998; 162(1):61–68. [PubMed: 9595664]
- Senn DB, Hemond HF. Nitrate controls on iron and arsenic in an urban lake. *Sci*. 2002; 296(5577):2373–2376.
- Silver S, Phung LT. Genes and enzymes involved in bacterial oxidation and reduction of inorganic arsenic. *Appl Environ Microbiol*. 2005; 71(2):599–608. [PubMed: 15691908]
- Smedley PL, Kinniburgh DG. A review of the source, behaviour and distribution of arsenic in natural waters. *Appl Geochem*. 2002; 17(5):517–568.
- Sun WJ, Sierra-Alvarez R, Fernandez N, Sanz JL, Amils R, Legatzki A, Maier RM, Field JA. Molecular characterization and in situ quantification of anoxic arsenite-oxidizing denitrifying enrichment cultures. *FEMS Microbiol Ecol*. 2009; 68(1):72–85. [PubMed: 19187211]
- Sun WJ, Sierra-Alvarez R, Milner L, Field JA. Anoxic oxidation of arsenite linked to chlorate reduction. *Appl Environ Microbiol*. 2010; 76:6804–6811. [PubMed: 20729322]
- Tufano KJ, Reyes C, Saltikov CW, Fendorf S. Reductive processes controlling arsenic retention: revealing the relative importance of iron and arsenic reduction. *Environ Sci Technol*. 2008; 42(22):8283–8289. [PubMed: 19068807]
- Turner AW. Bacterial oxidation of arsenite. *Nature*. 1949; 164(4158):76–77. [PubMed: 18133543]
- USEPA. National primary drinking water regulations; arsenic and clarifications to compliance and new source contaminants monitoring. *Fed Regist*. 2001; 66(14):6975–7066.
- Wang YT, Suttigarn A. Arsenite oxidation by *Alcaligenes faecalis* strain O1201 in a continuous-flow bioreactor. *J Environ Eng-Asce*. 2007; 133(5):471–476.

Fig. 1.

As^V formation by pure cultures EC1, EC3 and MC for oxidation of As^{III} (0.5 mM) linked to NO₃⁻ (1.5 mM) reduction. Legends: panel A, As^V formation, complete treatments containing As^{III} and NO₃⁻ and inoculated with EC1 (■), EC3 (●) and MC (▲); biological controls (dashed lines) containing As^{III} but lacking NO₃⁻: EC1 (□), EC3 (○) and MC (△); non inoculated control (x); panel B, NO₃⁻ consumption; panel C, NO₂⁻ formation. Biological treatments supplied with NO₃⁻ and As^{III}, EC1 (■), EC3 (●) and MC (▲); biological controls (dashed lines) supplied with As^{III} but no NO₃, EC1 (□), EC3 (○) and MC (△); non inoculated control (x).

Fig. 2. As^V production by pure cultures EC1, EC3 and MC with O₂ as electron acceptor. Oxidation of 0.5 mM of As^{III} with 0.35 mmol O₂ L_{liq}⁻¹. Legends: panel A, As^V formation. Biological treatments supplied with O₂ and As^{III}, EC1 (■), EC3 (●) and MC (▲); controls (dashed lines) supplied with As^{III} but no O₂, EC1 (□), EC3 (○) and MC (△); non inoculated control (x); panel B, O₂ consumption. Full treatments supplied with O₂ and As^{III}, EC1 (■), EC3 (●) and MC (▲); controls (dashed lines) supplied with O₂ but no As^{III}, EC1 (□), EC3 (○) and MC (△); non inoculated control (x).

Fig. 3.

H_2 oxidation linked to NO_3^- reduction. Treatments with $0.35 \text{ mmol } H_2 L_{liq}^{-1}$ and 1.5 mM of NO_3^- . Legends: panel A, H_2 oxidation biological treatments supplied with NO_3^- and H_2 , EC1 (■), EC3 (●) and MC (▲); controls (dashed lines) supplied with H_2 but no NO_3^- , EC1 (□), EC3 (○) and MC (△); non inoculated control (x); panel B, decrease of NO_3^- concentration; panel C, NO_2^- formation. Full treatments supplied with NO_3^- and H_2 and inoculated with EC1 (■), EC3 (●) and MC (▲); controls (dashed lines) with only H_2 and no NO_3^- : EC1 (□), EC3 (○) and MC (△); non inoculated control (x).

Fig. 4. Ability of the pure cultures to oxidize As^{III} under denitrifying conditions after being incubated with: panel A, 0.3 mM NO₃⁻ and 0.4 mmol L_{liq}⁻¹ H₂ or panel B, 0.45 mmol H₂ L_{liq}⁻¹ and 0.3 mmol O₂ L_{liq}⁻¹. Treatment with 0.5 mM of As^{III} and 0.3 mM of NO₃⁻: EC1 (■), EC3 (●) and MC (▲). Controls (dashed lines) with only 0.5 mM of As^{III} and no NO₃⁻: EC1 (□), EC3 (○) and MC (△). Non inoculated controls (x).

Table 1

Stoichiometric calculations of the biological catalyzed reactions mediated by three pure culture strains EC1, MC and EC3^{*}

Part A	$\Delta(\text{NO}_3^-)$	$\Delta(\text{NO}_2^-)$	$\Delta(\text{As}^{\text{V}})$	$\frac{\Delta(\text{As}^{\text{V}})}{\Delta(\text{NO}_3^-)}$
	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

Part B	$\Delta(\text{O}_2)$	$\Delta(\text{As}^{\text{V}})$	$\frac{\Delta(\text{As}^{\text{V}})}{\Delta(\text{NO}_2)}$
	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

Part C	$\Delta(\text{H}_2)$	$\Delta(\text{NO}_2^-)$	$\Delta(\text{NO}_3^-)$	$\frac{\Delta(\text{H}_2)}{\Delta(\text{NO}_3^-)}$
	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 (Part A), and aerobic conditions (Part B). Molar relationship of the H₂ oxidation under denitrifying conditions (Part C)

Table 2

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

Pure Cultures	Electron acceptors ⁺					
	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)
EC1	0.496 ± 0.011	4	0.053 ± 0.052	54	0.473 ± 0.002	2.5
MC	0.496 ± 0.004	3	0.240 ± 0.008	24	0.500 ± 0.010	2.5
EC3	0.485 ± 0.028	3	0.284 ± 0.116	24	0.472 ± 0.018	2.5

^{*} As^{III} concentration was 0.5 mM.

⁺ Concentrations of the different electron acceptor tested were 1.5 mM KNO₃, 0.5 mM NaNO₂, 0.35 mmol O₂ L⁻¹ and 0.25 mM NaClO₃. Electron acceptors that have been positively utilized by the cultures to oxidize As^{III} are shown in bold text.